Host – Parasite Interactions

Population genetics of Host – Parasite interactions in *Lumbricus terrestris* and *Monocystis sp.* (Apicomplexa: Gregarinea)

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Velavan Thirumalaisamy Palanichamy

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Dekan:	Prof. Dr. Hanspeter A. Mallot
1.Berichterstatter:	Prof. Dr. Nico K Michiels
2.Berichterstatter:	Prof. Dr. Jürgen Tomiuk

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TABLE OF CONTENTS

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Population genetics of Host – Parasite interactions in *Lumbricus terrestris* and *Monocystis Sp*. (Apicomplexa: Gregarinea)

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Velavan T P

This thesis is based on the following articles and manuscripts:

II Velavan TP, Hinrich Schulenburg, Nico K Michiels (2007) **Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris* L)** Molecular Ecology Notes. 7 (6): 1060-1062

III Velavan TP, Suska Sahm, Hinrich Schulenburg, Nico K Michiels. **High Genetic diversity and heterogeneous parasite load in earthworm *Lumbricus terrestris* on a German meadow.** Submitted Soil Biology and Biochemistry

IV Suska Sahm, Velavan TP, Hinrich Schulenburg, Nico K Michiels. **Reconstruction of mating history – a retrospective analysis of *Lumbricus terrestris* mate choice criteria in natural populations.** unpublished manuscript.

V Velavan TP, Hinrich Schulenburg, Nico K Michiels. **Detection of multiple infections by *Monocystis* strains in a single earthworm host using ribosomal internal transcribed spacer sequence variation.** Submitted Parasitology.

VI Velavan TP, Nadine Timmermeyer, Hinrich Schulenburg, Nico K Michiels. **Diversity of *Monocystis* parasites in relation to earthworm host fitness and heterozygosity.** unpublished manuscript.

Contribution of authors:

Velavan TP: Development of original ideas (in part together with Nico Michiels and Hinrich Schulenburg), planning of all experiments and studies, data gathering (**II, III, IV, V** and **VI**), supervision, participation in data collection and on molecular work (**IV**), final data analysis (**II, III, V** and **VI**), preparation of chapters for publication (**II, III, V** and **VI**).

Nico Michiels: Initial ideas, participation in experimental design, in data analysis and in manuscript preparations, supervision of all studies (**II - VI**).

Hinrich Schulenburg: Initial ideas (**III** and **V**), supervision of molecular methodologies, participation in data analysis and in manuscript preparations, supervision of all studies (**II - VI**).

Suska Sahm: Development of ideas, planning of experiments, data gathering and final data analysis, preparation of chapter for publication (**IV**).

Nadine Timmermeyer: DNA extraction of *Monocystis sp*. parasites (**VI**).

Chapter I

Summary and Outline of thesis
General Overview on Host Parasite interactions
General biology of Model system

General Overview

Summary of dissertation:

The earthworm *Lumbricus terrestris* is one of the most studied organisms in varied aspects of biology especially in the field of soil ecology and ecotoxicology, relatively very few have worked on individual based, evolutionary ecological perspective. *L. terrestris* have been explored in various scientific fields however very little attention is paid to *Monocystis* parasites they harbour in them. This sort of host parasite interactions is believed to influence an array of evolutionary and ecological processes that includes population dynamics, evolution of diversity, sexual reproduction and of parasite virulence (Lopez-Pascua and Buckling 2008). In the following research project, I investigated the interactions between the natural populations of earthworm species *L. terrestris* and with its most common parasite *Monocystis sp.* of the apicomplexan genus. In particular, I focussed on the population genetic structure and genetic diversity of both host *L. terrestris* and *Monocystis sp.* parasites at a microgeographical scale and related these diversity measures to that of parasite virulence and host fitness respectively. In addition, we tried to reconstruct the recent mating history events of *L. terrestris* in natural field populations and tested if *L. terrestris* uses parasite concentration, body or vesicle weight and spatial distance as criteria to choose its mating partner.

Outline of this thesis:

This thesis is structured in six different chapters with methodological details and hypothesis of each study is described explicitly for each chapter. **Chapter 1** is a general introduction in which an overview on host parasite interactions at an evolutionary ecology perspective is explained and related background information on the *L. terrestris* - *Monocystis sp*. model system is described. Chapters 2- 6 are organized in manuscript form in which it is written as introduction, materials and methods, results and discussion. The outline of this thesis emphasizes on key research questions that I address in each of these chapters.

The preliminary objective of this project is to study the population genetic structure and genetic diversity patterns in natural host populations of *L. terrestris.* In order to study the above objective, we need molecular tools such as neutral DNA markers. Therefore in **chapter 2**, I developed 10 microsatellite markers explicitly for the host *L. terrestris*, an individual from Muenster population and validated these primer pairs across *L. terrestris* population from Canadian origin. Few of these markers based on their polymorphism levels facilitated us to study population genetic structure and genetic diversity patterns in natural host populations at a microgeographical scale.

In parasite-host dynamics, parasites exert frequency-dependent selection on their hosts by favouring rare alleles that may confer resistance against infection. Therefore host populations that suffer strong parasite stress should maintain higher levels of genetic variability. Given the fact that *Lumbricus terrestris* has very low mobility and lives in dense, but patchy populations, we anticipate seeing local patterns and genetic structuring across subpopulations at a microgeographical scale. As *L. terrestris* is likely to have a restricted choice of mating partners due to their strict association with a permanent burrow (Michiels et al. 2001), this may lead to high inbreeding and genetic differentiation among subpopulations. Genetic differentiation among subpopulations may be further augmented by variation in parasite prevalence. Therefore in **chapter 3**, using three polymorphic microsatellite loci across 26 different earthworm subpopulations (281 genotypes), we tested the relationship between parasite load and genetic variation in natural populations of the common earthworm *L. terrestris.*

Mate choice plays an important role in gonochorist and also in hermaphrodite evolution. In simultaneous hermaphrodite like *Lumbricus terrestris*, mate choice is usually expected because it involves multiple risks and costs during copulation. Given that the population density of *Lumbricus terrestris* is normally high, there is enough opportunity for choice. Parasite-mediated sexual selection (Hamilton and Zuk 1982) predicts the evolution and maintenance of active mate choice in presence of parasites. Investing their resources on a highly parasitized partner means reduced fecundity, and risk of producing low resistance offspring, hence we expect to see a

sexual selection on a low parasitized individual. Also we expect these worms to outbreed to increase their genetic diversity to keep optimal level of virulence hindered. Therefore in **chapter 4,** as a part of a student diploma project work, we genotyped the stored allosperm in their spermathecae and tissue using 3 different microsatellites and tried to reconstruct their recent mating history and tested if *L. terrestris* uses parasite concentration, body or vesicle weight and spatial distance as any criterion to choose its partner.

Given that parasite concentration donot have an significant association between parasite load and subpopulation genetic diversity as inferred in Chapter II and parasite load does not seem to be a mate choice factor as in Chapter III, we embarked on this study to determine whether the *L. terrestris* host populations are infected by one single species of *Monocystis* or by multiple genotypes of same species? Therefore in **chapter 5**, I developed *Monocystis* specific primer pairs that amplify the complete internal transcribed spacer region in which we obtained evidence for their genetic divergence within one single host based upon sequence analysis of the ITS-1, ITS-2 and the 5.8S rRNA gene.

In host parasite interactions, parasites play a significant role in enhancing biological diversity of host populations as parasites often represent a significant selective pressure. We assume that host populations revealing high parasite diversity should exhibit high levels of heterozygosity and reduced fitness. Therefore in this context in **chapter 6**, using primer pairs explicitly designed for internal transcribed spacer (ITS) regions of *Monocystis sp*. and using polyacrylamide gel electrophoresis (PAGE) approach, we studied the genetic structure and genetic diversity of *Monocystis sp*. at a microgeographical scale. We then related this genetic diversity measures of the parasite to host genetic diversity, their respective heterozygosity levels and of their fitness parameters.

Host - Parasite Interactions

There has been increasing interest in the field of host parasite interactions over decades. Coevolution is defined as the evolution in one species in response to selection imposed by a second species, followed by evolution in the second species in response to reciprocal selection imposed by the first species (Clayton et al. 1999). Every organism is infected by massive amount of different parasite species. In general, parasitism is a symbiotic relationship where a parasite benefits by successfully completing their lifecycle from a long-lasting association with the host. To act in response to parasitism, hosts invest their resources lot on their immune defence. This results in a trade off with life history traits such as growth, survival and reproduction (Simovka et. al 2008). Specificity of these interactions between hosts and parasites play a central role in reproductive success of an organism. The effect of parasites to host range from immediate fitness costs to reduced reproductive potential such as castration (Ebert and Herre 1996; Kover 2000, Field 2005), host death and local extinction (Boots and Sasaki 2002, 2003, Field 2005).

Without genetic variation, evolutionary change cannot function. The wide array of studies on host pathogen interactions deal with two hypotheses based on mutation - selection balance (i) Heterozygote advantage (ii) negative frequency-dependent selection. Heterozygote advantage takes place when selection acts against homozygous individuals which effectively mean the hosts are more resistant to more parasite genotypes. Such conditions are proposed to maintain high level of genetic variation in vertebrate's major histocompatibility complex (MHC). There are several studies especially in vertebrates where parasite mediated selection on major histocompatibility complex (MHC) have been reported (Penn and Potts 1999, Penn 2002, De Bellocq et al. 2008, Babik et al. 2008, Bos D H et al. 2008). In the second model, when alleles become more common in a population, the host organisms become less fit. Selection favours parasites that can trail the most common genotype, resulting in reduced fitness in common host genotype. Therefore negative frequency dependent selection acts that provides an intrinsic advantage to rare phenotypes or a new host genotype, resulting in frequency dependent oscillations and the maintenance of genetic variation in the population.

The evolutionary arms race between host and parasite can often result in strong selection that promotes genetic variability through sexual recombination (Hamilton et al. 1990). This is chiefly in the case when (1) the parasite infects the host in a genotype-specific way, (2) the parasite population adapts to the most common host genotype, through which negative-frequency dependent selection can result, and (3) clear fitness costs are imposed by the parasite. Sexual recombination benefits an outcrossing host individual by reproducing a variable offspring. In host parasite relationships, rare genotypes have inherent advantages and may be selected. Larger the different genotypes in a host population, lesser the frequency and smaller the chance that a parasite will encounter the same genotype in successive hosts (Ebert and Hamilton 1996). By producing offspring with increased genetic variability (Howard and Lively 1998; Goddard et al. 2005), sexual reproduction reduces the adaptive advantage, via shorter generation time, that parasites have the benefit by producing genetically dissimilar offspring (Hamilton 1980; Hamilton et al. 1990; Ebert and Hamilton 1996).

Parasite-mediated sexual selection (Hamilton and Zuk 1982) predicts the evolution and maintenance of active mate choice in presence of parasites. In separate sex species, mate choice is usually carried out by the female, simply due to the fact that females have to invest more in the offspring than the males (sperm donors). In addition, few mating events are sufficient for females to incorporate enough sperm to fertilise their eggs, whereas for a male individual each additional mating adds to his lifetime reproductive success (LRS). In contrast to such separate sex species, mate choice scenarios can look extremely different in simultaneous obligatory outcrossing hermaphrodites, because they combine male and female interests in one individual. However, mate choice decisions are under the control of both partners. The ability to allocate resources flexibly to male and female functions as well as arising conflicts over sexual roles complicate sexual strategies enormously (Michiels and Newman 1998). Although sexual selection on traits of one gender is always coupled with traits of the other gender, it could be shown that hermaphrodites have all necessary features for sexual selection (Morgan 1994). Empirical data on different aspects of mate choice in hermaphroditic invertebrates are relatively few, but

promising results are shown in snails (Haase and Karlsson 2004; Webster and Gower 2006), nematodes (Kleemann and Basolo 2007) and in flatworms. As known from other studies (Sauter and Brown 2001), the mating history of a certain individual influences the choice made in meticulous situations. Especially for long-living, sessile or territorial animals like *L. terrestris*, the mating history depends on their surrounding neighbors which have a high impact on their decisions for future reproductive strategies.

Extensive interest has been focused on parasite virulence in literature that has been studied on host parasite interactions. Virulence may be defined as the reduction in host fitness (lifetime reproductive success) as an effect of parasitic infection (Read, 1994). In evolutionary models, virulence is measured as parasite induced host mortality as a result of direct or indirect result of parasitic infection (Webster and Davies 2001). Indirect threats of virulence include predation risk, other pathogens susceptibility (Poulin et al. 1998). When we talk about virulence in a parasite perspective, the parasites agree on optimal levels of virulence with the host to strike a balance between parasite growth and reproduction with that of host survival. Virulence levels are increased when there is a higher risk of multiple infections especially in homogenous host populations and in addition when parasites are more host specific (Levin and Bull 1994, Mackinnon and Read 1999, Ebert 1999, Regoes et al. 2000, Kirchner and Roy 2002). On the other hand, one could expect reduced virulence to evolve when parasites are not highly host specific and in heterogeneous host populations where the populations are resistance to infection (Gandon and Michalakis 2000, Ganusov et al. 2002). In natural populations, optimal level of virulence is in a state of fluctuation because coevolution is an ongoing process whereby host genotype frequencies keep changing as a result of selection forced by parasites and in turn parasites requires certain timeframe to evolve on their optimal level of virulence (Dybdahl and Storfer 2003).

Local adaptation occurs when parasites become adapted to infecting hosts whereby they have coevolved in the past sympatrically. At the same time considering local adaptation of hosts,

they become locally adapted when they are resistant to parasite genotypes when they share a common history with them (Gandon et al. 1996; Kaltz and Shykoff 1998; Gandon and Michalakis 2002; Lively and Dybdahl 2000; Dybdahl and Storfer 2003; Kawecki and Ebert 2004). For local adaptation, spatial heterogeneity of the environment is a prerequisite (Gandon and Van Zandt 1998). In host-parasite interactions, the evolutionary outcome depends extensively on the quantity of gene flow among populations in both the host and the parasite (Dybdahl and Lively 1998). If the host does not evolve in response to the parasite, local adaptation occurs when the migration rate of parasite is very low (Gandon and Van Zandt 1998). Studies by Gandon and Michalakis 2002 predict that if parasites migrate more than the host, then parasites become locally adapted and if not the parasites will be locally maladapted. To be more precise if the extent of gene flow differs between host and parasite, either the host or parasite with a higher gene flow will have a selective advantage due to introduction of rare alleles into the local population (Gandon et al. 1996). Therefore local adaption is influenced by migration rates of either host or the parasite; however it also depends on gene specificity.

Genetic basis of infection is often assumed in many of the host-parasite models in evolutionary biology. There are two different genetic systems describing infectivity. One is gene for gene model (GFG) and the other corresponds to matching allele model. Gene for gene is the genetic system of interaction originally formulated for plant- pathogen interactions. Under this model a pathogen elicitor allele activates a specific host response allele (a dominant and universally resistant allele in the host, recessive and virulence allele in the pathogen), leading to a defense reaction in the host (Dybdahl and Storfer 2003) resulting in cross infectivity where one parasite genotype can infect multiple host genotypes. In case of matching allele model, a parasite infects a host if the alleles of the parasite match those of the host at the interaction loci (loci that encode traits that are recognized by specific molecular receptors). This is a kind of nonself recognition system where a host recognizes parasite genotypes that do not match host genotypes. In this case, no single parasite allele is universally infective to all host genotypes. There has been evidence for genetic basis of infection in snail-schistosome system (Webster and

Woolhouse 1998, Lively et al. 2004) and on Daphnia microparasite system (Decaestecker et al. 2003).

In conclusion Lumbricid – *Monocystis sp*. model system serves as a valuable model system to study host parasite interactions and meets necessary requirements to study them at different levels (as mentioned above) in an evolutionary ecological perspective.

The Host: *Lumbricus terrestris*

General Biology

Charles Darwin was the first scientist to acknowledge the significance of earthworms, which he described it as “natures plough” (http://www.uclan.ac.uk). Over decades, the earthworms have been explored and well studied for their beneficial effects on the physical and chemical nature of soils. The earthworm *Lumbricus terrestris* belongs to the most widely spread and best investigated Oligochaetes (Annelida: Clitellates) Fig 1. *L. terrestris are* native worms of Europe, but also distributed widely around the world, due to human introductions. It is considered to be a solemn pest species in few areas where it has been introduced, as it out-competes locally native worms. *Lumbricus terrestris* goes under a variety of common names such as Night crawler or Vitalis (North America) and dew worm (Canada). Soils that are moist and rich in organic matter are the preferred habitat and distribution of these worms depends on proximity to human habitation. *Lumbricus terrestris* is an anecic worm, which means they form deep permanent burrows and comes to the surface to feed. Visibility of these worms are high than most other earthworms as they have a characteristic feature of copulating on the surface at night. The other characteristic feature of this species is to pull leaves closer to the mouth of its burrow where they partially decay before being eaten. The potential life span of *Lumbricus terrestris* is unknown, though it has been kept in the laboratory for 6 years (http://en.wikipedia.org/wiki/Lumbricus_terrestris). The majority of the literature on *L. terrestris* relates to applied aspects (i.e. ecotoxicology and interactions with important crops) in agriculture as it is usually predictable that earthworms improve soil structure that in turn benefit crop productivity (Lee and Foster 1991; Edwards et al. 1995; Subler and Kirsch 1998, VandenBygaart et al. 2000) and in bioremediation (Edwards and Bohlen 1996; Giggleman et al. 1998). However, only a few authors have worked intensively with mating and

Fig 1: *Lumbricus terrestris*
From Dr. Joris Koene

reproduction in the laboratory (Grove 1925; Nuutinen and Butt 1997a, 1997b; Butt and Nuutinen 1998, Field et al. 2005).

Morphology

L. terrestris has a reddish-brown back, a yellowish underside and an often prominent orange-red 'saddle' region known as the 'clitellum', closer to the reproductive organs (http://www.arkive.org/species). One of the key morphological characteristic features to identify these worms is that, they have a flattened tail region irrespective of their cylindrical body (Fig 1). The body is segmented and each segment bears small hairs known as 'chaetae', which help the worm to move through the soil and facilitate anchorage between their partners during copulation (Koene et al. 2005). The gut comprises of foregut, midgut, and hindgut. Foregut and hindgut are ectodermal derivatives and are lined with cuticle. The mouth opens into the short, thin-walled buccal cavity in segments 1-3. The pharynx is posterior to the buccal cavity in segments 3-5. Posterior to the pharynx, the gut narrows to become the thin-walled oesophagus in segments 6-12 (http://webs.lander.edu/rsfox/invertebrates/lumbricus.html). Six large, creamy white seminal vesicles are hidden at the posterior end of the oesophagus. Posterior to the oesophagus and oesophageal pouches one can find two pairs of white calciferous glands which remove excess calcium and carbon dioxide from the blood. Calciferous glands play a vital role in the regulation of blood pH. Posterior to the last pair of calciferous glands, the oesophagus narrows again and then joins the large, bulbous, thin-walled crop in segment 12, which act as a food storage organ. Posterior to the crop the gut becomes the gizzard the site for mechanical digestion. The gut narrows again posterior to the gizzard and

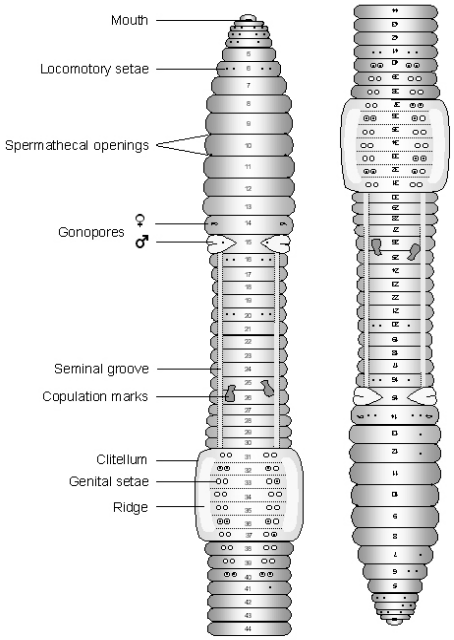


Fig 2: Schematic diagram of *L.terrestris* anatomy
From Dr. Nico Michiels

becomes the intestine which is the region for chemical digestion (hydrolysis) and absorption. The intestine has a greater extent of yellow chlorogogen tissue. The anterior intestine, or saccular intestine, is specialized for synthesis and secretion of enzymes and hydrolysis of food molecules (http://webs.lander.edu/rsfox/invertebrates/lumbricus.html). The extreme posterior end of the gut is the rectum or hindgut and opens to the exterior via the anus. The overall schematic view of ventral anatomy of the earthworm *L. terrestris* is depicted in Fig 2.

Mating aspects

Fig: 3 *L. terrestris* during copulation
From Dr. Nico Michiels

L. terrestris are obligate outcrossing hermaphrodites with complex male and female reproductive organs present in each individual. The precopulatory behaviour of *L. terrestris* is quite complex (Michiels et al. 2001) and involves repeated reciprocal burrow visits, in which the partners protrude their anterior segments into the burrow of their partner (Nuutinen and Butt 1997*a*). Copulation begins as partners line up with their ventro-ventral contact on (either side) and form the classic '*S*-shape' mating position (Fig.3). Normally the copulation duration lasts about 180 – 220 min (Nuutinen and Butt 1997*b*). The received sperm are temporarily stored in sperm receptacles while the clitellum secretes a mucous cocoon. The cocoon slides along the worm, picking up the eggs that are produced in ovaries and then the stored sperm from special reproductive pores (female and male gonophores), and then slips off the worm's head (http://io.uwinnipeg.ca/~simmons/lb7pg1.htm). The embryos develop within the cocoon. The reproductive structures of the earthworm start at segment nine. Seminal receptacles are found in segments nine and ten and in segments 9-12 three pair's seminal vesicles are found. Sperm are produced within testes i.e. inside the seminal vesicles and are transferred to the male

gonophores via the vas deferens. The female reproductive structures consist of a pair of ovaries segment 13) connected to the female gonophores via a series of small passageways (Fig 4). Since earthworms can produce ova year round, cocoon production is continuous. Thus cocoon production typically depends on the availability and quality of sperm within the spermathecae. Butt and Nuutinen (1998) report that viable cocoons are produced for at least six months following only a single mating and viable cocoons were produced for even longer. Cocoons (typically 6 x 4.5-5.0 mm) are lemon-shaped and can be deposited as deep as 0.4 m from the soil surface; however most are deposited in the top 0.05 m (Butt 2002). *L. terrestris* can store viable sperm for up to five months after mating and mate repeatedly, once every 11 days according to laboratory estimates (Michiels et al. 2001), making sperm competition likely.

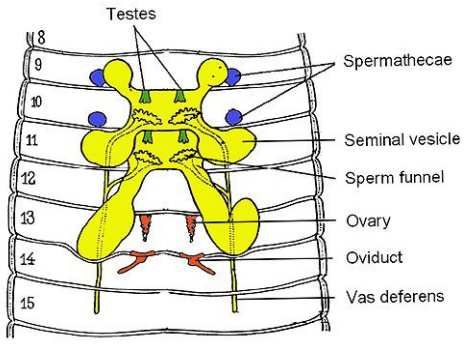


Fig 4 : Schematic diagram of the internal reproductive anatomy of *L. terrestris* (dorsal view)
From Dr. Stuart G Field

Dispersal and Predation

Normally *L. terrestris* leads a very sedentary lifestyle, as a permanence of burrow patterns shown by their middens. This likely shows inheritance or 'recycling' of burrows, as estimates of maximum *L. terrestris* lifespan have been put at 6-8 years. (Butt and Nuutinen 2005). There is obviously a preference for staying at the same site for as long as possible, by weighing risks and potential benefits of dispersal and settlement at a novel site. Over surface movements on moist, flat terrain have been recorded at a speed of 20 m/hr and, based on trail length measurements, nocturnal activity away from the burrow has been measured up to 19 m during a single surface foray (Butt and Nuutinen 2005). Dispersal of these worms relates more to sex and out-breeding. Avoidance of mating with very closely related animals is enhanced by movement of juveniles away from the natal burrow. Moreover the dispersal of many animals is often condition

dependent. Resource needs for *L. terrestris* may therefore be physical, in terms of a habitable burrow with associated food in close proximity, but also encompass the availability of non-closely related adult conspecifics. Other than organisms higher up on the food chain (owls, rodents, birds, etc.) earthworms are also predated in the UK by the New Zealand flatworm (*Arthurdendyus triangulates*), an exotic species first observed in the UK in 1963 (Lowe 2006). Another flatworm predator is the *Bipalium adventitium*, also invasive in North America (Ducey et al. 1999).

The Parasite: *Monocystis*

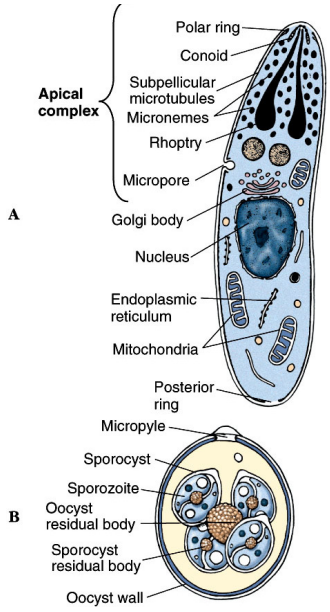


Fig: 5 A typical apicomplexan sporozoite illustrating apical complex and other intracellular structures
From: www.uprm.edu

L. terrestris is heavily parasitised. Among the many parasitic species, gregarine sporozoans (Apicomplexa: Gregarinea) are most common (Edwards and Bohlen 1996). The genus *Monocystis* (Fig. 5) is particularly numerous in the sperm vesicles, but also can occur in the spermathecae (sperm receiving organs). Stressed animals tend to show higher parasite loads than those from undisturbed environments (Pižl and Sterzynska 1991), indicating that immunocompetence may be impeded when stressed. High *Monocystis* loads can lead to castration (Sims and Gerard 1985). In extreme cases, *L. terrestris* will reabsorb the entire seminal vesicles to clear an infection (Breidenbach 2002), which must represent a heavy cost to an individual.

The lifecycle of *Monocystis*

Oocysts (sporocytes) are generally transmitted by ingestion, whereupon sporozoites emerge within the intestinal tract. Sporozoites then grow into trophozoites after penetrating the gut wall. Mobile mature trophozoites cross the gut wall, migrate to the seminal vesicles, where further

maturation occurs. Eventually mature trophozoites (~200 µm) pair encases themselves in a common envelope called a gametocyst. Within the gametocyst (Fig. 6), the two original trophozoites (now called gametocytes or gamonts), begin to produce gametes via budding, which unite to form many zygotes. Zygotes then secrete a thick extracellular wall and become sporocysts (~20 µm). Within these sporocysts, the zygote (the only diploid stage in the lifecycle) undergoes one meiotic division (haploid), and two mitotic divisions to produce 8 new sporozoites. At this point the gametocyst ruptures releasing the many sporocysts into the seminal fluid and eventually into the environment to repeat the lifecycle (Schmidt and Roberts 2000; Bush et al. 2001). See Fig. 7 for their complete lifecycle. Further development of cyst doesn't occur until another earthworm swallows the cysts. The cysts are also ingested by birds or other animals that eats the earthworm. The cysts are not digestible and are voided with the faeces. Another worm now swallows the cyst, and now the cyst coat is digested and the freed sporozoites migrate to the seminal vesicles by boring through the gut wall into the coelom of the earthworm. Cysts may live in the soil for a considerable period. The parasite feeds explicitly on the cytoplasm of sperm morula by extruding enzymes and absorbing the digested products through the pellicle. It will frequently move to another morula and consume the cytoplasm, before it is fully-grown. Often numerous sperm tails adhere to the pellicle, giving the *Monocystis* a ciliated appearance (http://www.microscopy-uk.org.uk/).

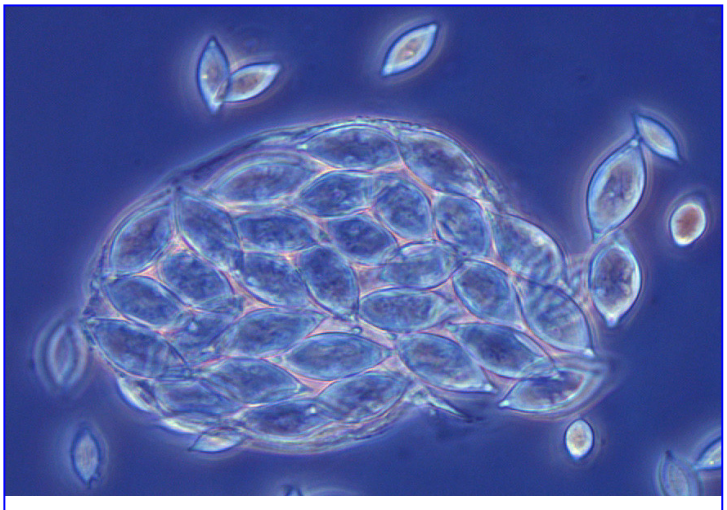


Fig. 6: Intact *Monocystis* gametocyst clearly showing numerous sporocysts. Magnification: at 40X under Phase contrast

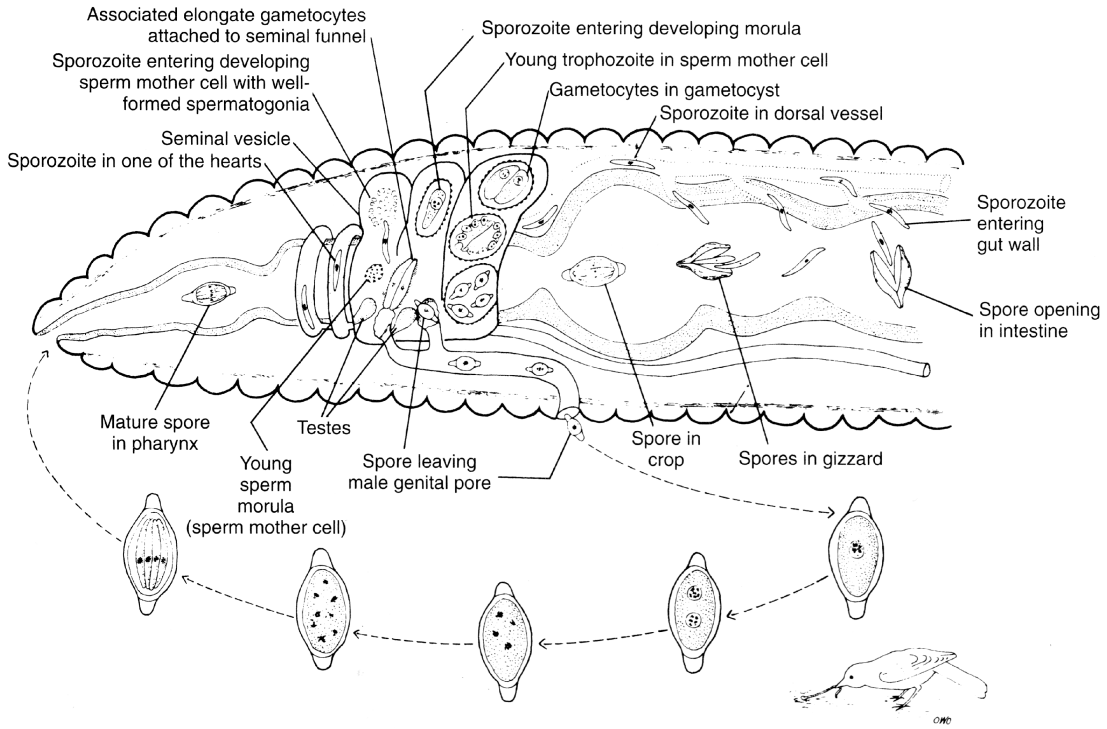


Fig. 7: Generalized life cycle of *Monocystis lumbrici* in a *Lumbricus terrestris* host.
From : Foundations of Parasitology, 7th edition, McGraw Hill Publications

Chapter II

Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris L*).

Velavan TP, Hinrich Schulenburg and Nico Michiels (2007)
Molecular Ecology Notes. 7 (6): 1060-1062

Keywords
Earthworms
Lumbricus terrestris
Microsatellite
Co-evolution

Abstract:

We developed and characterized ten highly polymorphic microsatellite loci from an SSR-enriched genomic DNA library of the common earthworm (*Lumbricus terrestris* L.). Characterization of these loci using 32 individuals revealed high levels of genetic diversity, 5 to 18 alleles per locus and a high observed and expected heterozygosity. These loci will be used for paternity analysis and population genetic studies of the coevolution between *L. terrestris* and its parasites.

Introduction

Genetic loci containing simple-sequence repeats (*i.e.*, microsatellites) represent powerful markers in population genetics primarily because they evolve rapidly, are found throughout the nuclear genome, generally have several alleles per locus, and are typically inherited in a codominant fashion (Jarne and Lagoda 1996). Our primary use of microsatellites will be to study population structure and fertilization in a mate preference context in *Lumbricus terrestris*. Conducting such studies requires development of highly polymorphic molecular markers such as microsatellites. Although microsatellite markers have been developed for *Lumbricus rubellus* no cross species amplification was described for *Lumbricus terrestris* (Harper et al. 2006).To the best of our knowledge, the microsatellites, which we describe in this paper are the first for the species *Lumbricus terrestris.*

Lumbricus terrestris is an obligate outcrossing hermaphrodite and represents the largest earthworm in northern and Western Europe. Infection of *Lumbricus terrestris* by *Monocystis sp.* is a well studied host-parasite system (Field SG, Michiels NK 2005). Molecular markers are essential to understand the evolutionary dynamics of this relationship (Field et al. 2007) and to assess the importance of other processes such as mate choice and habitat fragmentation, historical processes (e.g. bottlenecks, range expansions), and direct and indirect selective forces that shape genetic variation in natural populations (Avise 1994, 1995).

Materials and methods:

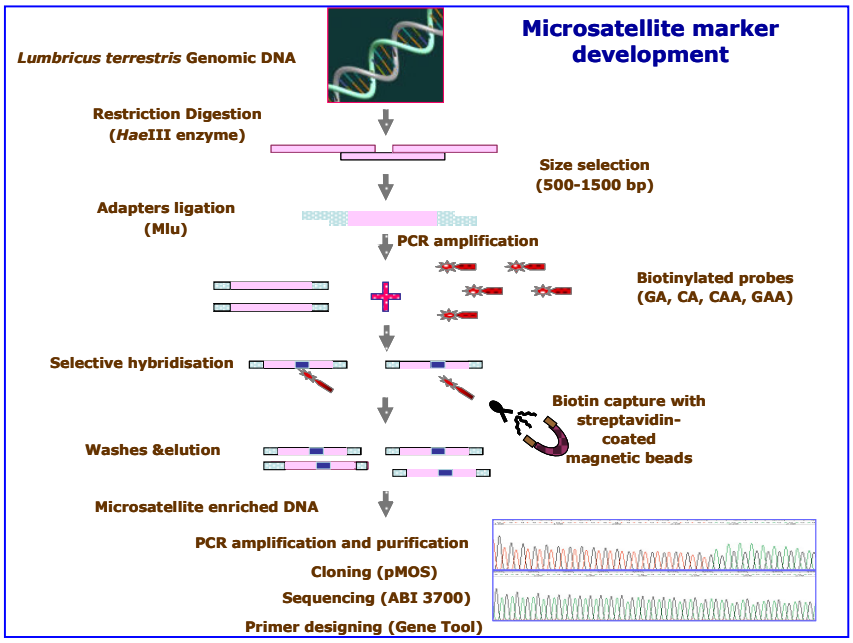
The individual used for developing microsatellites was collected at night from a sports field in Muenster (51° 58´ N, 7° 37´E), carefully cleaned from any soil debris and stored in ethanol at -20° C until DNA extraction. An SSR-enriched genomic library was constructed from this specimen following a modified protocol based on the methods of Bloor et al. (2001) and Edwards et al. (1996). Approximately 10 µg total genomic DNA was isolated from tissue using a modified CTAB method as described by Schulenburg et al. (2001). Briefly, the earthworm tissue was digested in CTAB buffer [2% (w/v) CTAB, 0.1 M Tris-HCl pH 8.0, 0.02 M EDTA, 1.4 M NaCl, 0.1% (v/v) β-mercaptoethanol], containing 5 µl proteinase-K (10 mg/ml), overnight at 50 ºC. DNA was extracted with two volumes of chloroform: isoamylalcohol (24:1) precipitated using isopropanol, washed with 70% ethanol, and resuspended in sterile H_2O. Extracted DNA was digested with *Hae*III restriction endonucleases (New England Biolabs) and fractionated on a 1.5 % agarose gel. Fragments of 0.5 - 1.5 kb were gel-eluted and ligated with *Mlu*I adaptors (Edwards et al. 1996). The adaptor-ligated SSR-rich DNA fragments were selected by hybridization to biotinylated oligonucleotides [$(GA)_{15}$, $(CA)_{15}$, $(AGA)_{10}$, $(CAA)_{10}$] and captured with streptavidin-conjugated magnetic beads (Dynabeads, DYNAL, Invitrogen). SSR-enriched DNA fragments were cloned into pMOS vector (Amersham Pharmacia) and transformed into competent *E. coli* DH5α cells, using electroporation. A total of 576 recombinant clones were recovered. Plasmids were isolated from 178 clones using QIAprep® Spin Miniprep Kit (Qiagen) and cloned inserts were amplified and sequenced for both strands using M13 universal primers and a commercial sequencing service (Macrogen Inc.). Eighty-eight sequences having SSR motifs were manually identified and primers were designed for twenty sequences that had >18 bp long repeat regions, using the program GENETOOL ver 1.0 (http://www.doubletwist.com) and synthesized with FAM, HEX and TET fluorescence labels at the 5' end (Invitrogen) (Table 1). Primers were labeled with reference to the *L. terrestris* microsatellite (LTM) locus and the orientation of the primer (forward "F" versus reverse "R"), e.g. "LTM 128 F" is the forward primer of locus LTM 128. Microsatellite PCR products can have additional bands at 1-base pair (bp) intervals from the nonadenylated allele, which can make it difficult to decide which peak to score (Smith et al. 1995). This problem

hampered reliable analysis of the five microsatellite loci LTM 128, LTM 163, LTM 193, LTM 026 and LTM 252. As a solution, we "PIG-tailed" the reverse primers for these loci by the addition of GTTTCTT to the 5' end of the primer, which is suggested to result in 100% adenylation of the 3' end of the synthesized strand (Brownstein et al. 1996) (Table 1). For the above loci, PIG-tailing permitted accurate and reproducible genotyping.

After optimization of PCR conditions, the utility of the different loci as genetic markers was tested on a panel of 32 individuals obtained from National Bait Inc. (www.nationalbait.com), which provides field-collected worms from a single location in Ontario, Canada. PCR amplifications were carried out in 20 µl reaction volumes with 5 ng of genomic DNA, 1x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl; Invitrogen), 2 mM of MgCl2, 2 mM of dNTPs, 2 pM of each primer and 1 U Taq DNA polymerase (Invitrogen) on a Master Cycler EP Gradient (Eppendorf). Thermal cycling parameters were: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C denaturation, 1 min at primer-specific annealing temperatures (Table 1), 1 min at 72 °C extension, followed by a final extension of 5 min at 72 °C. Amplified products were first checked on an 1.5 % agarose gel and then analysed on ABI 3130*xl* automated DNA sequencer (Applied Biosystems, USA), following manufacture's instructions. Resolved PCR-products were precisely sized using Genescan Rox 500 size standard and Genemapper 3.7 software (Applied Biosystems, USA) to calculate the number, range, and distribution of amplified microsatellite alleles. Population genetic parameters were calculated using ARLEQUIN version 2.0 (Schneider et al. 2000) and PIC (Polymorphism Information Content) using an online tool (http://www.agri.huji.ac.il/~weller/Hayim/parent/PIC.htm) (Aggarwal et al. 2004). Tests for linkage disequilibrium using Fischer's exact test were conducted in GENEPOP version 3.4 (Raymond and Rousset. 1995) with default Markov chain parameters.

Results and Discussion:

Ten microsatellite loci proved to be highly polymorphic and informative as to population genetic parameters. Analysis of linkage disequilibrium yielded two weakly significant cases (LTM 163 vs. LTM 165, $P = 0.0147$; LTM 165 vs. LTM 193, $P = 0.0255$) out of 45 pairwise comparisons.



None of the comparisons remained significant after Bonferroni correction (critical significance level of $P = 0.0011$), strongly indicating that the ten loci described are unlinked. Hardy-Weinberg equilibrium (tested using the Markov chain algorithm and Fischer's exact test in Arlequin version 2.0; Schneider et al. 2000) indicated no null allele for the loci LTM 128, LTM 163, LTM 165 and LTM 208, whereas the level of missing data for the locus LTM 193, LTM 026, LTM109, LTM 187, LTM 252 and LTM278 are 0.09, 0.09, 0.12, 0.18, 0.09 and 0.09 respectively. More details for these loci, i.e. locus designation, repeat motifs, primer sequences, allele attributes, PIC estimates and Genebank accession numbers, are summarized in Table.1.

In conclusion, the markers developed here have the degree of polymorphism and reliability that is required for earthworm paternity analysis and population genetics.

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Table 1: Details of the microsatellite markers developed in the study

Locus	Repeat motif	Primer sequence(5' - 3')	Tag[a]	Ta (°C)	Size range (bp)[b]	N	NG	NA	H_o	H_e	PIC	GenBank Accession
LTM 128	$(CA)_{11}(TCTG)_{20}$ †	F:CACGCTGTTGTTTCGCTCTTTGTT *R:CCGGGGACTGAGGAGAGAAAGACA	TET	60	192-268 (236)	32	20	14	0.84	0.88	0.86	AM182478
LTM 163	$(TGC)_{12}$	F:GCCGGAGCGTTAGGAGCGATAG *R:GGATACGCCCGACTCACCACTAA	FAM	60	138-216 (171)	32	19	12	0.69	0.86	0.84	AM182479
LTM 165	$(TCAC)_{15}$	F:TGACTGACACGCACCAACTAACTAACT R:TGGCTTAAGCTAGTGATTGAGTGAGTGA	FAM	60	152-212 (196)	32	15	14	0.94	0.91	0.89	AM182480
LTM 193	$(TGA)_{10}$	F:TCATTCCCCGACATCCAACAGA *R:TGCGTAAAGCCAATGAACCTGC	FAM	60	204-312 (285)	32	17	11	0.72	0.88	0.86	AM182481
LTM 026	$(GT)_{17}$	F:GTGCCTCTGTCTAATGTCTGCTCGTGTGTA *R:GCCGCTCTTTATACGCTCGTCGC	HEX	55	150-298 (208)	32	26	18	0.93	0.93	0.89	AM419426
LTM 109	$(GT)_{19}$	F:CGAACAAGATTACATACAACACAGGT R:TTGGAGTGTACAGAATATGGCATGCA	FAM	52	124-336 (124, 184)	32	18	15	0.50	0.91	0.90	AM419427
LTM 187	$(TC)_{17}$	F:CTTCGTTTTCTTAGCCTCAGCATATG R:CCGAATTGAAGACGTGCATCCA	FAM	60	190-388 (248)	32	12	11	0.50	0.82	0.79	AM419428
LTM 208	$(GA)_{23}$	F:AGGCAGGTAATCATTCAAGCAGAGAGAGA R:CGATTGTTTCTCCGTTTAGCGTTCTTAT	FAM	60	170-206 (180)	32	6	5	0.55	0.43	0.47	AM419429
LTM 252	$(CA)_{43}$	F:ACTCGTCAAAGGTACGCACTC *R:AGCAATGCAAAGTTGCAAACATACAC	FAM	52	156-368 (252)	32	17	15	0.48	0.94	0.92	AM419430
LTM 278	$(CA)_{40}$	F:TGGAATCTACAGAATATGGCATGC R:GCACCGAGCAATGGAAGTTT	FAM	52	188-298 (216, 298)	32	15	13	0.97	0.97	0.83	AM419431

[a]: Fluorescence label at 5´- end [b]: Figures in parenthesis are the most frequent allele(s) size **Ta**: Locus specific annealing temperature

N: Number of individuals analyzed *: Reverse primers pigtailed with GTTTCTT **NG**: Number of genotypes obtained per locus

H_o: Observed heterozygosity H_e: Expected heterozygosity **NA**: Number of alleles †: Microsatellites contain interruptions among repeats

Chapter III

High Genetic diversity and heterogeneous parasite load in earthworm *Lumbricus terrestris* on a German Meadow

Velavan TP, Suska Sahm, Hinrich Schulenburg and Nico Michiels

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Abstract:

In parasite-host dynamics, parasites exert frequency-dependent selection on their hosts by favouring rare alleles that may confer resistance against infection. Therefore host populations that suffer strong parasite stress should maintain higher levels of genetic variability. We studied the *Lumbricus terrestris – Monocystis sp*. host-parasite system at a microgeographical scale. Using three polymorphic microsatellite loci on one large earthworm population sampled at 26 different sites (281 genotypes), we tested the relationship between parasite load and genetic variation in natural samples of the common earthworm *Lumbricus terrestris*. Our analysis yielded the following: (1) parasite load varied significantly across sites in this population; (2) there was no consistent evidence for heterozygote deficiency (observed heterozygosities ranged between 0.74 to 0.87), indicating a low level of inbreeding; (3) there was no significant genetic structuring among sample sites; (4) we could not identify a significant association between parasite load and population genetic diversity; (5) there was considerable population differentiation (15.17%) between our German samples and a Canadian *L. terrestris* reference population. Our study provides insight into the population genetics of one of the most economically important soil organisms on a microgeographic scale.

Introduction:

Genetic variability allows populations to respond to selection exerted by a dynamic environment (biotic and abiotic). This includes selection by parasites on host populations. Natural host populations often exhibit significant genetic variability that allows them to coevolve with evolving parasites (Webster and Woolhouse 1999). Maintaining such diversity often results in trade-off between fitness costs and to that of resistance (May and Anderson 1983, Fritz and Simms 1992, Frank 1994, Webster and Woolhouse 1999, Schmid-Hempel 2003). For demographic reasons host populations harboring high parasite load is associated with reduced genetic diversity that effectively means lower host genetic diversity is associated with lower host fitness (see e.g., Coltman et al. 1999, Jarne and Théron 2001, Little 2002). In addition, parasites themselves possibly affect levels of genetic diversity by direct selection against non-resistant genotypes

(Field et al. 2007). Understanding the genetic diversity of host population diversity in relation to parasitism has significant impact for understanding the dynamics of host-parasite coevolution (Morand et al. 1996, Dybdahl and Lively, 1998, Webster and Woolhouse, 1998, 1999, Little 2002, Duncan and Little. 2007, Wilfert et al. 2007).

Several studies inferred that infections by parasites increases inbreeding depression, and that inbreeding leads to reduced resistance to parasites (e.g. Coltman et al. 1999, West et al. 1999, O'Brien 2000, Hedrick et al. 2001, Carr and Eubanks 2002, Keller and Waller 2002). Several studies also have concentrated on the implicit relationship between inbreeding and parasitism (Reid et al. 2003); however experimental evidence from natural populations is restricted. Numerous studies, particularly in vertebrates, have accounted for a positive relationship between inbreeding and infection (Coltman et al. 1999, Cassinello et al. 2001, Carr and Eubanks, 2002, Penn et al. 2002, Wiehn et al. 2002, Spielman et al. 2004); however various other studies, particularly in invertebrates, no significant effect was inferred (Stevens et al. 1997, Ouborg et al. 2000, Haag et al. 2003, Trouvé et al. 2003, Puurtinen et al. 2004). Recent studies have exposed the potential utility of molecular markers for understanding the amount and partitioning of genetic diversity within and between host populations (Simpson et al. 1993, Nadler 1995, Kautenburger 2006, Field et al. 2007).

Lumbricus terrestris – Monocystis sp, is a well studied host-parasite system (Field and Michiels 2005). Given the fact that *Lumbricus terrestris* lives a very sedentary life style with very low mobility (Butt and Nuutinen 2005) and lives in dense, but patchy populations, we anticipate genetic structuring across sampled sites at a microgeographical scale. As *L. terrestris* is likely to have a restricted choice of mating partners due to their strict association with a permanent burrow (Michiels et al. 2001), this may lead to high inbreeding and genetic differentiation. Genetic differentiation within a population may be further augmented by variation in parasite prevalence.

The population genetic structure of the host *Lumbricus terrestris* has been studied in a few cases. However, inferences about the selective forces acting on population structure have been limited for several reasons, including e.g. limited variability (RAPD and mtDNA markers) and limited number of populations sampled (Kautenburger 2006, Field et al. 2007). Contrary to other studies, we used microsatellites that are codominant single locus genetic markers which typically have many alleles per locus, high heterozygosity and they usually evolve neutrally (Bruford and Wayne 1993, Estoup et.al 1995, Jarne and Lagoda 1996). In this study, we analysed three highly variable microsatellite markers (Velavan et al. 2007) for *Lumbricus terrestris* individuals sampled on a meadow at 26 different sites. Our aim was to characterize the population genetic structure and genetic diversity of the host at a microgeographic scale and to relate this information to the level of parasitic infections by *Monocystis sp.*

Materials and methods

Study system

Lumbricus terrestris is an obligatory outcrossing hermaphroditic earthworm that is common in pastures, parks, agricultural fields and lawns. As an anecic species, *L. terrestris* is characterized by permanent vertical burrows, descending down to 2 m into the soil, relatively large body size and long lifespan (Edwards and Bohlen, 1996). Natural dispersal of *L. terrestris* is reported to be limited, i.e. approximately 4 m y-1 (Hoogerkamp et al. 1983). Mating occurs on the soil surface (Michiels et al. 2001). Since individuals remain anchored in their burrows, they have a restricted choice of mating partners. The seminal vesicles of *L. terrestris,* where self-sperm develop and are stored, are often strongly infected by the gregarine *Monocystis* sp. (Alveolata: Apicomplexa). Apicomplexa are mostly parasitic, with the gregarines exclusively infecting invertebrate hosts. Earthworms become infected by ingestion of sporocysts with soil. For details of the *Monocystis* life cycle, see Schmidt and Roberts (2000) and Bush et al. (2001). Recent studies have shown large variation in parasite concentration among individuals that is correlated with reduced growth (Field and Michiels 2005). Strong infections are known to result in destruction, resorption and

regeneration of the seminal vesicle, which effectively means that individuals are temporarily castrated (Breidenbach 2002).

Sampling

L. terrestris individuals were sampled at a microgeographical scale on a meadow next to the Biological Institute of the University of Tuebingen, Baden-Wuerttemberg, Germany (9° 00' E, 48° 30' N). The origin of a circular plastic hoop with a diameter of 84 cm (0.55 m²) was placed on a hand mowed site. An aqueous solution comprising of 170 g mustard powder per 10 litres of tap water (Gunn, 1992) was poured onto the hand mowed lawn to extract worms. Twenty litres of mustard solution were repeatedly poured onto the circumference of the hoop to extract worms. Each of these sites was resampled with the above mentioned procedure on the next subsequent day to sample as many worms as possible. All worms emerging within the same hoop were considered part of the same, arbitrary site. Twenty-six such sites approx. 1 to 2 m apart were sampled on this one large meadow. The number of worms per site varied from 7.27/m^2 to 54.54/m^2 individuals across each sites. Unfortunately, our landmarks of the sampling sites were lost when the lawn was mowed by gardeners making an analysis of distance effects impossible. Immediately after collection, worms were washed free of soil particles and mustard solution, dried on paper tissue and weighed ± 0.01 g. The individual was then sacrificed and cut anterior to the clitellum. The front part, which contains the seminal vesicles, was placed in an Eppendorf tube and stored in 100% Ethanol at -20°C until dissection. A tissue sample for DNA extraction was taken from the body wall of the tail end. DNA was isolated using DNeasy tissue kit (Qiagen). All worms were sampled within 5 weeks: In total 337 worms from 26 different sites were sampled.

Parasite concentration estimates

Samples were processed randomly. Seminal vesicles were dissected out, weighed and put into an equal volume (weight) of earthworm ringer solution (i.e. 1:2 dilution) (Ringer solution: 25mM NaCl, 4mM KCl, 6mM $CaCl_2$, 1mM $MgCl_2$, 26mM Na_2SO_4, 2mM Tris and 55mM Sucrose) and

homogenized using a sonicator (Bandelin sonopuls, Model UW 2070), further diluted (final = 1:10) and counted using a haemocytometer (Hecht-Assistent, ThomaNeu model, Sondheim, Germany) (Field et al. 2003). Absolute parasite load was calculated as the number of sporocysts/µl multiplied by their seminal vesicle weight in mg (assuming a density of 1 mg/µl). Parasite loads were determined for worms collected across all 26 sites.

Microsatellite analysis

The three selected loci LTM 128, LTM 163 and LTM 208 (Table 1) showed at least 10 uninterrupted di- and trinucleotide repeats each and were therefore considered to exhibit sufficient potential polymorphism for population genetic analyses. PCR conditions, electrophoresis details and methods for scoring amplification products followed those described by Velavan et al. (2007).

Table 1: Details of microsatellite marker used for this study

Locus	Repeat motif	Primer sequence (5' - 3')	Taga	nA	Ho	He	Size range (bp)	GenBank Accession
LTM 128	(CA)11 (TCTG)20	F : CACGCTGTTGTTTCGCTCTTTGTT R : CCGGGGACTGAGGAGAGAAAGACA	TET	19	0.74	0.79	146-236	AM182478
LTM 163	(TGC)12	F : GCCGGAGCGTTAGGAGCGATAG R : GGATACGCCCGACTCACCACTAA	FAM	14	0.75	0.81	132-183	AM182479
LTM 208	(GA)23	F : AGGCAGGTAATCATTCAAGCAGAGAGAGA R : CGATTGTTTCTCCGTTTAGCGTTCTTAT	FAM	18	0.87	0.85	152-212	AM419429

[a]: fluorescence label at 5´ end of primer, nA: number of alleles, H_o Observed heterozygosity and H_e expected heterozygosity

Genetic analysis

Genetic diversity measures were calculated for each locus with Arlequin version 2.0 (Schneider et al. 2000), including the number of alleles per locus (*NA*) and the observed (H_O) and the expected heterozygosity (H_e). Tests for deviations from Hardy–Weinberg equilibrium (HWE) were

performed for each locus–site combination using an exact test where the *P*-values were estimated without bias using a Markov chain method following the algorithm of Guo and Thompson (1992). Genotypic linkage disequilibrium was evaluated with GENEPOP 3.1 for each pair of loci in each site and significance was determined through a log-likelihood based exact test (Goudet et al. 1996). For all Markov chain tests, default parameters in GENEPOP for dememorization number, batches and iterations were invoked. Assessments of genetic parameters were made with the computer program FSTAT version 2.9.3 (Goudet 2001). Genetic variability within different sites was estimated as mean allelic richness (*A*; a measure of allele number independent of sample size; see Petit et al. 1998), and mean expected heterozygosity (H_e) at the three microsatellite loci. The degree of nonrandom mating within sampling sites (F_{IS}), and pairwise differentiation among sites (F_{ST}), were estimated with Weir and Cockerham (1984) estimators of *F*-statistics. Genetic population structure of the host population was investigated with a hierarchical analysis of molecular variance (AMOVA) as described in Michalakis and Excoffier (1996), which estimates how genetic diversity is partitioned among sampling sites, among individuals within sites and within individuals. The significance of the variance components associated with the different levels of genetic structure was tested using nonparametric permutation procedures as implemented in Arlequin 2.000 (Schneider et al. 2000). Using AMOVA, we examined population differentiation with our dataset to a population (n=32) of Canadian origin to ensure that the microsatellite markers used in this study are useful in distinguishing different earthworm populations. We also examined the relationship of pairwise differences in parasite load and genetic distances between 26 sites (as measured by F_{ST} values) with a Mantel test using the Arlequin (v2.0) software.

Statistical analyses

Data are reported as means ± SD. Parasite load values were transformed with natural log to achieve normally distributed data. Since different sites were sampled over a 5 week period, we had to correct for seasonal effects before comparing variation in body weight as well as parasite load between sites. Therefore, for each of these two traits, we first performed a polynomial

regression analysis on sampling date (quadratic for body weight $R^2 = 0.0834$, $F_{4,273} = 12.60$, $P < 0.001$, quadric for parasite load $R^2 = 0.1983$, $F_{4,273} = 16.88$, $P < 0.001$). The residuals of these regressions were then compared between sites using Kruskal Wallis ANOVA. Genetic parameters such as inbreeding coefficient (F_{IS}) mean expected heterozygosity (H_e) and mean allelic richness (A) were correlated with mean absolute parasite load of each site by Pearson correlation coefficient (r). Statistical analyses were carried out using JMP® v5.1.

Results

Population genetic parameters

We observed considerable variation at the three microsatellite loci studied (Table1). The total number of detected alleles per locus across all sites ranged from 14 to 19 (Table1). Overall observed heterozygosities across three different loci ranged from 74% to 87% (Table 1). The degree of nonrandom mating (F_{IS}), average allelic richness (A), and mean expected heterozygosity (H_e) are summarized in Table 2. Tests for conformity to HWE indicated no consistent heterozygote deficiency after correction for multiple testing using the false-discovery rate. Tests for genotypic linkage disequilibrium yielded no significant cases out of 78 pairwise comparisons strongly indicating that the three loci are unlinked. The estimates of within population inbreeding (F_{IS}; Table 2) indicated low inbreeding. The AMOVA analysis indicated no significant variation among sampled sites, with less than 0.41% of the microsatellites variation being explained by differences between sites (Table 3). We found significant population differentiation between our host populations and a reference population of Canadian origin (AMOVA, $F_{ST} = 0.16$, $P = 0.04$, 15.17% variation explained).

Table 2: Genetic and fitness parameters estimates across parasite load.

Parasite load	Genetic Parameters			Fitness	Sampling	
Mean Absolute parasite number ± SD (X10^6)	*A*	H_e	F_{IS}	Mean body weight ± SD in g	Site Nr.	n
2.06 ± 1.74	4.78	0.81	-0.064	2.61 ± 0.93	N17	7
2.54 ± 3.16	4.50	0.81	0.118	3.65 ± 0.72	N04	15
3.92 ± 3.09	4.50	0.78	-0.118	1.67 ± 0.61	N20	5
4.26 ± 5.29	4.59	0.80	0.023	2.75 ± 0.67	N14	9
4.30 ± 3.54	4.66	0.83	0.009	2.67 ± 0.82	N16	11
4.33 ± 3.54	4.62	0.81	-0.059	3.00 ± 0.84	N08	12
4.64 ± 6.85	4.49	0.80	0.091	3.36 ± 0.76	N05	17
5.74 ± 5.30	4.55	0.79	-0.134	3.27 ± 0.24	N18	13
5.93 ± 3.97	4.63	0.79	-0.039	2.83 ± 0.70	N07	13
7.02 ± 7.86	4.46	0.80	0.021	3.10 ± 0.76	N06	11
7.11 ± 8.22	4.79	0.83	0.08	2.49 ± 0.84	N15	10
7.81 ± 6.90	4.82	0.84	0.111	2.64 ± 0.80	N11	12
8.81 ± 9.99	4.52	0.79	-0.051	2.71 ± 0.67	N19	14
9.09 ± 4.78	4.69	0.83	0.116	2.45 ± 0.72	N21	10
9.62 ± 3.18	5.33	0.86	-0.065	2.63 ± 0.19	N23	4
10.6 ± 10.0	3.99	0.78	-0.032	2.17 ± 0.44	N22	5
11.1 ± 8.80	5.06	0.84	0.079	2.62 ± 0.43	N24	6
12.0 ± 13.1	4.79	0.83	0.001	2.96 ± 0.78	N29	16
14.1 ± 9.75	4.91	0.82	0.104	3.45 ± 0.73	N09	20
14.5 ± 9.75	4.77	0.82	0.035	3.28 ± 0.72	N10	11
14.5 ± 11.5	4.55	0.79	0.038	2.92 ± 0.81	N27	11
14.6± 12.2	4.82	0.85	-0.009	2.91 ± 0.67	N12	7
15.8 ± 9.56	4.62	0.85	0.059	2.47 ± 0.58	N25	5
15.9 ± 17.9	4.36	0.78	0.078	3.65 ± 0.93	N28	13
17.4 ± 17.5	4.87	0.85	0.000	2.94 ± 0.70	N30	13
21.1 ± 16.7	4.29	0.77	0.053	2.80 ± 0.54	N26	11
9.61 ± 10.6	4.70	0.81	0.024	2.96 ± 0.80	Overall	281

n, number of genotyped individuals; A, average allelic richness; H_e, expected heterozygosity; F_{IS}, within-population inbreeding coefficient (P = 0.0006).

Table 3: AMOVA comparing genetic variation in microsatellite data

Source of variation	d.f.	Sum of squares	Variance components	Fixation indices	*P* value	Percentage of variation
Among sites	25	33.95	0.00506	0.00414	1.0000	0.41
Among individuals within sites	255	318.60	0.03219	0.02645	0.04203	2.63
Within individuals	281	333.00	1.8505	0.03048	0.02835	96.95

Genetic variability and fitness measures

Individual earthworms varied considerably in weight from 0.95 g to 5.43 g (mean = 2.96 ± 0.80; n = 281). After correcting for sampling date, worm mass varied significantly among collected sites (Table 2; Kruskall–Wallis, χ^2 = 51.42, df =25, $P < 0.0014$). Among the 26 sites the overall *Monocystis sp.* infection prevalence was 99.3%. All sites harbored infected individuals, but parasite load varied greatly ranging from 0 to 6.94 x 10^7 (mean = 9.61X10^6 ± 1.06 X10^7; n = 281). Overall, parasite load showed significant variation among sites after correcting for seasonal effects during sampling period (Table 2; Kruskall–Wallis, χ^2 = 55.0, df = 25, $P < 0.0005$). Average earthworm weight was unrelated to average parasite load per sampled site (r = 0.091, df = 26, P = 0.66). All correlations between measures of genetic variability such as inbreeding coefficient (F_{IS}), average allelic richness (A), mean expected heterozygosity (H_e) with absolute parasite load were similarly insignificant ($r \leq 0.26$, df = 26, $P \geq 0.19$) (Table 4). The pairwise genetic distances between sampled sites (F_{ST}) ranged from 0.00 to 0.08. The Mantel test did not indicate a significant correlation between these pairwise measures of genetic distance (F_{ST}) and the corresponding pairwise differences in parasite load (r = 0.02, P = 0.44).

Table 4: Pearson correlation between measures of genetic variability, mean body mass and estimates of parasite load

Measures	r	df	*P*
Inbreeding coeffficient (FIS)	**0.2641**	**26**	**0.1924**
Average allelic richness (*A*)	**0.0663**	**26**	**0.7477**
Mean expected heterozygosity (He)	**0.1797**	**26**	**0.3798**
Mean body mass	**0.0912**	**26**	**0.6576**

Discussion

The original aim of the study was to consider different sampling sites as subpopulations. As *L.terrestris* has a low dispersal rate and restricted choice of mating partners, we expected to find a clear genetic structuring across different sampled sites at a microgeographical scale. However host population did not show clear genetic structuring as most of the total variance was present between individuals (rather than sampling sites). The high genetic diversity of the earthworms and the lack of genetic structuring strongly suggest that we are dealing with one large mating population rather than subpopulations. One possible explanation is that earthworms show higher dispersal and out-breeding between individuals than originally expected. Alternatively, it may be possible that earthworms from distant areas were introduced with parent soil to the university campus and subsequently spread to neighboring meadows. Future investigation of earthworm populations in older or less disturbed habitats may reveal the relevance of this factor. Another alternative explanation may be lack of sufficient resolution provided the used microsatellite markers. This is, however, unlikely, because we were able to infer substantial population differentiation of 15.17% variation between our host populations to animals of Canadian origin.

In contrast to genetic differences, earthworms varied significantly in parasite load between sampling sites suggesting differences in parasite prevalence or host resistance between

individuals from this meadow. Parasite-mediated selection may thus vary within the population. We expected that this variation associates with differences in host genetic diversity and/or genetic differentiation (see introduction). Microsatellites themselves are selectively neutral, yet they could be used as an indicator for heterozygosity although they may be linked to each other, so we expected an association due to (i) a general consequence whereby heterozygosity is related to an individual's inbreeding coefficient and consequently to heterozygosity throughout the genome, including at resistance loci, or (ii) a confined effect that the markers studied are in linkage disequilibrium with resistance loci, i.e. microsatellite heterozygosity and diversity are apparently associated with heterozygosity and diversity at functional loci that affect an individual's response to parasites. However, our analysis did not yield any indication for such an association, neither between parasite load and different measures of genetic diversity (inbreeding coefficient, F_{IS}, average allelic richness, A, and mean expected heterozygosity, H_e), nor between parasite load and genetic structuring. .

Two explanations may account for the absence of the expected effect: (i) Our initial assumption of a direct influence of this castrating parasite on host genetic differentiation is simple but not realistic. *L. terrestris* is host to a diverse array of parasites (Edwards and Bohlen 1996). Therefore, its population genetics may be influenced by a complex interaction network with different parasites. (ii) Individuals within this one large mating population are not really genetically separated. Moderate or little bit of genetic exchange may then prevent genetic manifestation of parasite-mediated selective differences between them. The latter alternative seems particularly likely considering that we could not infer significant genetic structuring. As such, the current results are consistent with our previous findings of absence of a relationship between parasite defense and mtDNA genetic diversity in a fragmented urban metapopulation of earthworms (Field et al. 2007). A prerequisite for finding such a relationship between parasite load and genetic variability is that the parasite infection should remain stable over seasonal variations. It was not possible to validate how stable these infections are seasons and time in general. In this study, we have corrected for the sampling date on individuals parasite load. Therefore we acknowledge that

sampling date had significant fluctuations on individuals parasite load. The probable means to offer facts on the stability of parasite infection is by sampling individuals over seasons. However a destructive sampling effect (sacrificing worms to obtain parasite load) still pose a hurdle to conclude the temporal or seasonal variation on infection rate. Further assessment in earthworm populations of different origins (such as Canadian population) may help understanding the importance of parasite-mediated selection on host populations.

Acknowledgments

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Chapter IV

Reconstruction of mating history – a retrospective analysis of *Lumbricus terrestris* mate choice criteria in natural populations

Suska Sahm, Velavan TP, Hinrich Schulenburg and Nico K Michiels

Unpublished Manuscript

Keywords

Mate-choice

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Monocystis sp.

Parasites

Microsatellites

Abstract

The theory of parasite-mediated sexual selection predicts mate choice in the presence of parasites. Parasite-resistant individuals should be preferred because they promise high-quality offspring. Other mate choice factors can be body size due to size-related fecundity or the distance to the partner. Most mate choice theories were developed for gonochorists with the resource-limited gender as the chooser, but situations can be much more complicated in hermaphrodites. The earthworm *Lumbricus terrestris* L. is an obligatory outcrossing simultaneous hermaphrodite. It is highly parasitized by the protozoan *Monocystis* sp. (*Apicomplexa*, *Eugregarinorida*) which lives in the seminal vesicles and is able to castrate the earthworm. To investigate the criteria *L. terrestris* L. uses for mate choice, a retrospective neighbourhood design was applied and we determine the most likely partner of a central focal individual using established microsatellites.

Introduction

Parasite-mediated sexual selection (Hamilton and Zuk 1982) predicts the evolution and maintenance of active mate choice in presence of parasites. However, common mate choice theories highlight that in a separate sex species, gender plays a vital role in choosing a partner due to its limited availability of resources that it can allocate for reproduction. In contrast to such separate sex species, mate choice scenarios can look extremely different in simultaneous obligatory outcrossing hermaphrodites, because they combine male and female interests in one individual. The ability to allocate resources flexibly to male and female functions as well as arising conflicts over sexual roles complicate sexual strategies enormously (Michiels and Newman 1998). Although sexual selection on traits of one gender is always coupled with traits of the other gender, it could be shown that hermaphrodites have all necessary features for sexual selection (Morgan 1994).

Considering risk factors posed by earthworms the major risk factor they pose is being pulled out of its own burrow. Earthworm mating takes place on the surface, with the animals

caudal end anchored in the burrow while the frontier ends are attached in a typically S-shaped position with the mating partner. During mating, special copulatory setae or bristles are pierced into the partners body to enhance further bonding between the pairs (Koene et al. 2005). Retraction into the burrow is thus much slower in mating pairs than in single individuals. At the end of a mating session, the pair has to detach by force, often resulting in one (usually the smaller) of them becoming pulled out of its burrow. As copulations usually start at dawn and last about 2-6 hours, mating pairs and individuals stranded on the surface are conspicuous for predators, e.g. birds (Michiels et al. 2001). Therefore, mating over long distances or with bigger individuals poses a serious risk to the worm. Partners that are far away should therefore promise high quality or quantity offspring to outweigh the risk of predation.

Considering mate choice aspects in earthworms, *Lumbricus terrestris* exhibits extensive precopulatory reciprocal burrow visits that is interpreted as a kind of mate assessment or courtship behaviour (Nuutinen and Butt 1997). In theory, mate choice should include the following factors: first, the partner should be big enough to provide female fecundity as cocoon production is strongly associated with body size. Second, the pull-out risk should be decreased by either choosing a partner close to its own burrow or by choosing a smaller partner. Obviously, the first and the latter are contradictory to each other. When both factors are balanced, the expectation would be that earthworms choose partners that are as similar in size as possible, leading to size - assortative mating. In laboratory experiments, pairs of same-sized individuals paired earlier than pairs of differently-sized individuals. (Michiels et al. 2001) Parasite concentration provides another mate choice factor. It is known that skin colour correlates positively with parasite concentration. Although *L. terrestris* possesses light receptors in its head region, it needs to be proven to what degree it is a useful cue to determine the partners parasite load (Field et al. 2003). Taking into account fitness costs posed by parasites, a direct fitness cost due to *Monocystis* infection levels could not be detected in earthworms. However Field and Michiels 2005 could show that strong *Monocystis* infections have a negative effect on *L. terrestris* growth, that could lead to fitness costs as fecundity is often positively associated to body size. Also previous studies

showed that cocoon production is strongly linked to size (Field et al. 2003). In addition, it is also shown in other hermaphrodites that mating success and mate choice depend on body size (Angeloni 2003).

So far, lab experiments that confirm different aspects of mate choice in hermaphroditic invertebrates are still rare, but promising results could be shown in snails (Haase and Karlsson 2004; Webster and Gower 2006), nematodes (Kleemann and Basolo 2007) and flatworms. Nevertheless, those experiments can only get a glimpse of what happens in natural populations. As known from other studies (Sauter & Braun 2001), the mating history of a certain individual influences the choice made in meticulous situations. Especially for long-living, sessile or territorial animals, the mating history depends on their surrounding neighbors which have a high impact on their decisions for future reproductive strategies. In invertebrates that are capable to store sperm, we have the extraordinary possibility to identify possible sperm donors from a surrounding community by using neutral markers such as microsatellites. For sessile or semi-sessile species one can scan for a complete set of potential partners, thus giving an insight into a period of recent mating events. In this study, we genotyped the stored allosperm in their spermathecae and tissue using 3 different microsatellites (LTM128, 163 and 208) to reconstruct their recent mating history and tested if *L. terrestris* uses parasite concentration, body or vesicle weight and spatial distance as criteria to choose its partner.

Material and Methods:

Sampling

Earthworms were sampled on a lawn of the Morgenstelle Campus of the Eberhard Karls University Tuebingen, Germany (GPS Data: 48°32'13.40''N, 9°02'12.87''O, 459 m height over German reference surface) . An aqueous solution comprising of 170g mustard powder per 10 litres of tap water (Gunn, 1992) was poured onto the hand mowed lawn that refuge worm casts. The earliest emerging sexually mature worm with well differentiated clitellum was considered as the focal individual. The origin of a circular plastic hoop (r = 42 cm) was centred at the focal

individual's burrow that was marked with a numbered flag. 20 litres of mustard solution were repeatedly poured onto the circumference of the hoop to extort as many sexually mature worms as possible. These were considered potential mating partners of the focal individual. All the extorted worm burrows were marked with numbered flags. The distances between the extorted worms were measured. Two nails A and B with an inter distance of one meter apart were fixed to the ground outside the circular hoop. The positions of all burrows that were marked with numbered flags were determined by measuring the distance to A and B, in addition to their distance to the focal individuals burrow. These measurements were used to create maps of each neighborhood by Pythagorean calculations (Fig 1). The number of worms per neighborhoods varied from 7.27/m^2 to 54.54/m^2 individuals. Immediately after collection, worms were washed free of soil particles and mustard solution, dried on paper tissue and weighed ± 0.01 g. The individual was then sacrificed and cut anterior till the clitellum. The front part, which contains the seminal vesicles, was placed in a falcon tube and stored in 100% Ethanol at -20°C until dissection. All worms were sampled within 5 weeks: In total 337 worms from 28 different neighborhoods were sampled.

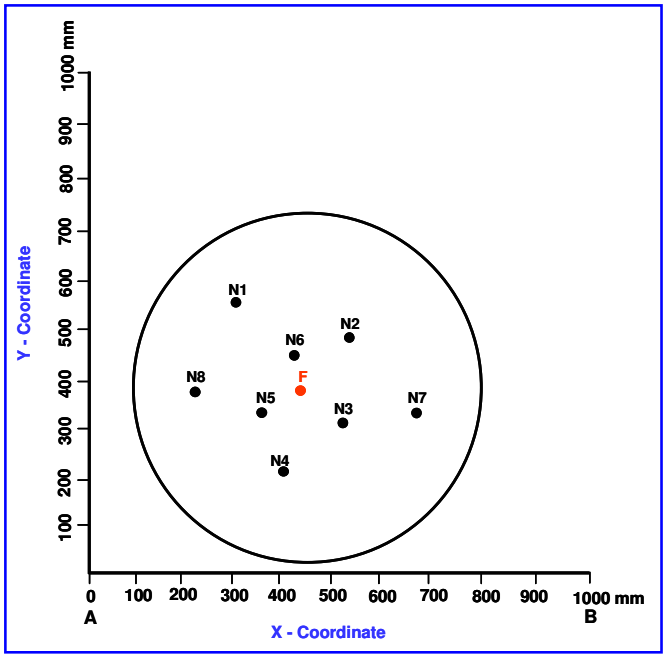

Fig. 1: A neighborhood map.

Earthworm dissection

The two pair of spermathecae (Receptaculae seminis) identified laterally on the ventral side as light orange to yellowish, pinhead like structures on the ninth and tenth segment were removed and pooled. To the pooled spermathecae, 200 µl of DNA extraction buffer (Qiagen) was added. The 3 pairs of seminal vesicles in the region right behind from segment nine to 13 were dissected out and weighed ± 0.01 g. Ringer solution (1mg= 1µl) was then added to these seminal vesicles. A 25 mm^2 part of tail tip body wall was cut and stored in a 1.5 ml Eppendorf tube with 200 µl DNA extraction buffer (Qiagen). Spermathecae, seminal vesicles and tissue samples were then stored at -80°C till DNA extraction.

Parasite concentration estimates

Samples were processed randomly. Seminal vesicles were dissected out, weighed and put into an equal volume (weight) of earthworm ringer solution (i.e. 1:2 dilution) (Ringer solution: 25mM NaCl, 4mM KCl, 6mM $CaCl_2$, 1mM $MgCl_2$, 26mM Na_2SO_4, 2mM Tris and 55mM Sucrose) and homogenized using a sonicator (Bandelin sonopuls, Model UW 2070), further diluted (final = 1:10) and counted using a haemocytometer (Hecht-Assistent, ThomaNeu model, Sondheim, Germany) (Field et al. 2003). Parasite concentration was calculated as the number of sporocysts/µl. Parasite concentrations were determined for all sampled neighborhoods. *Monocystis* spores can easily be recognized by their fusiform shape (Fig. 2). For more details on protocol see (Field et al. 2003).

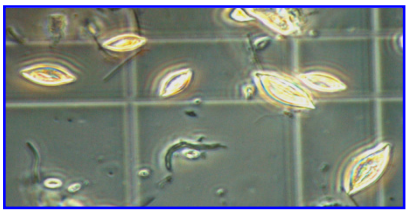

Fig. 2: *Monocystis* sporocysts visible under a light microscope in a Thoma Chamber.

Microsatellite amplification

Tissue and spermathecae DNA were isolated using the procedure as described in DNeasy tissue kit (Qiagen). Isolated DNA samples were stored at 8°C. DNA from the earthworm tissue and the

spermathecae (all four pooled) of each respective individual were amplified using three selected loci LTM 128, LTM 163 and LTM 208 (Table 1) considered to exhibit sufficient potential polymorphism. PCR conditions, electrophoresis details and methods for scoring amplification products followed those described by Velavan *et al.* (2007).

Table 1: Details of microsatellite marker used for this study

Locus	Repeat motif	Primer sequence (5' - 3')	Taga	nA	Ho	He	Size range (bp)	GenBank Accession
LTM 128	(CA)11 (TCTG)20	F : CACGCTGTTGTTTCGCTCTTTGTT R : CCGGGGACTGAGGAGAGAAAGACA	TET	19	0.74	0.79	146-236	AM182478
LTM 163	(TGC)12	F : GCCGGAGCGTTAGGAGCGATAG R : GGATACGCCCGACTCACCACTAA	FAM	14	0.75	0.81	132-183	AM182479
LTM 208	(GA)23	F : AGGCAGGTAATCATTCAAGCAGAGAGAGA R : CGATTGTTTCTCCGTTTAGCGTTCTTAT	FAM	18	0.87	0.85	152-212	AM419429

[a]: fluorescence label at 5´ end of primer, nA: number of alleles, H_o Observed heterozygosity and H_e expected heterozygosity

Calculation of mating likelihood

Alleles are not structured across different neighborhoods as inferred from the population genetic data analysis by AMOVA with Arlequin ver2.0 (0.4% variation across all neighborhoods). Hence, allele frequencies generated from the whole set of individuals were considered to calculate mating likelihoods. We then calculated the most likely donor to the focal (Table 2). Allele frequencies across all neighborhoods were calculated for both tissue alleles A_{Ti} and B_{Ti}. Presence or absence of these alleles across spermathecae of the focal individual is checked. Then donor probability of this allele P (A_{Ti}) is calculated by P (A_{Ti}) = [1 – Freq A_{Ti}] as to increase weightage of rare alleles. This is done for both alleles that are present in the spermathecae of the focal. The mean of the donor probabilities for both alleles yield the likelihood that an individual is a sperm donor (P (N→F) = [P (A_{Ti}) + P (B_{Ti})] / 2).

Table 2: Calculations for the most likely donor.

Allele in Spermathecae of Focal 1_F: 180					Allele in Spermathecae of Focal 2_F: 204				
ID	Tissue Allele (A_{Ti}) in neigbhor	Frequency of Tissue allele	Allele Presence (1) or absence (0) in Focal Spermathecae	Probability of allele A_{Ti}	Tissue Allele (B_{Ti}) in neigbhor	Frequency of Tissue allele	Allele Presence (1) or absence (0) in Focal Spermathecae	Probability of allele B_{Ti}	Overall probability (N to focal)
402	180	0.188	1	0.812	188	0.219	0	0	0.406
403	178	0.196	0	0	204	0.110	1	0.89	0.445
404	188	0.219	0	0	198	0.079	0	0	0

To determine the most likely receiver (Table 3), alleles that are present in the neighbors spermathecae were listed (allele 1_N, 2_N, 3_N). The focal is only able to donate sperm that contains its own alleles; therefore the neighbor spermathecae alleles were compared to the alleles that are present in the focals tissue (Focal Tissue Allele A_{FTi}/ B_{FTi}). The probability that an individual received the Allele A_{Ti} is then calculated by P (A_{FTi}) = [1-Freq A_{FTi}]. The mean of both receiver probabilities P (A_{FTi}) and P (B_{FTi}) gives the probability that an individual is a sperm receiver (P (F→N) = [P (A_{FTi}) + P (B_{FTi})] / 2). We count the allele for a homozygous individual, in this example the focal, only once. Here, P (B_{FTi}) is therefore always 0.

Table 3: Calculation for the most likely receiver

Allele in tissue of Focal A_{FTi}: 188					Allele in Spermathecae of Focal B_{FTi}: 188			
ID	Allele (1_N) in neigbhor spermathecae	Allele (2_N) in neigbhor spermathecae	Allele (3_N) in neigbhor spermathecae	Allele Presence (1) or absence (0) in Focal tissue	Probability of allele A	Allele Presence (1) or absence (0) in Focal tissue	Probability of allele B	Overall probability (Focal to neigbhor)
402	180	188	206	1	0.781	0	0	0.39
403	180	206		0	0	0	0	0
404	178	202	206	0	0	0	0	0

Finally, the overall mating probability is calculated by taking the mean of an individual donor and receiver probability (MP = [P (N→F) + P (F→N)] / 2).

Data Analysis

Neighborhoods 2 and 3 were excluded from the whole analysis due to small sample size. Values for vesicle weight and parasite concentration of neighborhoods 13, 31 and 32 had to be excluded due to scale problems. Body weight and vesicle weight were normally distributed, whereas parasite concentration was log-transformed to obtain normally distributed data. All following

statistics were carried out with JMP 2.0. Data were checked for time effects. No time effect could be detected for body weight (*nested ANOVA, d.f. = 20, F = 0.72, p = 0.80*), vesicle weight (*nested ANOVA, d.f. = 19, F = 1.10, p = 0.36*) and log parasite concentration (*nested ANOVA, d.f. = 19, F = 1.11, p = 0.34*). Neighborhoods differ significantly in body weight. *(Kruskal-Wallis Test, χ^2 = 83.3, d.f. = 28, p < 0.001)* The overall mean is 2907 mg in 337 individuals. The smallest individual weighed 950 mg, the biggest individual had 5430 mg. Neighborhoods also differ significantly in vesicle weight. *(Kruskal-Wallis Test, χ^2 = 134.16, d.f, = 26, p < 0.001)* The overall mean is 395 mg in 314 individuals. Vesicle weights range from 14 mg to 1038 mg. Neighborhoods differ significantly in log parasite concentration (spores / µl) *(Kruskal-Wallis Test, χ^2 = 67.3, d.f. = 25, p < 0.001)*. The overall mean is 20 769 spores / µl (log value 9.94) in 297 individuals. Parasite vesicle concentrations vary from 0 spores / µl in two individuals to 21 1935 spores / µl. However, it is very unlikely that the two individuals with a *Monocystis* count of 0 spores/µl had no parasites at all. It is more plausible that they had few parasites that were not detected in the small amount that is counted in a Thoma chamber.

Results

Distribution of probable mating partners

Individuals with a distance to focal > 300 mm were excluded because mating is very unlikely over larger distances. Ref Fig. 3 shows a distribution of the numbers of individuals the focal could choose. They range from 2 to 10 individuals, but most of the focals had 4 neighbors around them.

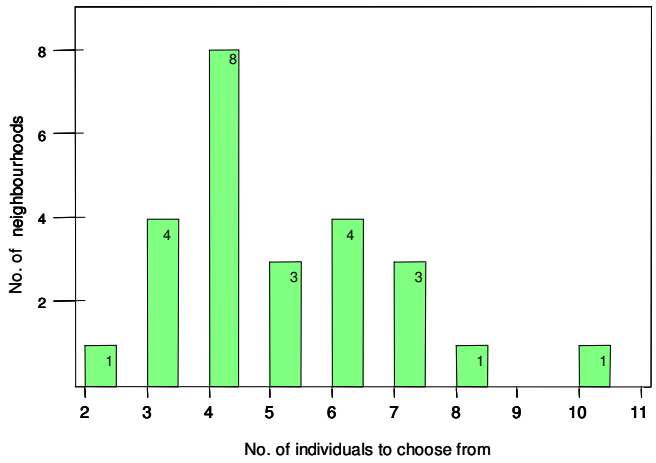

Fig. 3: Distribution of probable mating partners after exclusion (distance to focal > 300 mm).

Choice Patterns

To detect any choice patterns, pairwise comparisons of the most likely partner with the focal, the second most likely partner and the median of all accessible individuals in a neighborhood were carried out regarding body parameters, parasite concentration in vesicles and eventually distance to focal. The results are summarized in Table 4. Only the distance to the focal had a significant difference both to the second most likely partner and to the median of the accessible individuals in the 300mm range. Paired distributions of distance to the focal are shown in Fig. 4.

Table 4: Pairwise comparisons of most likely partner

paired t-test most likely partner / fitness-distance parameters	Focal			2nd Most likely partner			Median (Neigbhourhood)		
	d.f	t	*P*	d.f	t	*P*	d.f	t	*P*
Body weight	24	-0.97	0.34	24	-0.37	0.72	24	-0.65	0.52
Vesicle weight	22	0.08	0.94	22	-1.45	0.16	22	-1.85	0.08
Log parasite Conc.	22	1.14	0.26	22	-0.11	0.91	22	0.48	0.63
Distance to Focal	….	….	….	24	2.73	0.01	24	6.02	>0.001

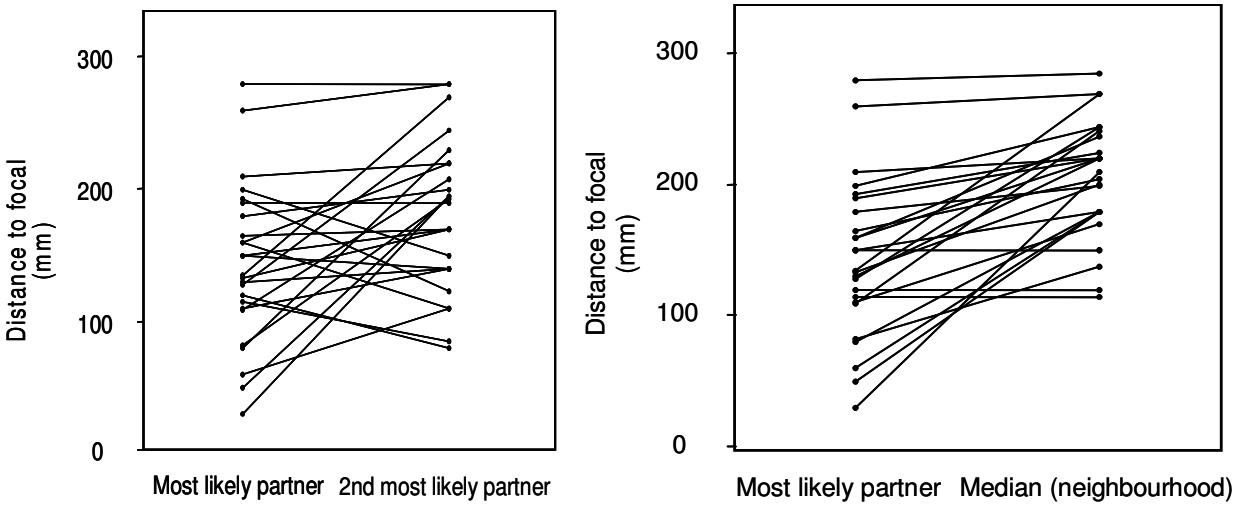

Fig. 4: Pairwise comparison of most likely partner and 2nd most likely partner and Pairwise comparison of most likely partner and median neighborhood.

Assortative Mating

To test for assortative mating, body weight, vesicle weight and parasite concentration of the focal individuals were correlated with the values for the most likely partner. The results are shown in Fig. 5, 6 and 7 respectively. None of these correlations remained significant.

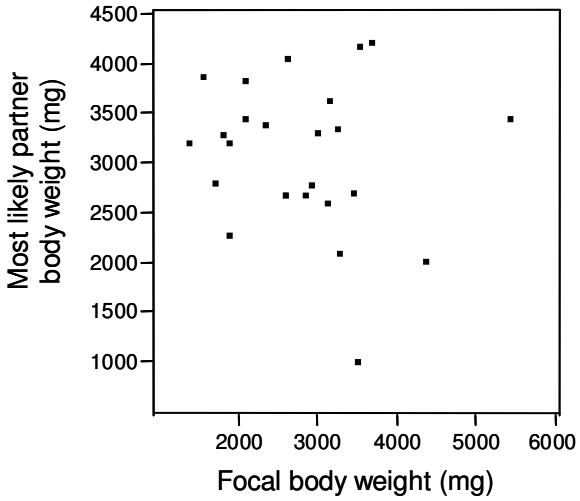

Fig. 5: Pearson correlation – body weight: *n = 25, r = -0.14, p = 0.51.*

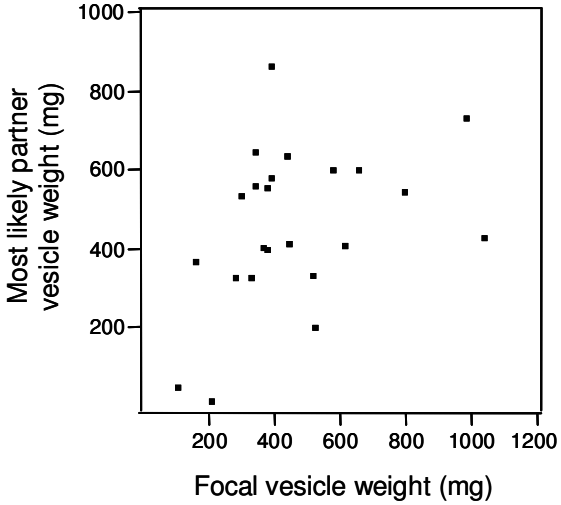

Fig. 6: Pearson correlation – vesicle weight: *n = 23, r = 0.39, p = 0.07.*

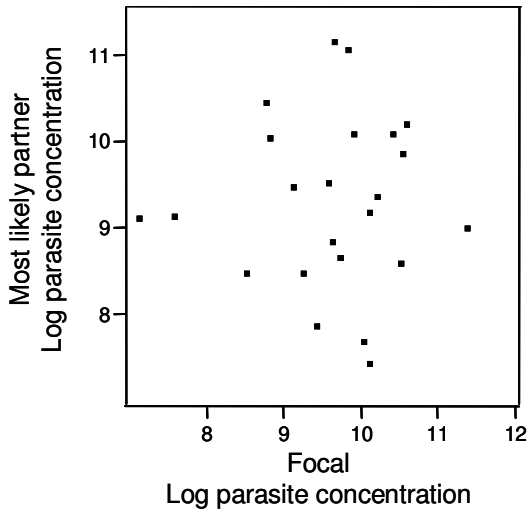

Fig. 7: Pearson correlation- Log parasite concentration: *n = 23, r = 0.02, p = 0.9.*

Mating Probabilities

Mating probabilities ranged from 16.4% to 66.7%. The mating probabilities for the most likely partners ranged from 37.4% to 66.7% (Fig.8)

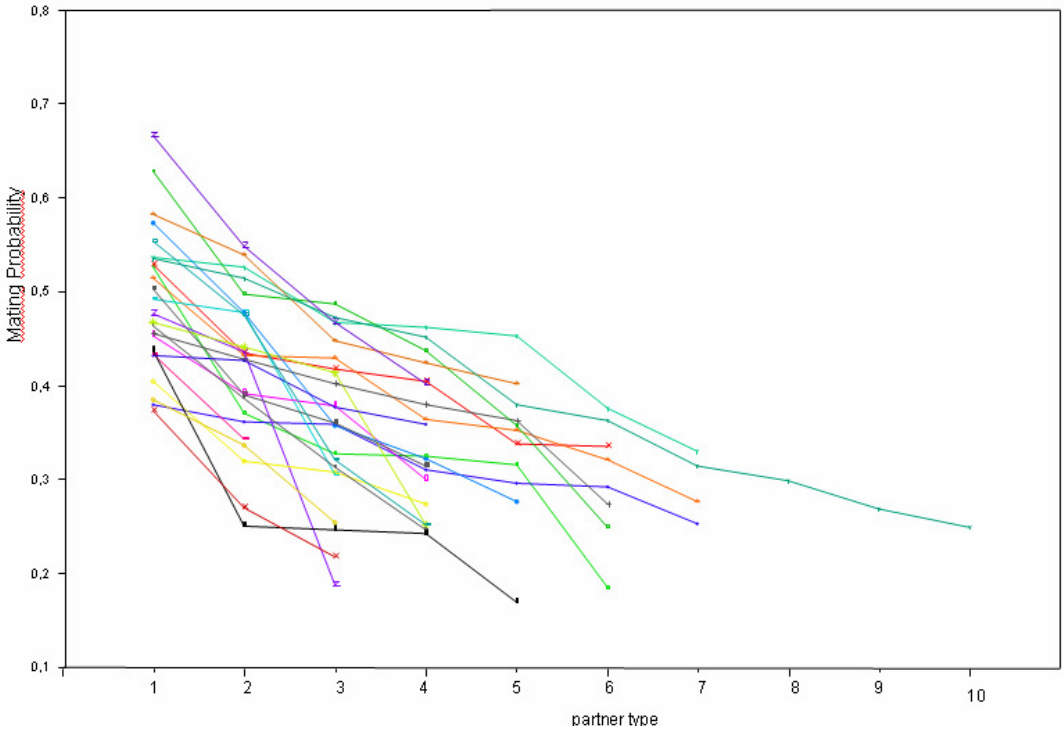

Fig.8: Sorted mating probabilities. Each line represents one neighborhood

Discussion

In principle, each focal individual of this study had the opportunity to choose its partner from a group of neighbors. As the group size varied greatly across neighborhoods, choice opportunities might have been diverse for each focal individual. If earthworms are able to adjust their choice criteria flexible based on quality and quantity of possible partners, any mate choice pattern will be hard to detect.

Parasite-mediated mate choice

In contrast to the expectation of parasite-mediated mate choice, parasite concentration did not have an influence on the choice of the focal. We have to take into account here that the effect of *Monocystis sp.* on earthworm fitness is not yet understood completely. The prevalence (no. of infected individuals / total no. of individuals (Schmidt and Roberts 2000) of *Monocystis sp*. is generally very high and almost all earthworms in natural populations harbor sporocysts. It seems that the association between *Monocystis* and *L. terrestris* is an old and benign one. The complicated journey a *Monocystis* cell undertakes through the earthworms body before propagating in the cells of the seminal vesicles might have taken some evolutionary time to develop. Tolerance and premunition (Incomplete immunity; parasites are held in check by the immune system, the host is asymptomatic) in the enduring presence of a parasite can therefore drive the choice parameter "parasite concentration" into neutrality. A study on earthworm immunocompetence, measured in PO active coelomocytes, and parasite concentration did not reveal any strong relationships between those factors (Field et al. 2004). This is suggestive for a wide range of plasticity between tolerance of the parasite and activation of the immune system. Other evidence for a long joint evolution is that an influence of *Monocystis* concentration on surface activity of the earthworms could not be detected. Here, it must be expected that parasite manipulation can increase its dispersal through predators. Previous studies in this system also failed to show short-term parasite effects on host copulation rates (Field et al. 2003). If any influence of parasite concentration on mate choice is present, the effect could be cryptic because choice strategies often depend on host and parasite life cycle status (early or later in life, before

or after propagation etc.) Since we did not know the current life history state of neither the host nor the parasite, we cannot infer from the data whether earthworms use parasite concentration as mate choice criteria. Furthermore, earthworms might use a combination of the given parameters or totally different, not investigated parameters to choose the optimal partner.

Predation risk and assortative Mating

However, distance to the focal had a significant effect. The focals distance to the second most likely partner as well as the median of the distance to all accessible individuals was larger than the distance to the most likely partner. Distance seems to be the factor that is most important for mate choice. The presence of predators drives direct selection pressures towards a fixed choice for the nearest partner. Even if a worm has a poorer paternal fitness through a smaller partner, it might be better to stay alive and achieve some maternal fitness through cocoon production. This situation gets more and more likely if we take the castrating effect of the *Monocystis* parasite into account. If an earthworm's paternal fitness is limited because it is not able to produce the normally excessive amounts of sperm, maternal fitness – which means surviving the mating and producing cocoons – becomes more important. Short-distance mating and predation avoidance could have another integrated side-effect: *Monocystis* sporocysts are usually descendants of one or few "founder" cells that infected the earthworm. As a consequence, the parasite population within the seminal vesicles should be highly inbred. Recombination between genetically different individuals i.e. outbreeding is an important tool in antagonistic host-parasite coevolution. Considering that earthworms do not disperse much, there is little chance for parasite dispersal. However, predation of these worms by birds may enhance dispersal of these notorious parasites through its faeces that in turn allows recombination with other populations. The pathway to recombinant parasite genotypes can be narrowed by the predation avoidance behaviour of the hosts. Further studies should therefore investigate the genetic composition of *Monocystis* populations. The results suggest that *L. terrestris* does not follow simple rules for assortative mating. We could neither confirm similarities for the investigated pairs nor a preference for partners with certain parameter conditions. As we mentioned before, this could also be due to the

lack of choice opportunities. Another alternative explanation is that earthworms choose their partner depending on their own condition. For an individual that has just mated recently, choice criteria could be totally different as for a virgin earthworm that has not mated yet, but is in the urgent need for sperm to fertilize its eggs.

Mating Probabilities

In contrast to our expectations, the calculated mating probabilites were very low (Fig.8). One possible explanation is that we have not sampled all adult worms that inhabit each neighborhood. The other possible explanation could be the high variation of the microsatellite loci itself. The calculation of mating probabilities assumes mendelian distribution of alleles across sperm cells, but this high variation hints to a brake of this rule.

Conclusion:

Overall *L. terrestris* exhibits mate choice for its closest partner. A hypothetic reason might be the enduring presence of predators. High variation both within and between neighborhoods may conceal the importance of other choice factors. All in all, the application of a neighborhood design is able to answer simple mate choice questions, but more detailed studies are necessary to expose all interactions in this Lumbricidae -Gregarine system.

Chapter V

Detection of multiple infections by *Monocystis* strains in a single earthworm host using ribosomal internal transcribed spacer sequence variation

Velavan TP, Hinrich Schulenburg and Nico K Michiels

Submitted Parasitology

Keywords

Monocystis sp.

ribosomal RNA

Lumbricus terrestris

Phylogeny

Polymerase chain reaction

Summary:

Monocystis sp. are sporocyst-forming apicomplexan parasites common in seminal vesicles of the earthworm *Lumbricus terrestris* where they may account for temporary castration. This study describes the internal transcribed spacer (ITS) region of the ribosomal cistron of *Monocystis sp*. This region, including ITS-1, the 5.8S ribosomal RNA gene, and ITS-2, was PCR amplified, cloned, and sequenced for *Monocystis* sp. isolated from the seminal vesicles of several wild-caught *L. terrestris*. Our analysis revealed substantial polymorphisms, also within single host organisms, indicating intra-host diversity of parasites. These genetic markers are the first that allow distinction of *Monocystis sp*. genotypes, opening new avenues for the study of parasite diversity within and between hosts.

Introduction

The interaction between *Lumbricus terrestris* and *Monocystis sp*. is a well studied host-parasite system (Field and Michiels 2005). The seminal vesicles of *L. terrestris,* where self-sperm develop and are stored, are heavily infected by the gregarine *Monocystis sp*. (Alveolata: Apicomplexa). Apicomplexa are mostly parasitic, with the gregarines exclusively infecting invertebrate hosts (Edwards and Bohlen 1996). Taxa of the genus *Monocystis* undergo three characteristic phases during their lifecycle (Schmidt and Roberts 2000; Bush et al. 2001): (i) Infection phase - Worms get infected by ingesting the oocysts that contain several sporozoites in the soil. These sporozoites enter the circulatory system and invade the sperm vesicle lumen where they mature as trophozoites. During this process, they destroy developing spermatocytes, (ii) Sexual phase - Gamonts undergo syzygy (two or more gamonts fuse with one another in tandem) and form a gametocyst (with a cyst envelope). Several nuclear divisions result in formation of a zygote that secretes an oocyst membrane. (iii) Dispersal phase - oocyst membrane hardens further resulting in sporocysts to form a typical fusiform shape. Two or three cell divisions follow to form eight sporozoits inside one spore. At this point the gametocyst ruptures releasing the many sporocysts into the seminal fluid and eventually into the environment to repeat the lifecycle. Recent studies have shown large variation in *Monocystis* concentration among individual earthworms that is

correlated with reduced growth (Field and Michiels 2005). Strong infections are known to result in destruction, resorption and regeneration of the seminal vesicle, which effectively means that individuals are temporarily castrated (Breidenbach 2002). Only few reports are available concerning biodiversity among *Monocystis* species based on morphological characterization (Bandyopadhyay and Mitra 2005; Bandyopadhyay et.al 2006). To date, diversity at the genetic level has not as yet been examined. Such molecular information may provide new insight into the examination of relationships between species and populations of *Monocystis* genus.

The ribosomal DNA (rDNA) of a eukaryotic cell typically contains a tandem, head-to-tail repetitive sequence with the structure 5´- IGS (Intergenic spacer region) -18S rDNA – ITS-1 - 5.8S rDNA – ITS-2- 28S rDNA – IGS - 3'. The repeat is normally transcribed by RNA polymerase 1 to produce a pre-rRNA which, in the nucleolus, is processed to remove the internal transcribed spacer (ITS) and intergenic spacer regions (IGS). Despite the fact that some sequence stretches within the ITS region are important because they are involved in the processing of the pre-rRNA (Goggin, 1994), this region is generally subject to high evolutionary rates (Gerbi 1986). Consequently, comparisons of ITS sequences have proven useful in studies on the evolutionary biology of populations and species (e.g. Daniela et al. 2007; Mes and Cornelissen 2004).

The ITS region have been studied in a few closely related species of apicomplexan taxa (Hnida and Duszynski 1999, Ellis et al. 1999); however the described primer pairs in these previous studies are not useful to amplify *Monocystis* DNA, because they are conserved with the ribosomal DNA of host taxon being studied here, thus leading to unwanted co-amplification of both parasite and host ITS. Therefore the objective of this study was to design new primers that allow specific amplification of the ITS region from *Monocystis sp.*. As it is as yet unknown whether *L. terrestris* is infected by a single *Monocystis* strain or several distinct genotypes or even species, our additional aim was to use these markers to assess intra-host diversity of *Monocystis* genotypes.

Materials and Methods

Parasites

Monocystis sp. were obtained from seminal vesicles of wild caught earthworm host *Lumbricus terrestris* originating from natural populations of Tuebingen. Based on the morphology of sporocysts under a light microscope *Monocystis sp*. were identified. Sporocysts of *Monocystis* vary in size depending upon species and their lifecycle stages. Sporocysts have a characteristic biconical shape with a mucoid plug at each end. (Mackinnon and Hawes 1961). The seminal vesicles of *L. terrestris* were dissected out and put into an equal volume (weight) of earthworm Ringer solution (i.e. 1:2 dilution) (Ringer solution: 25mM NaCl, 4mM KCl, 6mM $CaCl_2$, 1mM $MgCl_2$, 26mM Na_2SO_4, 2mM Tris and 55mM Sucrose) until used for DNA extraction.

Monocystis DNA Extraction

Genomic DNA of *Monocystis sp*. was extracted as follows: To the seminal vesicles in the ringer solution, 1 ml of 12% $NaClO_4$ was added and incubated overnight at room temperature to bleach the host tissue. After incubation, the bleached tissue of hosts along with intact sporocysts of *Monocystis sp.* was centrifuged at 13000 rpm for 10 min. The supernatant was discarded and the pellet was suspended in 50 µl of ATL buffer (Qiagen DNeasy tissue kit) and 100 µl of AL buffer (Qiagen DNeasy tissue kit). Excystation of sporocysts was achieved by using a sonicator (Bandelin sonopuls, Model UW 2070): the samples were homogenized three times for 25 sec at 9 cycles. The successful rupturing of cysts was checked under a light microscope (Fig.1). We proceeded with DNA isolation as described in the Qiagen DNeasy tissue kit protocol.

Polymerase Chain reaction and PCR product purification

The complete region of the ITS-1, 5.8S rRNA genes and the ITS-2 region were amplified from one Tübingen earthworm individual using an upstream primer located in the 3` end of the 18S rRNA and a downstream primer from the 5` end of the 28S rRNA. The upstream primer was designed using the published sequence of *Monocystis agilis* 18S rRNA gene (AH008869) and we ensured that this designed primer was not conserved with the *L. terrestris* 18S rDNA sequence

(AJ272183). The upstream primer consisted of 22 nucleotides: 5´-GAGAAGTCTTGTAAACCCAATT-3´. The downstream primer at the 28S rRNA region was designed using *Gregarina niphandrodes* sequence (DQ837379); however the designed primer was conserved with 28S rRNA region of the host *Lumbricus sp.* (DQ790041). The downstream primer consisted of 18 nucleotides: 5´- GTTAGTTTCTTTTCCTCC-3´. PCR amplifications were carried out in 20 µl reaction volumes with 5 ng of genomic DNA, 1x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl; Invitrogen), 2.5 mM of $MgCl_2$, 2 mM of dNTPs, 5 pM of each primer and 1 U Taq DNA polymerase (Taq DNA polymerase recombinant, Invitrogen) on a Master Cycler EP Gradient (Eppendorf). Thermal cycling parameters were: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C denaturation, 1 min at 52 °C annealing temperature, 2 min at 72 °C extension, followed by a final extension of 7 min at 72 °C. Several independently performed PCR reactions were combined before subsequent sequence analysis to minimize the impact PCR errors during amplification with *Taq* polymerase. PCR products (8 µl) was analysed by electrophoresis in 1.5% agarose gels, with a 100 bp DNA ladder molecular size marker (Invitrogen) and PCR -products were purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia).

DNA cloning, sequencing, and design of *Monocystis*-specific primers

The purified PCR fragment from one host individual was inserted into a TA cloning vector (TOPO cloning Kit, Invitrogen). The TA vector containing the ITS region were transformed into one shot *E. coli* (Invitrogen). Plasmids were isolated using QIAprep® Spin Miniprep Kit (Qiagen) and cloned inserts were sequenced for both strands using M13 universal primers using a commercial sequencing service (GATC Inc.). To ensure accuracy of the sequenced genes, several independent plasmids were sequenced in both directions and a consensus sequence was generated. The nucleotide sequences generated for the ITS-1, 5.8S rDNA and the ITS-2 of *Monocystis sp.* have been deposited in Genbank (FM174710). Using a nucleotide BLAST, the obtained clone revealed high similarity to ITS regions of other apicomplexan taxa, confirming that the sequence corresponds to *Monocystis sp.* and not the host individual. The ends of the 18S and

28S rRNA genes of *Monocystis* sp. were identified by homology when aligned with sequences from closer apicomplexan taxa. We designed a new primer pair (MITS-F and MITS-R) to obtain *Monocystis sp.* specific amplification. We ensured that the new primers show substantial differences to the *L. terrestris* ITS region. The sequences for these *Monocystis* - specific primers are as follows: MITS-F: 5´- GAGAATGGTCAAGTCGTAAC and MITS-R: 5´- GTTCAACGGGTATACTTGTTCAATTTCAGG. The primers were designed using the sequence submitted to the database (FM174710) and should yield a product size of 793 bp.

Genetic variation in *Monocystis sp.*

To assess the diversity of *Monocystis sp*. genotypes within hosts, we isolated DNA from two host individuals from Tuebingen and one from Rottenburg am Neckar (Baden-Württemberg, Germany) as well as from a commercially obtained individual originating from Canada. After DNA isolation (see above), we amplified *Monocystis* ITS rDNA using primers MITS-F and MITS-R. PCR amplifications were carried out in 20 µl volumes with the same reaction conditions as above and the following cycling profile: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C denaturation, 1 min 15 sec at 60 °C annealing temperature, 2 min at 72 °C extension, followed by a final extension of 7 min at 72 °C. The PCR products were subsequently purified, cloned, and sequenced as described above. In this case, we did not produce a consensus sequence per host individual, but instead used the various sequences obtained from independent clones for further evaluation of sequence diversity.

Sequence analysis

We aligned the obtained genotype sequences with previously published sequences from closely related taxa using Clustal X (Thompson et al. 1997). This alignment then served to identify the exact boundaries of 18S, ITS-1, 5.8S, ITS-2 and for 28S rDNA by their sequence homology. Thereafter, we used an alignment, which only contained the *Monocystis* genotypes isolated by us from *L. terrestris*, in order to perform population genetic and phylogenetic analyses. The mean genetic diversity across respective regions was computed using pairwise deletion and

proportional distances using MEGA ver 4.0 (Tamura et al. 2007). The phylogeny of the sequences was inferred using maximum likelihood (ML). We ran the program Modeltest to infer the optimal substitution model for the data set (Posada and Crandall 1998, Posada and Buckley 2004). The optimal substitution model, the Tamura-Nei model (Tamura and Nei 1993) with Gamma-rates across sites (TrN-G) (Yang, Z., 1993), was then employed to reconstruct a phylogenetic tree using the program PHYML with standard settings (Guindon and Gascuel 2003). Robustness of inferred relationships was assessed with non-parametric bootstrapping based on 500 replicate data sets (Felsenstein, 1985; Hillis and Bull, 1993). Phylogenies were rooted with the mid-point rooting method.

Results

Our first amplification permitted identification of the ITS region of *Monocystis* parasites from *L. terrestris* (FM174710). This sequence contained an ITS-1 with a length of 425 bp and a GC-content of 34%, the 5.8S rRNA gene with 155 bp in length and a %GC of 37, and an ITS-2 with 121 bp in length and a %GC of 31. Thereafter, we used the newly designed specific primers to obtain 12-15 *Monocystis* sequences from four different host individuals. The ITS regions from individual host organisms revealed the presence of multiple *Monocystis* genotypes. These genotypes showed considerable length variation and revealed high numbers of variable nucleotide positions (Table 1). Each individual host possessed 8-12 different *Monocystis* genotypes (Table 1). The rest of the clones screened were identical to respective individual genotypes. Exactly two identical sequences were found for three genotypes from Tuebingen host individual A, four genotypes from Tuebingen host individual B, three genotypes from the Rottenburg host individual and three genotypes from the Canadian host. We never obtained more than two identical sequences per identified genotype. The 34bp 28S region was completely conserved across genotypes and did not show any variable nucleotide positions, whereas the 18S region revealed a few variable nucleotide substitutions within three of the tested host individuals (Table 1). The ITS-1 region showed the highest amount of variation in both nucleotide substitutions and the incidence of indels, followed by ITS-2 and then 5.8S rDNA (Table 1). The

reconstructed ML tree (Log Likelihood = - 5569.8) is shown in Fig 2. Bootstrap support larger than 70% for individual branches is indicated. All Canadian genotypes clustered in one clade except for one genotype (C1_19). In contrast, genotypes from Tuebingen and Rottenburg hosts were scattered across the tree.

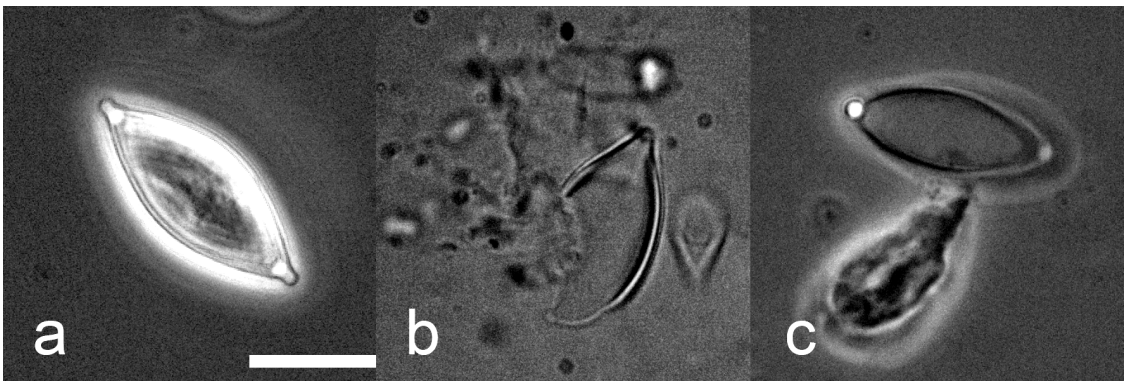

Fig 1: a: Encapsulated sporocyst before Excystation, **b and c:** Excysted sporocyst after Sonication (Scale: 1µm and Magnification is 100X using Phase contrast)

Discussion

Our study characterizes the primary structure of ITS-1, the 5.8S ribosomal RNA gene, and ITS-2 region for the parasite genus *Monocystis* and its use for the analysis of genotype diversity within and between host organisms. We have developed a reliable approach to break up the *Monocystis* sporocyst to isolate DNA. With a PCR-based approach, using our newly developed primer pairs MITS-F and MITS-R, we have shown that it is feasible to genetically detect the parasites in infected host individuals. Moreover our study demonstrates for the first time that multiple parasite genotypes infecting one single host is the rule rather than the exception (based on a small sample size). We reject the alternative explanation that variation among clones from a single host is exclusively due to PCR errors. The mutation frequency of an amplification reaction was determined by the formula: Mutation frequency = (error rate X d) where mutation frequency is expressed as mutations/kb, error rate is the “error rate/kb” of the Taq DNA polymerase used in this study ($8.9x10^{-5}$ errors per bp = 0.089 errors per kb (Cariello et. al.1991) and d is the number

of duplications during PCR (35 cycles). Based on the above formula we obtained the mutation frequency as 62.3 mutations/kb. The mutation frequency obtained for our amplicon (862bp) will then be 53.70 mutations/ 862bp. However, we observed 128 to 165 variable sites across the complete ITS region. Given that we observe more variable sites than expected by PCR errors (53.70 variations / 862bp), such variations is unlikely to be caused exclusively by PCR errors. Furthermore, sequence variation differs among the components of the ITS region (e.g. highest for ITS-1, then ITS-2, and lowest for the coding regions), which is not expected if variation is due to polymerase errors. Similarly, a considerable proportion of the variation is due to indels, which are unknown to result from polymerase errors during PCR. We are therefore confident that the nucleotide variation observed in our data set are true polymorphisms representing different *Monocystis* sp. strains. The level of diversity that we infer from this study will allow us to distinguish different genotypes from a single *Monocystis* strain. The observed level of high diversity is due to the fact that these infections are quite frequent which effectively means multiple genotypes being infected. Infections occur during the haploid stage of the parasite lifecycle; therefore we speculate that new infection means multiple alleles or newer genotypes. Given the fact that, parasites should impose a selection pressure favouring genetic diversity in their hosts (Altizer et al. 2001), the observed level of diversity in a single host will help us to understand the processes that shape genetic diversity in hosts and understanding dynamic coevolution between hosts and parasites i.e. specificity between host and parasite genotypes. Moreover the level of genetic diversity observed across *Monocystis* genotypes could potentially be used to infer about genetic structuring among host populations.

Our study revealed that parasite genotypes isolated from the same host individual or population do not necessarily form an exclusive monophyletic group. This is particularly unusual for the host populations from different continents, which are clearly separated geographically as well as genetically. In particular, *L. terrestris* microsatellite data revealed population differentiation of 15.17% (F_{ST} = 0.16, P = 0.04) as inferred from an analysis of molecular variance (Schneider et al. 2000) between the here included host populations from Tuebingen and Canada (Data submitted

for publication elsewhere). Such significant differentiation among host populations should be reflected by clear separation of the corresponding parasite lineages. One possible reason for absence of such separation is that the *L. terrestris* population from Canada is an invader population from Central Europe. Consequently the parasites *Monocystis sp.* have not diverged as much during the comparatively short evolutionary time scale. Evolutionary rates of parasites should usually be faster than those of their hosts (Hamilton et al., 1990). However, migration pattern and gene flow, long-distance host migration and host breeding ecology (Thompson, 1994; Gandon et al., 1996; Altizer 2001) can topple this asymmetry in evolutionary rates between host and parasite (Delmotte et al.1999). Moreover variation in life cycles of a parasite strongly affect parasite population genetic structure (Poulin and Morand 2000; Criscione and Blouin 2004) and local adaptive potential of the parasite (Gandon et al. 1996; Lively 1999). Given that *L.terrestris* hosts had migrated across continents and *Monocystis sp*. are highly dependent on hosts for their lifecycle (see introduction), we speculate that *Monocystis sp*. parasites have a coevolutionary disadvantage when compared to their host. Host specificity serves as a measure of ecological adaptation; however this will be difficult to demonstrate in hosts infected by many parasites. The other possible reason is that one can expect host-switch event that can be regular, in certain circumstances which later lead to rapid evolutionary radiation (Zietra and Lumme 2002).

To date, it is impossible to infer whether the observed sequence variation is indicative of different *Monocystis* species or only genotypes from a single species. One can achieve this by isolating trophozoites across species based on histological expertise, however relatively a few trophozoites can be isolated. Further differentiation could be inferred only if one could amplify the ITS region from morphologically distinct *Monocystis* parasites and compare the then observed sequence variation with that from our study. However, isolation and subsequent cultivation of *Monocystis sp*. from earthworms remains as yet an unresolved technical problem due to its complicated life cycle. Overall, the observed variation in ITS sequence and particularly length should provide a valuable tool for future analysis of *Monocystis* diversity.

Electronic Database Information

The GenBank Accession numbers for data presented herein are as follows: Accession Numbers for Tuebingen individual (A): FM174711 - FM174721, Tuebingen individual (B): FM174712 - FM174729, Rottenburg individual: FM174730 - FM174741 and for Canadian individual: FM174742 - FM174750.

Acknowledgments

I would like to thank Nils Anthes for assistance during phylogenetic analysis. This study was funded by a grant from the German Science Foundation (DFG MI 482/6-3).

	Tuebingen Individual (A)				Tuebingen Individual (B)				Rottenburg Individual				Canadian Individual			
	18s	ITS1	5.8s	ITS2	18s	ITS1	5.8s	ITS2	18s	ITS1	5.8s	ITS2	18s	ITS1	5.8s	ITS2
# alignment Sites (bp)	58	494	155	122	58	448	158	121	58	460	155	121	58	510	148	144
# Variable alignment sites	0	105	5	18	3	108	17	24	1	130	9	25	2	137	6	19
# indel regions	0	2	0	2	0	17	1	0	0	12	0	1	0	12	0	3
Maximum pairwise proportional differences	0	0.25	0.02	0.1	0.05	0.32	0.04	0.15	0.02	0.31	0.04	0.16	0.03	0.32	0.03	0.22
Genetic diversity indices	0	0.118	0.007	0.056	0.016	0.236	0.035	0.089	0.007	0.158	0.014	0.063	0.008	0.070	0.009	0.059
Total no of genotypes	11				8				12				9			
# sequences obtained per host	13				12				15				12			
Amplicon size range (bp)	723 - 862				608 - 790				640 - 790				790 - 811			

Table 1: Variation between 18S, ITS-1, ITS-2 and 5.8S rRNA sequences of *Monocystis sp.*

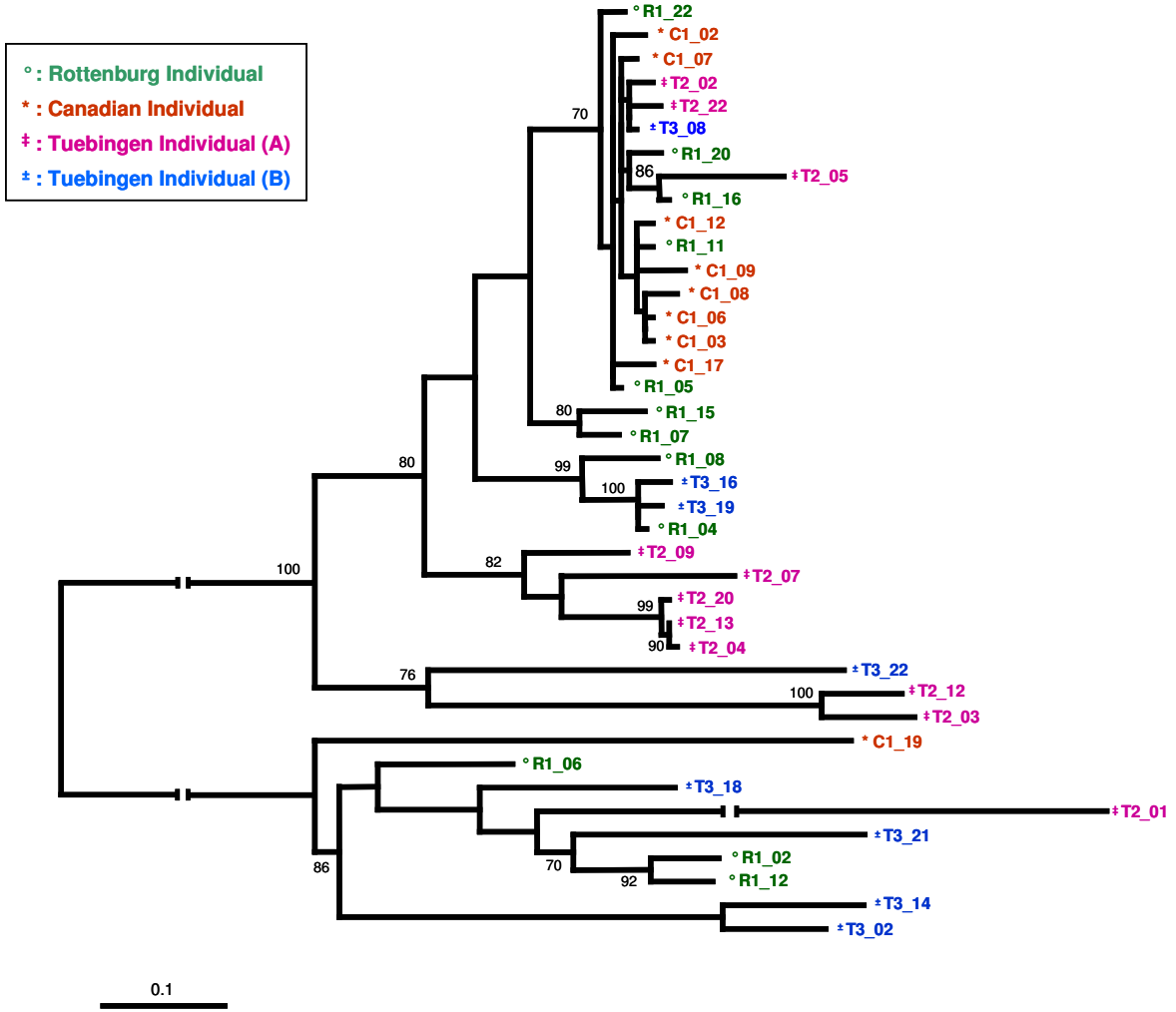

Fig 2: Maximum likelihood tree (Log Likelihood = - 5569.84232) inferred with the Tamura-Nei substitution model with Gamma rates across sites from the complete ITS region sequences (956 sites). ML bootstrap support was inferred from 500 replicates. Only values larger 70 are indicated.

Chapter VI

Diversity of *Monocystis* parasites in relation to earthworm host fitness and heterozygosity

Velavan TP, Nadine Timmermeyer, Hinrich Schulenburg and Nico K Michiels

Unpublished manuscript

Keywords

Monocystis sp.

Lumbricus terrestris

Genetic diversity

Parasite genotypes

Abstract:

Genetic basis of infection is often assumed in many of the host-parasite models in evolutionary biology. In host parasite interactions, parasite-mediated selection play a significant role in modulating genetic diversity of host populations as parasites often correspond to a significant selective pressure. We presume that host populations revealing high parasite diversity should exhibit high levels of heterozygosity and reduced fitness. Using ribosomal DNA markers of parasite *Monocystis sp*., we inferred the intra-host genetic diversity of the parasite populations by a polyacrylamide gel electrophoresis (PAGE) approach. We further evaluated for genotype by genotype interactions between the parasite diversity measures to that of host genetic diversity, their respective heterozygosity levels and of their fitness. We test this assumption using data on *L.terrestris* host species and their apicomplexan parasites *Monocystis sp*. we inferred (i) dispersal of *Monocystis sp* parasites were relatively low (ii) a significant positive association between mean observed parasite genotypes in relation to host gene diversity (iii) a significant negative correlation of both mean observed parasite genotypes and mean parasite gene diversity in relation to parasite load and vesicle weight in host subpopulations (iv) a statistical weak trend that mean number of genotypes increases with mean observed heterozygosity in host populations (v) we could not identify a significant association between parasite gene diversity in relation to average allelic richness, inbreeding coefficient or the body weight of the host subpopulations.

Introduction:

Parasites negatively influence host fitness, resulting in a large investment on immune defence by hosts ensuing in a trade off with life history traits such as survival, growth and reproduction (Simovka *et al.* 2008). The selection pressure on the host to overcome infection often results in an evolutionary arms race between parasite and host (Wegner *et al.* 2008). Parasites that remain as a ubiquitous part of environment tend to show a strong influence on individual hosts as well as on entire host populations (Price 1980, Hudson and Greenman 1998, Poulin 1998, Ebert 2005). In addition, parasites themselves can influence host invasion success (Mitchell and Power 2003,

Torchin *et al.* 2003) and on gene flow between host populations (Telschow *et al.* 2006). To overcome infection by parasites, one can expect outcrossing in host individuals resulting in high genetic diversity among the offsprings (Ebert *et al.* 2007). Moreover heterozygote advantage can facilitate hosts to reduce parasite success. This can happen when selection acts against homozygous individuals which effectively mean the hosts are more resistant to more parasite genotypes. There are several studies especially in vertebrates where parasite mediated selection on major histocompatibility complex (MHC) have been reported (Penn and Potts 1999, Penn 2002, Babik *et al.* 2008, Bos D H *et al.* 2008, De bellocq *et al.* 2008). However on the other hand, parasites also impose a selection pressure on their hosts whenever resistance to parasites is greater among genetically diverse individuals (Altizer *et al.* 2001, Schakelton *et al.* 2005). Overall, as a consequence of coevolution with parasites, genetic diversity in host populations is assumed to be in a state of flux. Knowledge on the process that shape parasite genetic diversity and their respective functional consequences of different parasite genotypes on hosts are vital (Hamilton *et al.* 2005), because it is likely to have consequences for the spread of parasites through host populations (Curtis *et al.* 2002, Springbett *et al.* 2003, Grenfell *et al.* 2004). In addition, genetic structure in parasites could potentially reveal the extent of genetic structuring in their respective hosts.

Genetic basis of infection is often assumed in many of the host-parasite models in evolutionary biology. Host parasite interactions are predominantly specific to the host and parasite genotype (i.e.) in the resistance of host genotypes to particular parasite genotypes and the infectivity of parasite genotypes for particular host genotypes (Haldane 1949). A single gene to complex number of genes is believed to be involved in these interactions between host resistance and parasite infectivity (Sorci *et al.* 1997). On the basis of gene for gene interaction, we expect two outcomes, either susceptibility which means high parasite success in invading the host or resistance which means parasite failure to establish in the host. In yet another model namely the matching allele model claims that each host allele confers resistance to each parasite allele, which means parasites can successfully establish in the hosts when there is no match between

host and parasite alleles. Therefore gaining knowledge on these interactions has important repercussion for understanding the mechanisms of host-parasite coevolution.

The interaction between *Lumbricus terrestris* and *Monocystis sp*. is a well studied host-parasite system (Field and Michiels 2005) yet relatively little is known about the costs incurred and a consensus on the pathogenicity of *Monocystis* infection is yet to be reached. Recent studies have shown large variation in *Monocystis* concentration among individuals that is correlated with reduced growth (Field and Michiels 2005). Genetic differentiation among host subpopulations is further augmented by variation in parasite prevalence. We hypothesize that host taxa harbouring high parasite diversity should exhibit high levels of heterozygosity and reduced fitness. In this study, we analysed the parasite *Monocystis sp.* intra host diversity collected across 26 different sites based on their length variation in ribosomal internal transcribed spacer region using PAGE (Poly Acrylamide Gel Electrophoresis) approach. Our aim was to characterize the population genetic structure and genetic diversity of the parasite at a microgeographic scale and to relate this information to fitness, genetic diversity and respective heterozygosity levels in host populations.

Materials and methods

Study system

Lumbricus terrestris is an obligatory outcrossing hermaphroditic earthworm that is common in pastures, parks, agricultural fields and lawns. As an anecic species, *L. terrestris* is characterized by permanent vertical burrows, descending down to 2 m into the soil, relatively large body size and long lifespan (Edwards and Bohlen, 1996). Natural dispersal of *L. terrestris* is reported to be limited, i.e. approximately 4 m/yr (Hoogerkamp *et al.* 1983). Mating occurs on the soil surface (Michiels *et al.* 2001). The seminal vesicles of *L. terrestris,* where self-sperm develop and are stored, are often strongly infected by the gregarine *Monocystis sp*. (Alveolata: Apicomplexa). Apicomplexa are mostly parasitic, with the gregarines exclusively infecting invertebrate hosts (Edwards and Bohlen 1996). Taxa of the genus *Monocystis* undergo three characteristic phases

during their lifecycle namely the infection phase, sexual phase and a dispersal phase. For details of the *Monocystis* life cycle, see Schmidt and Roberts (2000) and Bush *et al.* (2001). Strong infections are known to result in destruction, resorption and regeneration of the seminal vesicle, which effectively means that individuals are temporarily castrated (Breidenbach 2002).

Sampling

L. terrestris individuals were sampled at a microgeographical scale on a meadow next to the Biological Institute of the University of Tuebingen, Baden-Wuerttemberg, Germany (9° 00' E, 48° 30' N). The origin of a circular plastic hoop with a diameter of 84 cm (0.55 m²) was placed on a hand mowed site. An aqueous solution comprising of 170 g mustard powder per 10 litres of tap water (Gunn, 1992) was poured onto the hand mowed lawn to extract worms. Twenty litres of mustard solution were repeatedly poured onto the circumference of the hoop to extract worms. Each of these sites was resampled with the above mentioned procedure on the next subsequent day to sample as many worms as possible. All worms emerging within the same hoop were considered part of the same, arbitrary site. Twenty-six such sites approx. 1 to 2 m apart were sampled on this one large meadow. The number of worms per site varied from 7.27/m^2 to 54.54/m^2 individuals across each sites. Unfortunately, our landmarks of the sampling sites were lost when the lawn was mowed by gardeners making an analysis of distance effects impossible. Immediately after collection, worms were washed free of soil particles and mustard solution, dried on paper tissue and weighed ± 0.01 g. The individual was then sacrificed and cut anterior to the clitellum. The front part, which contains the seminal vesicles, was placed in an Eppendorf tube and stored in 100% Ethanol at -20°C until dissection. All worms were sampled within 5 weeks: In total 337 worms from 26 different sites were sampled.

Monocystis DNA Extraction

The seminal vesicle of the *L.terrestris* were dissected out and put into an equal volume (weight) of earthworm Ringer solution (i.e. 1:2 dilution) (Ringer solution: 25mM NaCl, 4mM KCl, 6mM $CaCl_2$, 1mM $MgCl_2$, 26mM Na_2SO_4, 2mM Tris and 55mM Sucrose). Genomic DNA of *Monocystis*

sp. was extracted as follows: To the seminal vesicles in the ringer solution, 1 ml of 12% $NaClO_4$ was added and incubated overnight at room temperature to bleach the host tissue. After incubation, the bleached tissue of hosts along with intact sporocysts of *Monocystis sp.* was centrifuged at 13000 rpm for 10 min. The supernatant was discarded and the pellet was suspended in 50 µl of ATL buffer (Qiagen DNeasy tissue kit) and 100 µl of AL buffer (Qiagen DNeasy tissue kit). Excystation of sporocysts was achieved by using a sonicator (Bandelin sonopuls, Model UW 2070): the samples were homogenized three times for 25 sec at 9 cycles. The successful rupturing of cysts was checked under a light microscope (Fig.1). We proceeded with DNA isolation as described in the Qiagen DNeasy tissue kit protocol.

Poly Acrylamide Gel Electrophoresis

The complete ITS-1, 5.8S rRNA genes and the ITS-2 region of *Monocystis sp.* were amplified from DNA of isolated *Monocystis* strains across 26 sites using primer pairs MITS-F: 5´-GAGAATGGTCAAGTCGTAAC and MITS-R: 5´- GTTCAACGGGTATACTTGTTCAATTTCAGG. (data submitted for publication elsewhere). PCR amplifications were carried out in 20 µl reaction volumes with 5 ng of genomic DNA, 1x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl; Invitrogen), 2.5 mM of $MgCl_2$, 2 mM of dNTPs, 5 pM of each primer and 1 U Taq DNA polymerase (Taq DNA polymerase recombinant, Invitrogen) on a Master Cycler EP Gradient (Eppendorf). Thermal cycling parameters were: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C denaturation, 1 min 15 sec at 60 °C annealing temperature, 2 min at 72 °C extension, followed by a final extension of 7 min at 72 °C. The PCR Products were processed randomly and run on 10% polyacrylamide gels (Novex® TBE Gel, Invitrogen) in 1X TBE buffer (Novex®, Invitrogen). Following manufacturer's instructions, electrophoresis was performed at a constant voltage of 200 V for 1 hour and 30 minutes. After electrophoresis, the gels were stained with SYBR Gold (Invitrogen, Molecular Probes Ltd., Karlsruhe, Germany) and subsequently examined under UV light. The banding pattern was scored using the program GeneSoft ver 3.08 (VWR International, Leuven) from the corresponding gel profiles. Length variability was inferred corresponding to fragments from 600 to

900 bp across each single host. We considered each prominent band to be a genotype with differences in length.

Genetic analysis

Each sample were scored for presence (1) or absence (0) of bands and entered into a binary matrix representing the phenotype of each individual genotype. Fingerprints for each profile were obtained using JMP® v5.1. Genetic population structure of the parasite populations was investigated with a hierarchical analysis of molecular variance (AMOVA) as described in Michalakis and Excoffier (1996), which estimates how genetic diversity is partitioned among and within sites. For this analysis, the 0/1 matrix was transformed into a squared Euclidean distance matrix between all individuals. The significance of the variance components associated with the different levels of genetic structure was tested using nonparametric permutation procedures as implemented in Arlequin 2.000 (Schneider *et al.* 2000). We also examined the relationship of genetic distances of parasite and genetic distances between 26 host sites (as measured by F_{ST} values) with a Mantel test using the Arlequin (v2.000) software. Assessments of genetic parameters of the hosts were made with the computer program FSTAT version 2.9.3 (Goudet 2001). Genetic variability within host populations was estimated as mean allelic richness (*A*; a measure of allele number independent of sample size; see Petit *et al.* 1998), and mean host gene diversity at the three microsatellite loci. The degree of nonrandom mating within host populations (F_{IS}), and pairwise differentiation among populations (F_{ST}), were estimated with Weir and Cockerham (1984) estimators of *F*-statistics.

Statistical analyses

Data are reported as means ± SD. Since different subpopulations were sampled over a 5 week period, we had to correct for seasonal effects before comparing variation in body weight as well as parasite load between sites. Therefore, for each of these two traits, we first performed a polynomial regression analysis on sampling date (quadratic for body weight $R^2 = 0.0834$, $F_{4,273} = 12.60$, $P < 0.001$, quadric for parasite load $R^2 = 0.1983$, $F_{4,273} = 16.88$, $P < 0.001$). The residuals

of these regressions were then compared between subpopulations using Kruskal Wallis ANOVA. Mean number of parasite genotypes and mean parasite gene diversity across collection sites were correlated with host measures such as gene diversity, observed heterozygosity, allelic richness (*A*), inbreeding coefficient (F_{IS}), body weight, vesicle weight and parasite load for each host subpopulation by Spearman correlation coefficient (r). Statistical analyses were carried out using JMP® v5.1. *P*-values for correlations are provided after correction for false detection rate (FDR).

Results

Population genetic parameters

In total 161 different fingerprints were obtained across twenty six sites. 27 different genotypes were detected for the whole population based on their length variation ranging from 603bp to 890bp. The total number of detected genotypes per individual across all populations ranged from 1 to 8. All parasite measures such as mean number of genotypes, average gene diversity and host measures such as mean body weight, mean absolute parasite number, mean host genetic diversity, average allelic richness and inbreeding coefficient values (F_{IS}) within subpopulations are summarized in Table 1. The AMOVA analysis indicated significant variation among sites, as 28.6% variation being explained by differences between parasite subpopulations (Table 2). The Mantel test did not indicate a significant correlation between pairwise measures of parasite genetic distance (F_{ST}) and the corresponding pairwise differences of host genetic distance (r = -0.15, P = 0.89).

Table 1: Genetic and fitness parameters estimates of host and parasite across 26 sites.

		Parasite		Host				
Population	n	Mean number of genotypes	Average Gene diversity	Mean body weight ± SD in g	Mean absolute parasite number ± SD	Mean host gene diversity	*A*	F_{IS}
N04	14	3.2	0.215 ± 0.122	3.65 ± 0.72	$2.54 X10^6 \pm 3.16 X10^6$	0.81	4.50	0.118
N05	17	2.9	0.197 ± 0.112	3.36 ± 0.76	$4.64 X10^6 \pm 6.85 X10^6$	0.80	4.49	0.091
N06	9	2.7	0.172 ± 0.106	3.10 ± 0.76	$7.02 X10^6 \pm 7.86 X10^6$	0.80	4.46	0.021
N07	12	2.6	0.171 ± 0.101	2.83 ± 0.70	$5.93 X10^6 \pm 3.97 X10^6$	0.79	4.63	-0.039
N08	12	3.0	0.197 ± 0.115	3.00 ± 0.84	$4.33 X10^6 \pm 3.54 X10^6$	0.81	4.62	-0.059
N09	7	2.3	0.137 ± 0.090	3.45 ± 0.73	$1.41 X10^7 \pm 9.75 X10^6$	0.82	4.91	0.104
N10	4	3.0	0.209 ± 0.151	3.28 ± 0.72	$1.45 X10^7 \pm 9.75 X10^6$	0.82	4.77	0.035
N11	7	3.7	0.232 ± 0.144	2.64 ± 0.80	$7.81 X10^6 \pm 6.90 X10^6$	0.84	4.82	0.111
N12	3	2.7	0.197 ± 0.162	2.91 ± 0.67	$1.46 X10^7 \pm 1.22 X10^7$	0.85	4.82	-0.009
N14	7	3.0	0.186 ± 0.118	2.75 ± 0.67	$4.26 X10^6 \pm 5.29 X10^6$	0.80	4.59	0.023
N15	8	2.6	0.181 ± 0.112	2.49 ± 0.84	$7.11 X10^6 \pm 8.22 X10^6$	0.83	4.79	0.08
N16	6	3.5	0.219 ± 0.141	2.67 ± 0.82	$4.30 X10^6 \pm 3.54 X10^6$	0.83	4.66	0.009
N17	6	4.2	0.269 ± 0.169	2.61 ± 0.93	$2.06 X10^6 \pm 1.74 X10^6$	0.81	4.78	-0.064
N18	11	2.6	0.180 ± 0.107	3.27 ± 0.24	$5.74 X10^6 \pm 5.30 X10^6$	0.79	4.55	-0.134
N19	14	2.9	0.186 ± 0.108	2.71 ± 0.67	$8.81 X10^6 \pm 9.99 X10^6$	0.79	4.52	-0.051
N20	5	3.4	0.200 ± 0.135	1.67 ± 0.61	$3.92 X10^6 \pm 3.09 X10^6$	0.78	4.50	-0.118
N21	8	2.6	0.175 ± 0.109	2.45 ± 0.72	$9.09 X10^6 \pm 4.78 X10^6$	0.83	4.69	0.116
N22	5	2.6	0.177 ± 0.121	2.17 ± 0.44	$1.06 X10^7 \pm 1.00 X10^7$	0.78	3.99	-0.032
N23	3	3.3	0.172 ± 0.144	2.63 ± 0.19	$9.62 X10^6 \pm 3.18 X10^6$	0.86	5.33	-0.065
N24	3	2.7	0.197 ± 0.162	2.62 ± 0.43	$1.11 X10^7 \pm 8.80 X10^6$	0.84	5.06	0.079
N25	2	3.0	0.111 ± 0.064	2.47 ± 0.58	$1.58 X10^7 \pm 9.56 X10^6$	0.85	4.62	0.059
N26	5	2.2	0.140 ± 0.099	2.80 ± 0.54	$2.11 X10^7 \pm 1.67 X10^7$	0.77	4.29	0.053
N27	11	2.6	0.180 ± 0.107	2.92 ± 0.81	$1.45 X10^7 \pm 1.15 X10^7$	0.79	4.55	0.038
N28	13	2.5	0.166 ± 0.098	3.65 ± 0.93	$1.59 X10^7 \pm 1.79 X10^7$	0.78	4.36	0.078
N29	11	2.5	0.175 ± 0.104	2.96 ± 0.78	$1.20 X10^7 \pm 1.31 X10^7$	0.83	4.79	0.001
N30	11	3.4	0.216 ± 0.126	2.94 ± 0.70	$1.74 X10^7 \pm 1.75 X10^7$	0.85	4.87	0.000
Overall	214	2.9	0.187 ± 0.031	2.96 ± 0.80	$9.61X10^6 \pm 1.06 X10^7$	0.81	4.70	0.024

n : number of host individuals F_{IS} : Inbreeding coefficient *A* : Average allelic richness

Table 2: Results of hierarchial analysis of molecular variance (AMOVA) comparing genetic variation among Monocystis parasite populations.

Source of variation	d.f.	Sum of squares	Variance components	Fixation indices	*P* value	Percentage of variation
Among populations	**25**	**294.23**	**0.61637**	**0.286**	**< 0.001**	**28.6**

Genetic variability and fitness measures

Individual earthworms varied considerably in weight from 0.95 g to 5.43 g (mean = 2.96 ± 0.80; n = 214). After correcting for sampling date, worm mass varied significantly among sites (Table 3; Kruskall–Wallis, χ^2 = 51.42, df =25, $P < 0.0014$). Among the 26 sites the overall *Monocystis sp.* infection prevalence was 99.3%. All sampled sites harbored infected individuals, but parasite load varied greatly ranging from 0 to 6.94 x 10^7 (mean = 9.61X10^6 ± 1.06 X10^7; n = 214). Overall, parasite load showed significant variation within sites after correcting for seasonal effects during sampling period (Table 3; Kruskall–Wallis, χ^2 = 55.0, df = 25, $P < 0.0005$). Average earthworm weight was unrelated to average parasite load per sampling site (r = 0.091, df = 26, P = 0.66). Correlations of mean number of parasite genotypes, mean parasite gene diversity with observed heterozygosity, allelic richness, inbreeding coefficient (F_{IS}), and residual body weight of host remained insignificant ($r \leq 0.26$, df = 26, $P \geq 0.18$) (Table 3). However, we could infer a positive correlation trend between mean number of parasite genotypes and mean observed heterozygosity of host populations (r = 0.3465, n = 26, P = 0.0829) (Table 3). Correlations between mean number of parasite genotypes and mean host gene diversity yielded a significant positive correlation (r = 0.3943, n = 26, P = 0.0462) (Table 3). We inferred a significant negative correlation between mean number of parasite genotypes with absolute parasite load and vesicle weight of host populations ($r \geq -0.5374$, df = 26, $P \leq 0.0261$) (Table 3). Also we inferred a significant negative correlation between mean parasite gene diversity with absolute parasite load and vesicle weight of host populations ($r \geq -0.6792$, df = 26, $P \leq 0.0111$) (Table 3).

Table 3: Spearman correlation between parasite and host measures

Host /Parasite measures	Mean number of Parasite genotypes		Mean Parasite gene diversity	
	r	*p*	r	*p*
Mean host gene diversity	**0.3943**	**0.0462***	**0.2163**	**0.2886**
Mean Observed heterozygosity	**0.3465**	**0.0829**	**0.2235**	**0.2724**
Average Allelic richness	**0.2699**	**0.1823**	**0.2118**	**0.2989**
Mean host FIS	**-0.1782**	**0.3838**	**-0.0925**	**0.653**
Mean residual body weight	**-0.1803**	**0.3782**	**-0.2697**	**0.1827**
Mean residual log parasite load	**-0.4356**	**0.0261****	**-0.6792**	**0.0001*****
Mean vesicle weight (mg)	**-0.5374**	**0.0046*****	**-0.4897**	**0.0111*****

df=26
P<0.05 *
P<0.1 after correction for False Detection Rate (FDR)**
P<0.05* after correction for FDR**

Discussion

Our aim of this study was to infer intra host diversity of *Monocystis sp.* parasites and to investigate the relationship between parasite genetic diversity measures in relation to host fitness and heterozygosity levels. The diversity of *Monocystis sp*. parasites as inferred from most of the total variance among populations (28.6%) suggests that dispersal of these parasites is relatively low. Studies by Blouin *et al.* 1995 and McCoy 2003 compared the genetic structures of parasites having hosts with different dispersal capabilities supported movement of hosts as a key criterion for parasite gene flow. Given that *Lumbricus terrestris* host dispersal is reported to be limited, i.e. approximately 4 m/yr (Hoogerkamp et al. 1983) and moreover these parasites are not vertically transmitted (Field and Michiels 2006), we observe a relatively low dispersal of *Monocystis*

parasites. This trend is further supported by diversity of the hosts which relatively had a very low diversity (0.41%) (Data submitted for publication elsewhere). Therefore host vagility and their complicated life cycle with the host may be a possible reason for this relatively low dispersal. We expect to see substantial increase in seminal vesicles weight in proportion to amount of genotypes they harbour. Nevertheless we found a significant negative correlation for both mean numbers of parasite genotypes and for mean parasite gene diversity when correlated with vesicle weight of the host individuals (Table 3). These correlations suggest that these parasite genotypes are believed to be involved in destruction of the seminal vesicle which goes in accordance with findings by Breidenbach 2002 in which he emphasizes that strong infections result in destruction, resorption and regeneration of the seminal vesicle leading to temporary castration of the host individual. We inferred a negative correlation that absolute number of parasite in host decreases with increase in parasite genotypes or parasite gene diversity (Table 3). One likely reason for this is that parasites may out compete each other to establish their specific genotypes thus resulting in reduced parasite load. We found a positive correlation when mean number of parasite genotypes correlated with mean host gene diversity (Table 3). This would explain the fact that both susceptibility and resistance is in a state of flux in this coevolution process. Moreover we found a weak trend that mean number of genotypes increases with mean observed heterozygosity in host populations (Table 3). As expected, host genetic diversity increases with increase in parasite diversity. This may explain frequency dependent oscillations and the maintenance of genetic variation in both host and parasite. Heterozygote advantage occurs when selection acts against homozygous individuals, thus avoiding that fixation of any one allele. If host alleles convey resistance to infection, heterozygotes may be better buffered against infection, as they would be resistant to more parasite genotypes. Such a condition has been suggested to maintain the unusually high degree of genetic variation in the vertebrate major histocompatibility complex (MHC) (Penn & Potts 1999; Penn 2002, Wegner 2008). However we cannot infer a significant correlation between the observed host heterozygosity to parasite diversity. One possible reason is that the host heterozygosity could be associated with specific parasites. The other likely reason could be as Klein and O'Huigin (1994) suggested that parasite species are

associated with the history of their hosts and that they should have co-evolved with the host immune system. Ilmonen *et al.* (2007) further argued that MHC heterozygosity provides no immunological benefits when resistance is recessive, and can actually reduce fitness in the hosts. Neither the parasite diversity nor the mean number of genotypes influences the parasite fitness as such except for seminal vesicle. Overall our study provides insights on genotype- genotype interactions on a well studied host parasite system.

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REFERENCES

References

A

Aggarwal RK, Velavan TP, Uday Kumar D, Hendre PS, Karthik Sanker, Lalji Singh (2004). Development and characterization of novel microsatellite markers from the olive ridley sea turtle (*Lepidochelys olivacea*). Molecular Ecology Notes 4 (1): 77-79.

Altizer S, Foufopoulos J, Gager A. (2001). Conservation and diseases. In Encyclopedia of biodiversity, Vol. 2 (ed.S. A. Levin), pp. 109–126, San Diego, CA: Academic Press.

Angeloni Lisa (2003). Sexual selection in a simultaneous hermaphrodite with hypodermic insemination: body size, allocation to sexual roles and paternity. Animal Behaviour.Vol. 66 (3), 417-426.

Avise JC (1994). Molecular Markers, Natural History, and Evolution. Chapman & Hall, New York.

Avise JC (1995). Mitochondrial DNA polymorphism and a connection between genetics and demography of relevance to conservation. Conservation Biology, 9, 686–690.

B

Babik W, Pabijan M, Radwan J. (2008) Contrasting patterns of variation in MHC loci in the Alpine newt. Molecular Ecology: 17(10) 2339-2355.

Bandyopadhyay PK, Mitra AK, (2005). Observations on two new species of Monocystis Stein, 1848 (Protozoa: Apicomplexa: Monocystidae) *Monocystis darjeelingensis* sp.n. and *M. ranaghatensis* sp.n. from earthworms (Annelida: Oligochaeta) of West Bengal, India Animal Biology 55 (2): 123-132.

Bandyopadhyay PK, Bhowmik B, Mitra AK (2006). Biology of *Monocystis clubae* sp nov (Apicomplexa: Eugregarinida) from an Indian earthworm *Lampito mauritii* (Annelida: Oligochaeta) of India Zootaxa 1120: 51-55

Bloor PA, Barker FS, Watts PC, Noyes HA, Kemp SJ (2001). Microsatellite libraries by enrichment. http://www.genomics.liv.ac.uk/animal/research/MicrosatelliteEnrichment.pdf.

Blouin MS, Yowell CA, Courtney CH, Dame JB (1995) Host movement and the genetic structure of populations of parasitic nematodes. *Genetics*, 141, 1007–1014.

Boots M, Sasaki A. (2002). Parasite-driven extinction in spatially explicit host-parasite systems. American Naturalist 159: 706-713.

Boots M, Sasaki A. (2003). Parasite evolution and extinctions. Ecology Letters 6: 176-182.

Bos DH, Gopurenko D, Williams RN, DeWoody JA (2008) Inferring population history and demography using microsatellites, mitochondrial DNA, and major histocompatibility complex (MHC) genes. Evolution: 62 (6), 1458-1468.

Breidenbach J. (2002). Normalanatomie und- histologie des Lumbriciden *Lumbricus terrestris* L. (Annelida: Oligochaeta). Doctoral Thesis. University of Münster, Münster, Germany.

Brownstein MJ, Carpten JD, Smith JR (1996). Modulation of nontemplated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. Biotechniques, 20, 1004 - 1010.

Bruford MW, Wayne RK. (1993). Microsatellites and their application to population genetic studies. Curr Opin Genet Dev. Dec; 3(6):939–943.

Bush AO, Fernández JC, Esch GW, Seed RJ. (2001). Parasitism: the diversity and ecology of animal parasites. Cambridge: Cambridge University Press.

Butt KR. (2002). Depth of cocoon deposition by three earthworm species in mesocosms. European Journal of Soil Biology 38: 151-153.

Butt K and Nuutinen V (2005). The dawn of the dew worm, Biologist Vol. 52 (4), 218-223.

C

Cariello NF, Swenberg TA, Skopek TR. (1991). Fidelity of the *Thermococcus litoralis* DNA polymerase (Vent) in polymerase chain reaction determined by denaturing gradient gel electrophoresis.*Nucleic Acids Res.* 19: 4193-4198.

Carr DE, Eubanks MD. (2002). Inbreeding alters resistance to insect herbivory and host plant quality in *Mimulus guttatus* (Scrophulariaceae). Evolution 56, 22–30.

Cassinello J, Gomendio M, Roldan ERS. (2001). Relationship between coefficient of inbreeding and parasite burden in endangered gazelles. Conserv. Biol. 15, 1171–1174.

Clayton DH, Lee PL, Tompkins DM, Brodie ED III. (1999). Reciprocal natural selection on host parasite phenotypes. American Naturalist 154: 261-270.

Coltman DW, Pilkington JG, Smith JA, Pemberton JM. (1999). Parasite-mediated selection against inbred Soay sheep in a free-living, island population. Evolution 53, 1259–1267.

Criscione CD, Blouin MS. (2004). Life cycles shape parasite evolution: comparative population genetics of salmon trematodes. Evolution 58:198-202.

Curtis J, Sorensen RE, Minchella DJ. (2002). Schistosome genetic diversity: the implications of population structure as detected with microsatellite markers. Parasitology 125(Suppl.), S51–S59.

D

Daniela GS, Barton E, Bunbury N, Lunness P, Bell DJ, Kevin MT. (2007) Molecular identity and heterogeneity of trichomonad parasites in a closed avian population Infection, Genetics and Evolution 7, (4), 433-440.

De Bellocq, Gouey J, Charbonnel N, Morand S. (2008). Coevolutionary relationship between helminth diversity and MHC class II polymorphism in rodents, J. Evol. Biol: 21(4)1144-1150.

Decaestecker E, Vergote A, Ebert D, De Meester L. (2003). Evidence for strong host clone parasite species interactions in the *Daphnia* microparasite system. Evolution 57: 784- 792.

Delmotte F, Bucheli E, Shykoff J. (1999). Host and parasite population structure in a natural plant-pathogen system. Heredity, 82, 300–308.

Ducey PK, Messere M, Lapoint K, Noce S. (1999). Lumbricid Prey and Potential Herpetofaunal Predators of the Invading Terrestrial Flatworm *Bipalium adventitium* (Turbellaria: Tricladida: Terricola). The American Midland Naturalist 141:305-314.

Duncan AB, Little TJ. (2007). Parasite-driven genetic change in a natural population of *Daphnia*. Evolution 61, 796-803.

Dybdahl MF, Lively CM. (1998). Host-parasite coevolution: evidence for rare advantage and time lagged selection in a natural selection. Evolution 52, 1057-1066.

Dybdahl MF, Storfer A. (2003). Parasite local adaptation: Red Queen versus suicide king. Trends in Ecology and Evolution 18: 523-530.

E

Ebert D, Herre EA. (1996). The evolution of parasitic diseases. Parasitology Today 12: 96-100.

Ebert D, Hamilton WD. (1996). Sex against virulence: The coevolution of parasitic diseases. Trends in Ecology and Evolution 11: A79-A82.

Ebert D. (1999). The evolution and expression of parasite virulence. In Evolution in Health and Disease (Stearns, S., ed.), pp. 161–172, Oxford University Press.

Ebert D (2005). Ecology, epidemiology, and evolution of parasitism in Daphnia. National Library of Medicine (US), National Center for Biotechnology Information, Bethesda, MD.

Ebert D., Altermatt F, Lass S. (2007). A short term benefit for outcrossing in a *Daphnia* metapopulation in relation to parasitism. J. R. Soc. Interface 4:777-785.

Edwards KJ, Barker JHA, Daly A, Jones C, Karp A (1996). Microsatellite libraries enriched for several microsatellite sequences in plants. Biotechniques, 20: 758-760.

Edwards CA, Bohlen PJ, Linden DR, Subler S. (1995). Earthworms in agro-ecosystems. In: Earthworm Ecology and Biogeography in North America, (Hendrix PF, Ed.). Lewis Publishers, Boca Raton. pp. 185-213.

Edwards CA, Bohlen, PJ. (1996). Biology and ecology of earthworms. 3rd edn. London: Chapman & Hall.

Ellis JT, McMillan D, Ryce C, Payne S, Atkinson R, Harper PAW. (1999). Development of a single tube nested polymerase chain reaction assay for the detection of *Neospora caninum* DNA, International Journal for Parasitology, 29 (10), 1589-1596.

Estoup A, Garnery L., Solignac M, Cornuet JM. (1995). Microsatellite variation in honeybee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models, Genetics 140 679-695.

F

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791.

Field SG, Schirp HJ, Michiels NK. (2003). The influence of *Monocystis* sp. infection on growth and mating behaviour of the earthworm *Lumbricus terrestris*. Canadian Journal of Zoology 81: 1161-1167.

Field SG, Kurtz J, Cooper EL, Michiels NK. (2004). Evaluation of an innate immune reaction to parasites in earthworms. Journal of Invertebrate Pathology 86: 45-49.

Field SG, Michiels NK (2005). Parasitism and growth in the earthworm Lumbricus terrestris: fitness costs of the gregarine parasite Monocystis sp. Parasitology 130: 1–7.

Field SG. (2005). The Influence of the Sporozoan Parasite *Monocystis* sp. on the Reproductive and Population Biology of the Earthworm *Lumbricus terrestris* L. Doctoral Thesis. University of Münster, Münster, Germany.

Field SG, Michiels NK (2006). Acephaline gregarine parasites (Monocystis sp.) are not transmitted sexually among their lumbricid earthworm hosts. Journal of Parasitology 92(2): 292-297.

Field SG, Lange M, Schulenburg H, Velavan TP, Michiels NK (2007) Genetic diversity and parasite defense in a fragmented urban metapopulation of *Lumbricus terrestris*. Animal. Conservation. 10 (2) 162–175.

Frank SA. (1994). Coevolutionary genetics of hosts and parasites with quantitative inheritance. Evol. Ecol. 8, 74-94.

Fritz RS, Simms EL (ed.) (1992). Plant resistance to herbivores and pathogens. University of Chicago Press.

G

Gandon S, Capowiez Y, Dubois Y, Michalakis Y, Olivieri I. (1996). Local adaptation and gene for-gene coevolution in a metapopulation model. Proceedings of the royal Society of London B 263: 1003-1009.

Gandon S, Van Zandt PA (1998). Local adaptation in host-parasite interactions. Trends in Ecology and Evolution, 13, 215-216.

Gandon S, Michalakis Y. (2000). Evolution of parasite virulence against qualitative and quantitative host resistance. Proc. R. Soc. Lond. Ser. B 267, 985–990.

Gandon S, Michalakis Y. (2002). Local adaptation, evolutionary potential and host-parasite coevolution: interactions between migration, mutation, population size and generation time. Journal of Evolutionary Biology 15: 451-462.

Ganusov VV, Bergstrom CT, Antia R. (2002). Within-host population dynamics and the evolution of microparasites in a heterogeneous host population. Evolution 56, 213–223.

Gerbi S A. (1986). Chapter 7 - Evolution of ribosomal DNA. Pp. 419-517 In: Molecular evolution, ed. McIntyre, R.

Giggleman MA, Fitzpatrick LC, Goven AJ, Venables BJ. (1998). Effects of pentachlorophenol on survival of earthworms (*Lumbricus terrestris*) and phagocytosis by their immunoactive coelomocytes. Environmental toxicology 17: 2391-2394.

Goddard, M. R., Godfray, H. C. J., Burt, A. (2005). Sex increases the efficacy of natural selection in experimental yeast populations. Nature 434, 636–640.

Goggin CL. (1994). Variation in the two internal transcribed spacers and 5.8S ribosomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). Molecular and Biochemical Parasitology 65: 179-182.

Goudet J, Raymond M, de Meeüs T, Rousset F (1996). Testing differentiation in diploid populations. Genetics, 144, 1933– 1940.

Goudet J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www.unil.ch/izea/softwares/fstat.html.

Grenfell BT, Pybus OG, Gog JR., Wood J LN., Daly J.M., Mumford JA., Holmes, E. C. (2004). Unifying the epidemiological and evolutionary dynamics of pathogens. Science 303, 327–332.

Grove AJ. (1925). On the reproductive processes of the earthworm, *Lumbricus terrestris*. Quarterly Journal of Microscopical Science 69: 245-290.

Guindon S, Gascuel O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52(5): 696-704.

Gunn A, (1992). The use of mustard to estimate earthworm populations. Pedobiologia 36:65-67.

Guo SW, Thompson EA (1992). Performing the exact test for Hardy–Weinberg proportions for multiple alleles. Biometrics, 48, 361–372.

H

Haag CR, Sakwin´ ska O, Ebert D. (2003). Test of synergistic interaction between infection and inbreeding in *Daphnia magna*. Evolution 57, 777–783.

Haase M, Karlsson A. (2004). Mate choice in a hermaphrodite: you won't score with a spermatophore. Animal Behaviour 67: 287-291.

Haldane JBS. (1949). Disease and evolution. Ric. Sci. 19 (Suppl. A), 168–174.

Hamilton R, Boots M, Paterson S (2005).The effect of host heterogeneity and parasite intragenomic interactions on parasite population structure. Proc Biol Sci. 22; 272(1573): 1647–1653.

Hamilton WD. (1980). Sex versus non-sex versus parasite. Oikos 35: 282-290.

Hamilton WD, Zuk M. (1982). Heritable true fitness and bright birds: a role for parasites? 218: 384-387.

Hamilton WD, Axelrod R, Tanese R. (1990). Sexual reproduction as an adaptation to resist parasites (a review). Proceedings of the National Academy of Sciences USA 87: 3566- 3573.

Harper GL, Cesarini S, Casey SP, Morgan AJ, Kille P, Bruford MW (2006). Microsatellite markers for the Earthworm *Lumbricus rubellus*. Molecular Ecology Notes. Vol. 6, 325-327.

Haase M, Karlsson A. (2004). Mate choice in a hermaphrodite: you won't score with a spermatophore. Animal Behaviour 67: 287-291.

Hedrick PW, Kim TJ, Parker KM. (2001). Parasite resistance and genetic variation in the endangered *Gila topminnow*. Anim. Conserv. 4:103–109.

Hillis DM, Bull JJ. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42: 182-192.

Hnida JA, Duszynski DW. (1999). Taxonomy and systematics of some *Eimeria* species of murid rodents as determined by the ITS1 region of the ribosomal gene complex. Parasitology 119: 349 – 357.

Hoogerkamp M, Rogaar H, Eijsackers HJP. (1983). Effect of earthworms on grassland on recently reclaimed polder soils in the Netherlands. In Earthworm ecology: from Darwin to vermicultur: 85–105. Satchell, J.E. (Ed.). London: Chapman & Hall.

Howard RS., Lively CM (1998). The maintenance of sex by parasitism and mutation accumulation under epistatic fitness functions. Evolution 52 (2):604-610, 1998.

Hudson P, Greenman J (1998). Competition mediated by parasites: biological and theoretical progress. Trends Ecol Evol 13:387–390.

I J

Ilmonen P, Penn DJ, Damjanovich K, Morrison L, Ghotbi L, Potts WK. (2007). Major Histocompatibility Complex heterozygosity reduces fitness in experimentally infected mice. Genetics 176: 2501–2508

Jarne P, Lagoda PJ L., (1996). Microsatellites, from molecules to populations and back. Trends ecol. Evol. 11:424-430.

Jarne P, Théron A (2001). Genetic structure in natural populations of flukes and snails: a practical approach and review. Parasitology, 123, 27-40.

K

Kaltz O, Shykoff J. (1998). Local adaptation in host-parasite systems. Heredity 81: 361-370.

Kawecki TJ, Ebert D. (2004). Conceptual issues in local adaptation. Ecology Letters 7: 1225-1241.

Kautenburger R. (2006). Genetic structure among earthworms (*Lumbricus terrestris L.*) from different sampling sites in western Germany based on random amplified polymorphic DNA. Pedobiologia 50 (3): 257-266.

Keller LF, Waller DM. (2002). Inbreeding effects in wild populations. Trends Ecol. Evol. 17:230–241.

Kirchner JW, Roy BA (2002). Evolutionary implications of host– pathogen specificity: fitness consequences of pathogen virulence traits. Evol. Ecol. Res. 4, 27–48.

Kleemann GA, Basolo AL. (2007). Facultative decrease in mating resistance in hermaphroditic *Caenorhabditis elegans* with self-sperm depletion. Animal Behaviour 74: 1339-1347.

Klein J, O'Huigin C (1994) MHC polymorphism and parasites. Phil Trans R Soc Lond B 346:351–358.

Koene JM, Pfortner T, Michiels NK (2005). Piercing the partner's skin influences sperm uptake in the earthworm *Lumbricus terrestris*. Behavioral Ecology and Sociobiology 59(2): 243-249.

Kover PX. (2000). Effects of parasitic castration on plant resource allocation. Oecologia 123: 48-56.

L

Lee K, Foster RC. (1991). Soil fauna and soil structure. Australian Journal of Soil Research 29: 745-776.

Levin BR, Bull JJ. (1994). Short-sighted evolution and the virulence of pathogenic micro-organisms. Trends Microbiol. 2, 76–81.

Little TJ (2002). The evolutionary significance of parasitism: do parasite-driven genetic dynamics occur ex silico? J. Evolutionary Biology 15 (1), 1-9.

Lively CM. (1999). Migration, virulence, and the geographic mosaic of adaptation by parasites. Am. Nat., 153: S34–S47.

Lively CM, Dybdhal MF. (2000). Parasite adaptation to locally common host genotypes. Nature 405: 679-681.

Lively CM, Dybdhal MF, Jokela J, Osnas EE, Delph LF. (2004). Host sex and local adaptation by parasites in a snail – trematode interaction. *American Naturalist* 164: S6-S18.

Lopez-Pascua LDC, Buckling A (2008). Increasing productivity accelerates host-parasite coevolution Journal of Evolutionary Biology 21 (3), 853–860.

Lowe C. University of Central Lancashire. Earthworm Research Group: FAQ. 18 Feburary 2003. http://www.uclan.ac.uk/facs/science/erg/faqs.htm Accessed 9 October 2006.

M

Mackinnon DL, Hawes, RJS. 1961. Class Sporozoa. In: An Introduction to the Study of Protozoa. Oxford University Press, London, pp. 156–213.

Mackinnon MJ, Read AF. (1999). Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. Evolution 53, 689–703.

May RM., Anderson RM. (1983). Epidemiology and genetics in the coevolution of parasites and hosts. Proc. R. Soc. Lond. B 219, 281-313.

McCoy KD (2003). Sympatric speciation in parasites — what is sympatry? *Trends in Parasitology*, 19, 400–404.

Mes THM, Cornelissen AWCA. (2004) Non-homogenized ITS regions in the parasitic nematode *Cooperia oncophora* Parasitology, 129, (2), 213-222.

Michalakis Y, Excoffier L (1996). A generic estimation of population subdivision using distances between alleles with special references to microsatellite loci. Genetics, 142, 1061–1064.

Michiels NK, Newman LJ. (1998). Sex and violence in hermaphrodites. Nature 391(6668): 647-647.

Michiels NK, Hohner A, Vorndran IC. (2001). Precopulatory mate assessment in relation to body size in the earthworm *Lumbricus terrestris*: avoidance of dangerous liaisons? Behav. Ecol. 12, 612–618.

Mitchell CE, Power AG (2003). Release of invasive plants from fungal and viral pathogens. Nature 421:625–627.

Morand S, Manning SD, Woolhouse MEJ (1996).Parasite-host coevolution and geographic patterns of parasite infectivity and host susceptibility. Proceedings of the Royal Society of London, Series B: Biological Sciences, 263, 119-128.

Morgan T. (1994). Models of Sexual Selection in Hermaphrodites, Especially Plants. The American Naturalist Vol. 144: 100-125.

N

Nadler SA (1995). Microevolution and the genetic structure of parasite populations. Journal of Parasitology, 81, 395-403.

Nuutinen V, Butt KR. (1997a). The mating behaviour of the earthworm *Lumbricus terrestris* (Oligochaeta: Lumbricidae). Journal of Zoology (Lond) 242: 783-798.

Nuutinen V, Butt KR. (1997b). Pre-mating behaviour in the earthworm *Lumbricus terrestris* L. Soil Biology and Biochemistry 29: 307-308.

Nuutinen V, Butt KR. (1998). The mating behaviour of the earthworm *Lumbricus terrestris* (Oligochaeta: Lumbricidae). Journal of Zoology (Lond) 242: 783-798.

O

O'Brien, S. J. (2000). Adaptive cycles: parasites selectively reduce inbreeding in Soay sheep. Trends Ecol. Evol. 15:7–9.

Ouborg NJ, Biere A., Mudde CL. (2000). Inbreeding effects on resistance and transmission-related traits in the Silene-Microbotryum pathosystem. Ecology 81, 520–531.

P

Penn DJ, Potts WK. (1999). The evolution of mating preferences and major histocompatibility complex genes. American Naturalist 153: 145-164.

Penn DJ. (2002). The scent of genetic compatibility: sexual selection and the major histocompatibility complex. Ethology 108: 1-21.

Penn DJ, Damjanovich K, Potts WK. (2002). MHC heterozygosity confers a selective advantage against multiple- strain infections. Proc. Natl. Acad. Sci. USA 99, 11260–11264.

Petit RJ, Mosaudik AEl, Pons O. (1998). Identifying populations for conservation on the basis of genetic markers. Conserv. Biol. 12:844–855.

Pižl V, Sterzynska M (1991). The influence of urbanization on the earthworm infection by Monocystid gregarines. Fragmenta Faunistica *(Warsaw)* 35 (9-14).

Posada D, Crandall KA (1998). Modeltest: testing the model of DNA substitution. Bioinformatics 14 (9): 817-818

Posada D, Buckley TR. (2004). Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. Systematic Biology 53: 793-808.

Poulin R (1998). Evolutionary ecology of parasites: from individuals to communities. Chapman & Hall, London.

Poulin R, Hecker K, Thomas F. (1998). Hosts manipulated by one parasite incur additional costs from infection by another parasite. Journal of Parasitology 84, 1050-1052.

Poulin R, Morand S. (2000). The diversity of parasites. Q. Rev. Biol. 75:277–293.

Price PW (1980). Evolutionary Biology of Parasites. Princeton University Press, Princeton, NJ.

Puurtinen M, Hytönen M, Knott KE, Taskinen J, Nissinen K, Kaitala V. (2004). The effects of mating system and genetic variability on susceptibility to trematode parasites in a freshwater snail, *Lymnaea stagnalis*. Evolution 58, 2747–2753.

QR

Raymond M, Rousset F (1995). GENEPOP 3.4, Population genetics software for exact tests and ecomenicism. Journal of Heredity, 86, 248-249.

Read AF. (1994). The evolution of virulence. Trends Microbiol. 2, 73–76.

Regoes RR. (2000). Evolution of virulence in a heterogeneous host population. Evolution 54, 64–71

Reid JM, Arcese P, Keller LF. (2003). Inbreeding depresses immune response in song sparrows (*Melospize melodia*): direct and inter-generational effects. Proc. Roy. Soc. Lond. Ser. B 270, 2151–2157.

S

Sauter A, Brown JFM (2001). To copulate or not? The importance of female status and behavioural variation in predicting copulation in a bumblebee. Animal Behaviour Vol. 62(2), 221-226.

Shackleton E A. MacDougall, Derryberry EP, Foufopoulos J, Dobson PA, Hahn PT. (2005) Parasite-mediated heterozygote advantage in an outbred songbird population. Biol. Lett. 1, 105–107.

Schmid-Hempel P, Variation in immune defence as a question of evolutionary ecology. Proceedings of the Royal Society London B, (2003) 270, 357-366.

Schmidt GD, Roberts LS. (2000). Foundations of parasitology. 6th edn. Boston: McGraw-Hill Publishing.

Schmidt GD, Roberts LS. (2005). Foundations of parasitology. Boston: McGraw-Hill Publishing.

Schneider S, Roessli D, Excoffier L (2000). *ARLEQUIN* ver: 2000 a software for population genetic data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Schulenburg, JH, Hancock JM, Pagnamenta A, Sloggett JJ, Majerus MEN, Hurst GDD. (2001). Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). Molecular Biology and Evolution 18:648-660.

Šimková A, Lafond T, Ondračková M, Jurajda P, Ottová E, Morand S. (2008) Parasitism, life history traits and immune defence in cyprinid fish from Central Europe, *BMC Evolutionary Biology* (8) 1-11.

Simpson AJG, Dias Neto E, Johnston DA., Kaukas A, Rollinson, D. (1993). Recent molecular approach to the study of schistosome genetics. Experimental Parasitology 77, 376-379.

Sims RW, Gerard BM. (1985). Earthworms: Synopses of the British Fauna (New Series), No. 31. Linnean Society London and The Estuarine and Brackish-Water Sciences Association, Brill & Backhuys, London.

Smith JR, Carpten JD, Brownstein MJ, Ghosh S, Magnuson VL, Gilbert DA, Trent JM, Collins FS (1995). Approach to genotyping errors caused by nontemplated nucleotide addition by Taq DNA polymerase. Genome Research, 5, 312-317.

Sorci G, Moller AP, Boulinier T (1997). Genetics of host-parasite interactions. *TREE* Vol 12(5), 196-200

Spielman D, Brook BW, Briscoe DA, Frankham R. (2004). Does inbreeding and loss of genetic diversity decrease disease resistance? Conserv. Genet. 5, 439–448.

Springbett AJ, MacKenzie K, Woolliams JA, Bishop SC. (2003). The contribution of genetic diversity to the spread of infectious diseases in livestock populations. Genetics 165, 1465–1474.

Stevens L, Yan GY, Pray LA. (1997). Consequences of inbreeding on invertebrate host susceptibility to parasitic infection. Evolution 51, 2032–2039.

Subler S, Kirsch A. (1998). Spring dynamics of soil carbon, nitrogen, and microbial activity in earthworm middens in a no-till cornfield. Biology and Fertility of Soils 26: 243-249.

T U V

Tamura K, Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10: 512–526.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.

Telschow A, Engelsta¨dter J, Yamamura N, Hammerstein P, Hurst GDD (2006). Asymmetric gene flow and constraints on adaptation caused by sex ratio distorters. J Evol Biol 9:869–878.

Thompson JN. (1994). The Coevolutionary process. Univ. of Chicago Press, Chicago, IL.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876 -4882.

Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM (2003). Introduced species and their missing parasites. Nature 421:628–630.

Trouvé S, Degen L, Renaud F, Goudet J. (2003). Evolutionary implications of a high selfing rate in the freshwater snail *Lymnaea truncatula*. Evolution 57, 2303–2314.

VandenBygaart AJ, Fox CA, Fallow DJ, Protz R. (2000). Estimating earthworm-influenced soil structure by morphometric image analysis. Soil Science Society of America Journal 64: 982-988.

Velavan TP, Schulenburg H, Michiels NK (2007). Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris L*) Molecular Ecology Notes. 7 (6): 1060-1062.

W

Webster P, Woolhouse MEJ (1998). Selection and strain specificity of compatibility between snail intermediate hosts and their parasitic schistosomes. Evolution 52, 1627-1634.

Webster P, Woolhouse MEJ (1999). Cost of resistance: relationship between reduced fertility and increased resistance in a snail-schistosome host parasite system. Proceedings of the Royal Society of London, B 266, 391-396.

Webster JP, Davies CM (2001). Coevolution and compatibility in the snail-schistosome system. Parasitology. 123 Suppl: S41-S56.

Webster JP, Gower CM (2006). Mate choice, frequency dependence, and the maintenance of resistance to parasitism in a simultaneous hermaphrodite. Integrative and Comparative Biology 46(4): 407-418.

Wegner KM, Kalbe M, Milinski M, Thorsten BHR (2008) Mortality selection during the 2003 European heat wave in three-spined sticklebacks: effects of parasites and MHC genotype BMC Evolutionary Biology 8:124

Weir BS, Cockerham CC. (1984). Estimating *F*-statistics for the analysis of population structure. Evolution 38:1358–1370.

West SA., Lively CM, Read AF. (1999). A pluralist approach to sex and recombination. J. Evol. Biol. 12:1003–1012.

Wiehn J, Kopp K, Rezzonico S, Karttunen S, Jokkela J. (2002). Family-level covariation between parasite resistance and mating system in a hermaphroditic freshwater snail. Evolution 56, 1454–1461.

Wilfert L, Gadau J, Baer B, Schmid- Hempel P. (2007). Natural variation in the genetic architecture of a host-parasite interaction in the bumblebee *Bombus terrestris*. Molecular Ecology 16(6), 1327-1339.

X Y Z

Yang Z. (1993). Maximum likelihood estimation of the phylogeny from DNA sequences when substitution rates differ over sites. Mol. Biol. Evol. 10:1396–1401.

Zietara MS, Lumme J. (2002). Speciation by host switch and adaptive radiation in a fish parasite genus Gyrodactylus (Monogenea: Gyrodactylidae). Evolution 56, 2445–2458.

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Appendix

LEBENSLAUF

Name	VELAVAN THIRUMALAISAMY PALANICHAMY
Born	15.05.1980 in Oddanchatram, India
Maritalstatus	Married
Nationality	Indian

School Education

June 1994 – April 1995	Junior High school: Christian. Matriculation school.
June 1995 – April 1997	High School: T.M.H.N.U.Higher secondary school.

University Education

June 1997 – June 2000	Madurai-Kamaraj University, Tamilnadu, India. Bachelors in Microbiology spl. (*Immunology)*
July 2000 – June 2002	Barkatullah University, Madhya Pradesh, India Masters in Microbiology
July 2002 – May 2004	Junior research fellow (Project) Centre for Cellular and Molecular biology (CCMB), India. **Project 1:** Conservation genetics of marine turtles on the mainland and island coasts of India. **Project 2:** Understanding Amphibian endemism in Western Ghats of India.
July 2004 – June 2005	Doctorate student of Prof.Dr. Nico Michiels at Institute for Animal Evolution and Ecology, Faculty of Biology at Westphalen – Wilhelms University, Muenster.
July 2005 – July 2008	Doctorate student of Prof.Dr. Nico Michiels at Department of Animal Evolutionary Ecology, Zoological Institute, Faculty for Biology at Eberhard Karls University, Tuebingen.

Publications

Novo M., **Velavan T.P**., Díaz-Cosín D.J., Almódvar A, Hinrich Schulenburg and Michiels N.K. (2008): Microsatellite markers for the drought- resistant earthworm *Hormogaster elisae*. Molecular Ecology Resources. 8 (4): 901-903. (Equal Authorship)

Ramesh K Aggarwal, Albert Lalremruata, **Velavan TP**, Pavani Sowjanya, Lalji Singh (2008): Development and characterization of ten novel microsatellite markers from olive ridley sea turtle (*Lepidochelys olivacea*). Conservation Genetics (9) :981-984

Velavan TP, Hinrich Schulenburg, Nico Michiels (2007): Development and characterization of novel microsatellite markers for the common earthworm (*Lumbricus terrestris L*) Molecular Ecology Notes. 7 (6): 1060-1062

Field SG, Lange M, Schulenburg H, **Velavan TP**, Michiels NK (2007): Genetic diversity and parasite defense in a fragmented urban metapopulation of *Lumbricus terrestris*. Animal Conservation 10 (2) 162–175.

Gururaja KV, Aravind NA, Sameer Ali, Ramachandra TV, **Velavan TP**, Krishnakumar V , Aggarwal RK. (2007): Morphogenetic description of a new frog species from the Central Western Ghats of India: An Indicator species of Forest Fragmentation. Zoological Science 24 (5): 525-534.

Thangaraj K, Sridhar V, Kivisild T, Reddy AG, Chaubey G, Singh VK, Kaur S, Agarawal P, Rai A, Gupta J, Mallick CB, Kumar N, **Velavan TP**, Suganthan R, Udaykumar D, Kumar R, Mishra R, Khan A, Annapurna C, Singh L. (2005): Different population histories of the Mundari- and Mon-Khmer-speaking Austro-Asiatic tribes inferred from the mtDNA 9-bp deletion/insertion polymorphism in Indian populations. Hum Genet. 116(6):507-17

Ramesh K Aggarwal, **VelavanTP**, D.Uday Kumar, Prasad.S.Hendre, Karthik Sanker, Lalji Singh (2004): Development and characterization of novel microsatellite markers from the olive ridley sea turtle (Lepidochelys olivacea). Molecular Ecology Notes. 4 (1): 77-79

Karthikeyani T.P., **VelavanTP**, Ramesh, M., (2002): Physico-Chemical and Biological Characterization of river Shanmughanadhi, TamilNadu. Environment and Ecology 20(2): 482-486.