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

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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.

Key words: *Monocystis* sp., ribosomal RNA, polymerase chain reaction.

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 3 characteristic phases during their life cycle (Schmidt and Roberts, 2000; Bush *et al*. 2001). (i) Infection phase – worms become 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 (2 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 forming a typical fusiform shape. Then 2 or 3 cell divisions follow to form 8 sporozoites inside 1 spore. At this point the gametocyst ruptures releasing the many sporocysts into the seminal fluid and eventually into the environment to repeat the life cycle. 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 the *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

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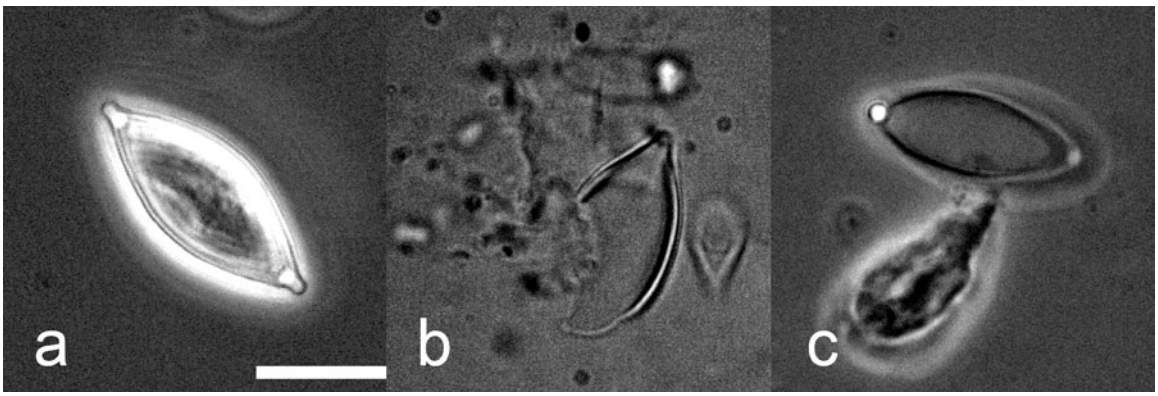

Fig. 1 (a) Encapsulated sporocyst before excystation; (b and c) excysted sporocyst after sonication. (Scale bar = 1 μm and magnification is 100 × using phase-contrast microscopy.)

(Gerbi, 1986). Consequently, comparisons of ITS sequences have proven useful in studies on the evolutionary biology of populations and species (e.g. Mes and Cornelissen, 2004; Gaspar da Silva *et al*. 2007).

The ITS region has 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 Tübingen, Rottenburg and an individual of Canadian origin obtained from National Bait Inc. (www.nationalbait.com), which provides field-collected worms from a single location in Ontario, Canada. Based on the morphology of sporocysts under light microscopy, *Monocystis* sp. were identified. Sporocysts of *Monocystis* vary in size depending upon species and their life-cycle 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: 25 mM NaCl, 4 mM KCl, 6 mM $CaCl_2$, 1 mM $MgCl_2$, 26 mM Na_2SO_4, 2 mM Tris and 55 mM 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 12 000 ***g*** 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 3 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 1 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′-GAGAAGTCTT-GTAAACCCAATT-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

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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, 1 × 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 of PCR errors during amplification with *Taq* polymerase. PCR products (8 μl) were 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 1 host individual was inserted into a TA cloning vector (TOPO cloning Kit, Invitrogen). The TA vector containing the ITS region was 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 specific amplification of the *Monocystis* sp. We ensured that the new primers showed 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 2 host individuals from Tübingen and 1 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, 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%,

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Table 1. Variation between 18S, ITS-1, ITS-2 and 5·8S rRNA sequences of *Monocystis* sp.

	Tübingen individual (A)				Tübingen 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	

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 each of 4 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). In addition, exactly 2 identical clones were found for 3 genotypes from the Tübingen host individual A, for 4 genotypes from Tübingen host individual B, 3 genotypes from the Rottenburg host individual and for 3 genotypes from the Canadian host. We never obtained more than 2 identical clones per identified genotype. The 34 bp 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 3 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% is indicated for individual branches. All Canadian genotypes clustered in 1 clade except for 1 genotype (C1_19). In contrast, genotypes from Tübingen and Rottenburg hosts were scattered across the tree.

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 parasite genetic 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 individual hosts 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 × 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 ($8{\cdot}9 \times 10^{-5}$ 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

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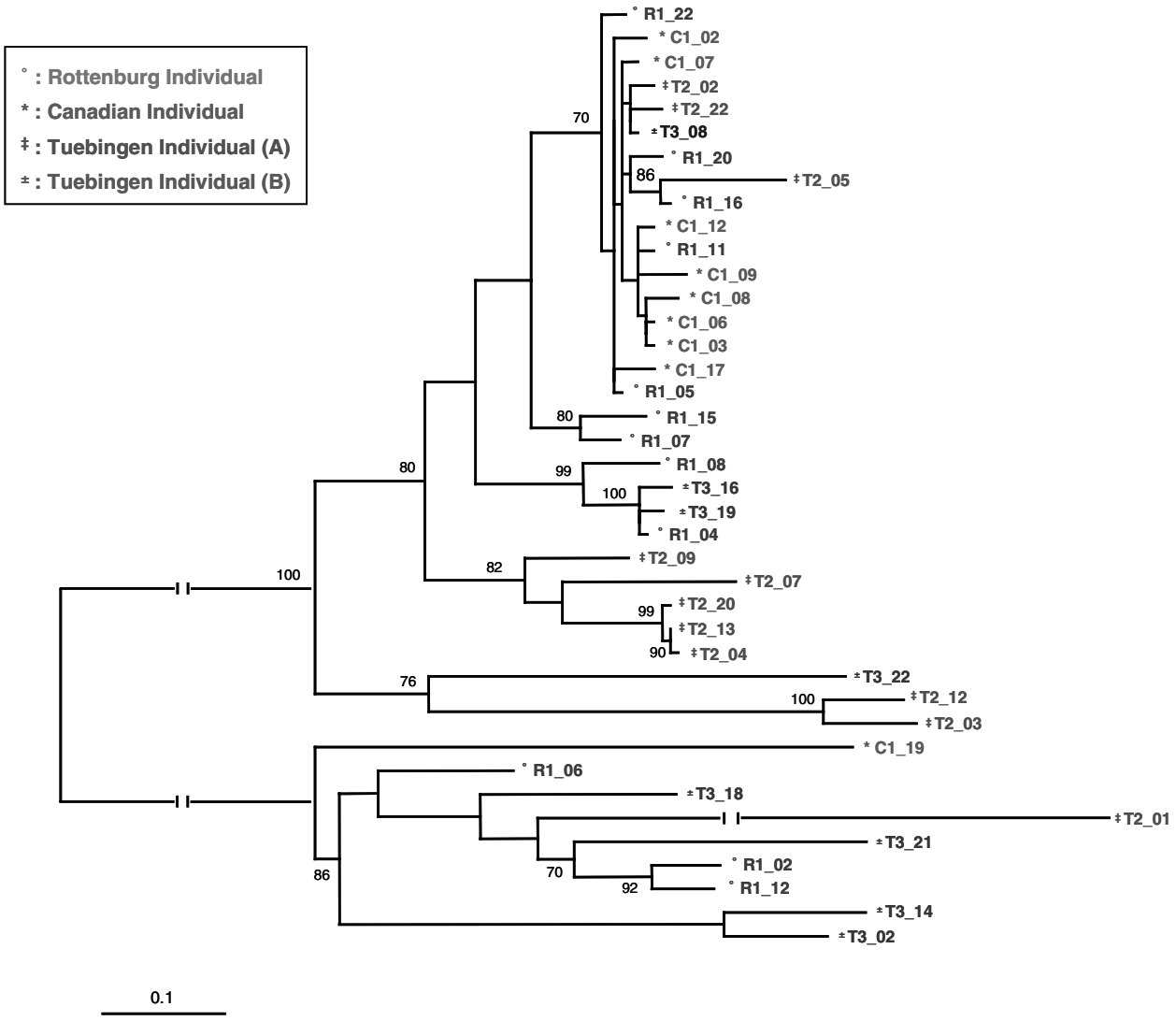

Fig. 2. Maximum likelihood tree (log likelihood = −5569·84232) inferred with the Tamura-Nei substitution model with Gamma rates across sites from the complete ITS region sequences (956 sites). ML bootstrap support was inferred from 500 replicates. Only values larger than 70 are indicated.

formula we obtained the mutation frequency as 62·3 mutations/kb. The mutation frequency obtained for our amplicon (862 bp) will then be 53·70 mutations/862 bp. However, we observed 128–165 variable sites across the complete ITS region. Given that we observe more variable sites than expected by PCR errors (53·70 variations/862 bp), such variations are 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 known to result from polymerase errors during PCR. We are therefore confident that the nucleotide variations observed in our data set are true polymorphisms. As another alternative, intra-host sequence diversity may be due to the fact that *Monocystis* sp. possesses more than one ribosomal cistron and that the different copies are not identical in response to homogenizing molecular processes based on unequal crossing-over (i.e. concerted molecular evolution; Dover, 1982; Grauer and Li, 2000). However, to our knowledge, protist genomes do not contain more than a handful (i.e. less than 5) copies of the ribosomal cistron (Flamant *et al*. 1984; Rooney, 2004). Therefore, it is unlikely that all sequence variants are caused by such variation within individual parasites. Furthermore, sporocysts contain the haploid product of meiosis, such that heterozygous parasites would produce 2 different alleles per locus after selfing. This process could lead to the presence of variation even if the host is only infected by a single parasite genotype. Nevertheless, as above, this process is unlikely to be sufficient to explain the level of sequence diversity observed by us within individual hosts. Therefore, we conclude that individual earthworms are host to at least more than one *Monocystis* genotype.

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At the same time, it is as yet impossible to infer whether the observed sequence variation is indicative of different *Monocystis* species or only genotypes from a single species based on sequence variation. Relatively few trophozoites are observed and one can achieve differentiation by isolating trophozoites across species based on histological expertise. This differentiation could be further inferred only if one could amplify the ITS region from individual and 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. This was the first study of this kind to characterize intra-host genetic variation in *Monocystis* infections of earthworms. This study may enable us to clarify ideas regarding evolution of virulence in relation to within-host competition. Moreover the level of clonal diversity may provide information on rates of transmission. The level of diversity that we infer from this study should allow us to distinguish possibly different genotypes from a single *Monocystis* strain. Infections are quite frequent, and this infection occurs in the host by ingesting soil containing the sporocysts. These sporocysts are at a haploid stage during this infection stage. This effectively means each new infection contributes to multiple genotypes (see the Introduction section for their life cycle). 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 also to understand dynamic coevolution between hosts and parasites. In brief, it will facilitate our study of intra-host diversity of these *Monocystis* genotypes across host populations and relate the observed genetic diversity measures of *Monocystis* to that of heterozygozity levels, inbreeding, allelic diversity and of fitness parameters across the host subpopulations. Moreover, the level of genetic diversity observed across *Monocystis* genotypes could potentially be used to infer the 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% ($F_{ST}=0{\cdot}16$, $P=0{\cdot}04$) as inferred from an analysis of molecular variance (Schneider *et al*. 2000) between the here included host populations from Tübingen 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, variations in the life cycle 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 life cycle (see the Introduction section), 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 multitude of parasites. 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 Tübingen individual (A): FM174711 – FM174721, Tübingen individual (B): FM174712 – FM174729, Rottenburg individual: FM174730 – FM174741 and for Canadian individual: FM174742 – FM174750.

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