Prevalence and dispersal of a facultative bacterial symbiont associated with an endemic metazoan host

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ABSTRACT

Co-dispersal is of primary importance in symbiotic relations between macro- and micro-organisms. Obligate symbionts generally follow the same dispersal pattern as their host, while it still remains unresolved whether hosts and their facultative symbionts follow the same dispersal patterns. Here we addressed the question of symbiont and host co-dispersal by analyses of the earthworm *Lumbricus terrestris*, and its facultative bacterial symbiont *Verminephrobacter*. We analyzed co-dispersal by direct sequence-based typing of the earthworm mitochondrial ND4, and the symbiont *adk* genes, respectively. A total of 96 earthworms from Norwegian ($n = 20$), German ($n = 20$), English ($n = 31$) and Canadian ($n = 25$) populations were analyzed. We found that the earthworms were mainly endemic, while the symbiont showed a more complex dispersal pattern. The symbiont showed a significant difference in prevalence between the geographic regions. In addition, we found a low degree of co-evolution between host and symbiont, with some of the symbiont sequence types being globally distributed. Future research, however, is needed to resolve whether the symbiont is truly globally distributed, in contrast to its endemic host.

Introduction

Symbiotic relations between macro- and microorganisms are frequent in nature. An important property in symbiosis is co-dispersal between host and symbiont. The co-dispersal mechanisms are relatively well characterized for obligate symbionts, while much less is known about facultative symbionts (Russell et al. 2003; Dale and Moran 2006; Chiel et al. 2009).

There is an ongoing debate of whether microorganisms, in contrast to macroorganisms, show a global dispersal pattern (Fierer and Jackson 2006; Martiny et al. 2006). Global versus endemic distribution is of fundamental importance for understanding dispersal of symbiotic microorganisms. For microorganism symbionts the basic question addressed is whether the dispersal is driven mainly by niche selection (Baas-Becking 1934), or by dispersal restrictions (Whitaker et al. 2003). If niche selection is the main driving force then horizontal transfer and global distribution patterns would be expected, while if dispersal is the main limitation then vertical dispersal by descent in the macroorganism host would be expected leading to an endemic distribution.

Earthworms are metazoan macroorganisms with a slow intrinsic dispersal rate (Marinissen and Vandenbosch 1992) that have a *Verminephrobacter* symbiont in their nephridia (Pinel et al. 2008). It has been shown that the symbionts are recruited very early during embryo development (Davidson and Stahl 2008). Studies suggest that *Verminephrobacter* has co-evolved with the lumbricid earthworms over approximately 100 million years, with an overall phylogeny corresponding to the host species (Lund et al. 2009). The intraspecies distribution, however, still remains unknown. Determining the intraspecies distribution pattern is of particular interest in unraveling the mechanism of the host–symbiont relation, since niche selection promotes a global distribution pattern, while direct transfer from the parents to the embryo would contribute to an endemic distribution of the symbiont.

The aim of the present work was to use the earthworm *Lumbricus terrestris* and its *Verminephrobacter* symbiont as a model to investigate the co-dispersal between a metazoan host with restricted distribution, and its bacterial symbiont. We addressed this by direct culture-independent genotyping of both host and symbiont of earthworms from four geographic regions, and through analyses of the stability of the *Lumbricus–Verminephrobacter* interaction in a laboratory microenvironment.

We present data supporting a complex distribution of a facultative symbiont in an endemic host. These findings are discussed in the light of general theories for microorganism and macroorganism evolution and dispersal.

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Materials and methods

Earthworm populations

Earthworms of the species *Lumbricus terrestris* from Canada, Britain, Germany and Norway were used in the experiments. The Canadian population was commissioned from the National Bait Inc. (Mississauga, Ontario, Canada). There were two British populations; the first British population was collected in Preston, United Kingdom by a Ph.D. student of Prof. K.R. Butt at the University of Central Lancashire, while the second population was ordered from The Recycle Works Ltd. (Ribchester, United Kingdom). The German earthworms came from a natural population in Saarbrücken and were collected by Prof. Dr. R. Klein at the University of Trier, while the Norwegian population was collected in Grue municipality, Hedmark, Norway.

Twenty individuals from each population, except the German, were placed in their respective 3 L box with approximately 2.5 L soil from Magic Products, Inc., Amherst Junction, USA, and fed once per week with the Magic Worm Food (Magic Products). The German earthworms died immediately after arrival in our laboratory from Trier, so these worms were not farmed for further sampling. The mortality for the rest of the populations was investigated by determining the fraction of the earthworms that died in a time-course over a period of approximately one year.

Stability analyses

To investigate if the number of symbiotic *Verminephrobacter* is stable over time, we took three samples approximately each month from 15 earthworms – five from each of the three populations (British, Canadian and Norwegian). The rationale for this number was our experimental capacity. We also tried to cure three earthworms by treatment with antibiotics. This was done by adding a 1:1 mix of kanamycin and ampicillin at a concentration for 1 mg/g feed. Antibiotics were administered for 17 days.

DNA purification

Approximately 50 mg samples of the anterior part of live earthworms were immediately dissected upon arrival. The samples were conserved in 96% ethanol and stored at −20 °C before further processing. Tissues were lysed by adding 10 μL lysis buffer (Qiagen, Hilden, Germany) and 0.08 μL Proteinase K (25 mg/mL) per 1 mg of tissue. The samples were subsequently incubated at 56 °C for approximately 2 h until the tissues were completely lysed. The lysate was diluted with lysis buffer at a 1:1 ratio before automated DNA isolation on a Genom-96 robot using the MagAttract DNA, Blood M96 Kit (Qiagen), or manual DNA isolation using the Qiagen Blood and Tissue Kit. DNA concentrations and purity were measured using a NanoDrop ND 1000 spectrophotometer from NanoDrop Products, Wilmington, DE, USA.

PCR amplification and DNA sequencing

We evaluated the four householding genes *adk, fumC, icd* and *rpoB* as genetic markers for the *Verminephrobacter* symbiont in *L. terrestris*. PCR primers were designed from the published *Verminephrobacter eiseniae EF01-2* genome sequence (GenBank accession number CP000542) using CLC Main Workbench software (CLCbio, Århus, Denmark). DNA isolated from *Verminephrobacter eiseniae* EF01-2 was utilized as template for the initial PCR optimization experiments. Thermocycling conditions were investigated using gradient-PCR with the following reaction mix: 1× AmpliTaq Gold reaction buffer, 2 mM MgCl₂, 2 mM dNTP's, 0.2 μM of each primer, and 0.3 U/μL AmpliTaq Gold DNA

Table 1
Oligonucleotides applied in this work.

Target[a]	Sequence
fumC F	5′-TGA GCA GAT CCA AAG CAA-3′
fumC R	5′-TTC GGG GCG ATC GAG ATT-3′
icd F	5′-GGT TGC GGT TGT TGT CCA G-3′
icd R	5′-AAG GCG CGT GAT GTG AAG-3′
rpoB F	5′-CTT ACA TCT CGC ACA CCC T-3′
rpoB R	5′-CCA TCA CCA CCA GTT CCT C-3′
adk F1	5′-GCA TCC CGC AAA TCT CCA-3′
adk R1	5′-CAG TGG CTG TAG TAG TCC-3′
adk F2	5′-GAC TGA TTC TGT TGG GCG-3′
adk R2	5′-CGC TTT CTG ACG GTT TCT-3′
adk F3	5′-ATC CCG CAA ATC TCC AC-3′
adk R3	5′-CGC TTT CTG ACG GTT TC-3′
ND4 F	5′-TTG GGT GTC AAA AAT CAC TTC-3′
ND4 R	5′-TAA ATT GTC AGC CAG AAT CAA AC-3′
adk qPCR F	5′-GGC TTT TTG TTT GAC GGC TT-3′
adk qPCR R	5′-GGC GTT TCT TGA CGG TGT-3′
adk qPCR P	5′-CTT CCT TGT CGT CTT CGC GCT-3′
18S qPCR F	5′-TCC CAG TAA GCG CGA GTC AT-3′
18S qPCR R	5′-ACG GGC GGT GTG TAC AAA G-3′
18S qPCR P	5′-AGC TCG CGT TGA TTA CGT CCC TGC-3′

[a] F, forward primer; R, reverse primer; P, probe.

polymerase (Applied Biosystems, Foster City, CA, USA). The thermocycling conditions tested were as follows: one cycle of 95 °C for 10 min; 40 cycles of 95 °C for 30 s, gradient 50–65 °C for 30 s, 72 °C for 90 s; one final extension cycle at 72 °C for 7 min. The marker *adk*, with the F1 and R1 primes (Table 1) was chosen for the main genotyping experiments using an annealing temperature of 55 °C. Blast searches in the NCBI Microbial Genomes database showed that the primers were specific to the *Verminephrobacter eiseniae EF01-2* genome. Two additional *adk* forward (F2 and 3) and reverse primers (R2 and 3) were also constructed and evaluated during the course of the work (Table 1). The rationale for choosing *adk* for genotyping was that this gene gave the strongest amplification in the evaluation. We only chose one gene from the symbiont based on the previous suggestion that the genome structure of *Verminephrobacter* is relatively clonal (Lund et al. 2009). The mitochondrial *ND4* gene was used for *Lumbricus terrestris* amplification and sequencing, as previously described by Field et al. (2007). All PCR primers used are shown in Table 1.

DNA was sequenced using the Applied Biosystems Big Dye Terminator sequencing kit v1.1 on an ABI 3100 Genetic Analyzer according to the manufacturer's instructions. The generated sequences were deposited in GenBank with accession numbers GU799332 to GU799396 for *ND4* and GU799397 to GU799415 for *adk*.

Real-time quantitative PCR

For symbiont quantification we designed *adk* gene primers and probes on the basis of six phylotypes of the gene found during the course of this work. Amplification of *adk* was normalized against earthworm DNA as determined by amplification of the nuclear 18S RNA gene using primers and probes designed from published sequences (accession numbers GQ337499). Q-PCR was carried out in 25 μL using the following reaction conditions: 1× AmpliTaq Gold reaction buffer, 0.1 μM TaqMan-probe, 0.2 μM of each primer, 200 μM dNTP, 2 mM MgCl₂ and 5U AmpliTaq Gold DNA polymerase (Applied Biosystems). The thermocycling conditions used were: one cycle at 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 60 °C for 1 min. The Q-PCR was performed using the Applied Biosystems 7500 Real-Time PCR system. The primers and probes employed are described in Table 1.

Phylogenetic reconstruction and population comparisons

The sequences were aligned using the Clustal application in the CLC Main Workbench software. Based on the alignments, maximum parsimony phylogenetic trees were constructed using the Mega 4.1 software (Kumar et al. 2001). Maximum parsimony trees were used as inputs to the Component 2.0 program (Roderic D.M. Page, The Natural History Museum, London, UK) for earthworms with both *ND4* and *adk* sequences. A Nelson consensus tree was constructed based the largest group of most frequently replicated clusters in both trees (Nelson 1979). Finally, we determined the nearest neighbor interchange between the two trees (Waterman and Smith 1978).

The population pair-wise genetic distances were computed as differences in fixation index (F_{ST}) distances using the implementation in Arlequin, where F_{ST} is a measure of population differentiation based on genetic polymorphism data (Hudson et al. 1992). Neighbor-joining phylogenetic trees were subsequently constructed based on the distance data (Saitou and Nei 1987).

The potential endemism of a particular phylotype was determined by comparing the observed geographic distribution to random permuted distributions. The significance of the endemic distribution is determined by the frequency of the random distributions showing equal or more geographic structure than that observed.

Results

Lumbricus terrestris population structure and phylogeography

The mitochondrial *ND4* gene sequence from 65 earthworms included in our work showed 16 different phylotypes (Fig. 1 A). Seven of the phylotypes showed an endemic distribution, and one a non-endemic pattern (Table 2). The non-endemic sequence type (nd4–12), however, was only found in Canadian and English earthworms. Endemism of the remaining 8 sequence types could not be classified due to limited sample size.

Taking into account both the sequence divergence and frequencies of the different phylotypes, the population structure showed significant differences for all pairwise comparisons (results not shown). A neighbor-joining tree based on these pairwise differences showed that the Canadian and English populations were most closely related, while the German population was the most distant (Fig. 1B).

Verminephrobacter prevalence, population structure and phylogeography

We identified *Verminephrobacter* in 76% of the earthworms using Q-PCR (Suppl. Table 1). The prevalence was highest in the German and Canadian populations with 90%, and 84%, respectively. The prevalence in the British population was 74%, while the Norwegian population had a prevalence of 50%. We found that there
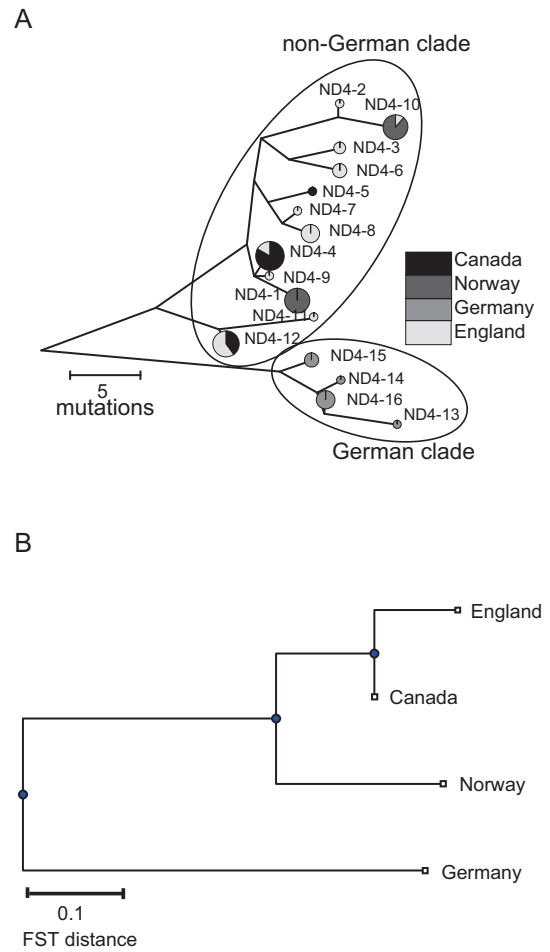
Fig. 1. Earthworm phylogeograpic dispersal patterns. (A) Maximum parsimony tree for the *ND4* gene. The size of the circle represents number of sequences in that sequence type. The smallest circle represents a single sequence, while the largest represents ten sequences. The grayscale represents the geographic origin. (B) Neighbor-joining tree describing the genetic structure of populations based on pair-wise F_{ST} distances.

were significant differences in the prevalence of bacteria in British and German populations (pB/T = 0.02), and between Norwegian and the German populations (pN/A = 0.01). There was no significant difference in bacterial prevalence between the rest of the populations (pB/C = 0.76, pB/N = 0.37, pC/N = 0.39, pC/T = 0.14).

We obtained good quality *adk* sequences from 20% of the earthworms in our dataset using the F1 and R1 primer pair (Table 1). The PCR products leading to low quality sequences consisted of multiple low molecular weight bands indicating misspriming. We therefore constructed two additional forward (F2 and 3) and reverse (R2 and 3) primers (Table 1). All combinations of *adk* forward and reverse primers were evaluated to obtain PCR products of expected size

Table 2
Phylogeograpic *ND4* sequence type distribution.

Country	Sequence types[a]															
	nd4-1	nd4-2	nd4-3	nd4-4	nd4-5	nd4-6	nd4-7	nd4-8	nd4-9	nd4-10	nd4-11	nd4-12	nd4-13	nd4-14	nd4-15	nd4-16
Canada	0	0	0	10	1	0	0	0	0	0	0	4	0	0	0	0
England	0	1	2	2	0	3	1	5	1	1	1	6	0	0	0	0
Germany	0	0	0	0	0	0	0	0	0	0	0	0	1	1	3	5
Norway	9	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0
Endemic	Yes	n.d	n.d	Yes	n.d	Yes	n.d	Yes	n.d	Yes	n.d	No	n.d	n.d	Yes	Yes

[a] The potential endemism of the sequence types are indicated at the $p < 0.05$ level. Endemism for the sequence types labeled n.d. could not be determined due to limited number of isolates.
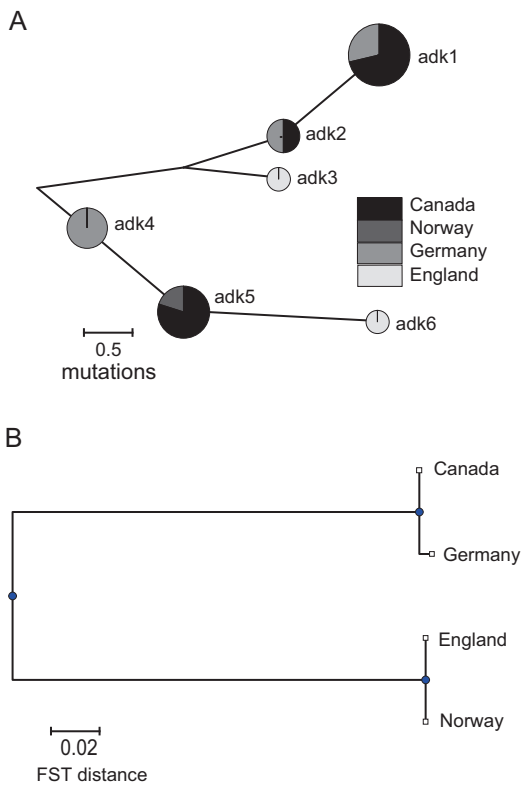
Fig. 2. Verminephrobacter phylogeograpic dispersal patterns. (A) Maximum parsimony tree for the *adk* gene. The size of the circle represents number of sequences in that sequence type. The smallest circle represents a single sequence, while the largest represents seven sequences. The grayscale represents the geographic origin (B) Neighbor-joining tree describing the genetic structure of populations based on pair-wise F_{ST} distances.
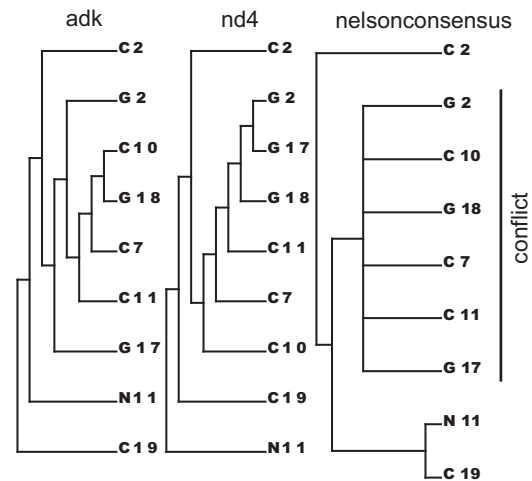

Fig. 3. Earthworm and *Verminephrobacter* co-evolution. Maximum parsimony trees were generated for earthworms with both *ND4* (A) and *adk* (B) sequence information. Based these trees were a Nelson consensus tree constructed with a nearest neighbor interchange distance between the *ND4* and *adk* trees of 9. Letter designations are as follows, C (Canadian), N (Norwegian) and G (German).

while the *adk* sequences did not show the same clustering pattern. This was also reflected in the consensus tree with a conflicting branching pattern for the German earthworms. The only three earthworms, two Canadian (C2 and C19) and one Norwegian (N11), showed congruency between *adk* and *ND4*.

Simulated microenvironment experiments

The temporal stability of symbiotic *Verminephrobacter* was analyzed in a selection of 15 earthworms over a period of approximately six months. These analyses revealed comparatively large fluctuations in the relative abundance of *Verminephrobacter*, but with no particular trends (Suppl. Table 2). In three of the earthworms, *Verminephrobacter* was not detected at any of the time-points, indicating the absence of *Verminephrobacter* ($p = 0.01$, given an assumption of lack of detection by chance). In an attempt to cure the earthworms of *Verminephrobacter*, we treated four earthworms using a combination of ampicillin and kanamycin for 17 days. All earthworms showed a reduction in their *Verminephrobacter* content (Suppl. Table 3).

With respect to our investigations of worm mortality and *Verminephrobacter* prevalence, we observed that individuals of the German population, which died shortly after arrival, showed the highest quantity and prevalence of *Verminephrobacter*. For the other populations, however, we did not detect correlation between the earthworms that died and *Verminephrobacter* content during the experimental period of approximately one year (results not shown).

Discussion

The phylogeographic distrubution of mitochondrial *ND4* sequences confirmed the endemism of *L. terrestris*, with all the German earthworms being on a relatively deep separate branch. Previously, geographic structuring of *L. terrestris* was also observed within Germany (Kautenburger 2006). On the other hand, we found that the English, Norwegian and Canadian sequence types were intermixed, with the British population having the highest diversity. An interpretation of this result is that the Canadian and Norwegian *L. terrestris* populations originated from England. This is likely, given significant human migration between these regions (Tiunov et al. 2006).

from the samples with low quality sequences. However, no additional products were obtained (results not shown).

We identified 6 phylotypes for the 19 earthworms with good quality *adk* sequences (Fig. 2A), with one showing an endemic pattern and three a non-endemic pattern (Table 3). The remaining 2 phylotypes could not be classified due to low frequencies. The pairwise comparison of population sequence type divergence and frequencies showed that none of the populations were significantly different (results not shown). Although the pair-wise distances were not significant, the neighbor-joining tree supported a clustering of English and Norwegian, and German and Canadian *Verminephrobacter*, respectively (Fig. 2B).

Co-evolution analyses

The potential co-evolution of earthworms and their symbionts was analyzed by comparing earthworm mitochondrial *ND4* and *Verminephrobacter adk* trees. This comparison showed that there were generally large discrepancies between the two trees (Fig. 3). The *ND4* sequences showed a clustering of the German earthworms,

Table 3
Phylogeographic *adk* sequence type distribution.

Country	Sequence types					
	adk-1	adk-2	adk-3	adk-4	adk-5	adk-6
Canada	5	1	0	0	4	0
England	0	0	1	0	0	1
Germany	2	1	0	3	0	0
Norway	0	0	0	0	1	0
Endemic	No	No	n.d.	Yes	No	n.d.

Unfortunately, we obtained too few sequences to draw rigorous conclusions about endemism or global distribution for *Verminephrobacter*. Interestingly, however, the dispersal of German *Verminephrobacter adk* sequence types showed that 2 of 3 sequence types also were found in other regions. Only one *adk* sequence type showed an endemic German distribution. Furthermore, the low degree of co-evolution between *Verminephrobacter* and *L. terrestris* further supports the conclusion that *Verminephrobacter* is not strictly vertically inherited. The degree of vertical dispersal, however, needs to be investigated in larger studies.

Recent evidence suggests that multiple bacterial species can colonize the nephridia of earthworms (Davidson et al. 2010). We can therefore not rule out multiple *Verminephrobacter* strain colonizations of a single earthworm. If this is the case, then the disconcordance between earthworm and *Verminephrobacter* evolution can be explained by a population of *Verminephrobacter* strains, rather than single strain, having co-evolved with the earthworm host.

It has been shown that it is possible to cure earthworms of *Verminephrobcater* (Davidson and Stahl 2006), supporting the hypothesis that this symbiont is facultative. This facultative symbiosis hypothesis is also supported by our results through the apparent absence of *Verminephrobacter* in a range of earthworms. Facultative symbionts have an increased frequency of recombination compared to obligate symbionts (Hoffmeister and Martin 2003). Recombination could potentially distort single gene phylogenies. Future studies should also include multiple *Verminephrobacter* genes to obtain rigorous phylogenetic information.

The maximum divergence for the neutral sites between two *Vernminephrobacter adk* sequences detected in our dataset was approximately 6%. Using the evolutionary rate determined by Lund et al. (2009) for 16S rRNA, and calibrating this by the expected 16S rRNA gene and synonymous site evolution rate (Kuo and Ochman 2009), the estimated age of the lumbricid symbiotic *Verminephrobacter* common ancestor is about 40 million years. This roughly corresponds to the origin of *L. terrestris* (Lund et al. 2009), so it seems likely that *Verminephrobacter* has co-evolved with the *L. terrestris* population. The data by Lund et al. (2009), however, support a model where *Verminephrobacter* does not cross species barriers. In future studies it would be interesting to determine the nature of these barriers, and to determine the detailed global distribution of *Verminephrobacter*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pedobi.2010.12.005.

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