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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 expermients. Thermocycling conditions were investigated using gradient-PCR with the following reaction mix: 1 × AmpliTaq Gold reaction buffer, 2 mM $MgCl_2$, 2 mM dNTP's, 0.2 μM of each primer, and 0.3 U/μL AmpliTaq Gold DNA 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 expermients 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.

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.

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 $MgCl_2$ 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.