McNair Scholars Journal

Volume 11 | Issue 1 Article 6

2007

# 16S rRNA DGGE Analysis of Gut and Hepatopancreas Microflora in the Isopod Oniscus asellus

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# 16S rRNA DGGE Analysis of Gut and Hepatopancreas Microflora in the Isopod Oniscus asellus

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**Abstract**

*The microflora (microbes) of the gastrointestinal tract have been found to play an important role in the health of animals. In addition to pathogenic microbes and their associated negative effects, beneficial microbes have important positive effects. However, the dynamics of the transmission and colonization of the microflora between mates and between parents and offspring are still largely unknown. Variation in the microflora between individuals suggests that selection can play a role in this transmission and colonization. As a preliminary step to investigate the transfer of microflora between male and female isopods, microbial DNA from* Oniscus asellus, *washed and unwashed gastrointestinal tracts and hepatopancreas glands were isolated and characterized using 16S rRNA primers and DGGE analysis. Males and females showed considerable variation between microflora in gastrointestinal tracts while hepatopancreas samples were more similar.*

**Introduction**

The microflora of the gastrointestinal tract plays an important role in the health of its host. In addition to pathogenic microbes, beneficial microbes have several positive effects on the health of the host (Rastall et al., 2005). The microflora (all the microbial inhabitants) provide metabolic functions, protection from invasion by pathogenic microbes, and modulation of the immune systems. The resident or indigenous microflora (authochthonous) is often specialized for the gastrointestinal tract while the transient microflora (allochthonous) that is passing through is not. A classic example is the specific microbial communities found in ruminants. Agriculture has taken advantage of these beneficial effects by feeding microbes (probiotics) to domestic animals. While the majority of the microflora studies have concentrated on vertebrate animals, the microflora in invertebrates is equally important (Dillon and Dillon, 2004). The role of the microflora in the digestion of cellulose in termites is one striking example.

The role and transmission of microbes is an important aspect in understanding how microflora affect host health, but outside of specific pathogenic organisms our understanding of how microbes are transmitted between individuals is limited. It is possible that this transmission of beneficial microbes is favored by natural selection (Lombardo et al., 1999). Model systems for the study of the transmission of microflora in a variety of organisms would be helpful in understanding their roles.

The terrestrial isopoda are potential systems for studying microflora, their effects, and their transmission between individuals (Hassal et al., 2005). Terrestrial isopods provide an important role in the breakdown and processing of detritus (Zimmer, 2002). How much isopod nutrition comes from digestion of organic material in detritus versus digestion of microbes growing on the detritus is unclear. However, the isopod gastrointenstinal tract, like all tracts, is specialized for

the maintenance of a specific indigenous microflora (Zimmer and Brunne, 2005). The isopoda gastrointestinal tract is simple, with a midgut and short hindgut. Two paired hepatopancreas glands empty into the anterior end of the midgut and aid in digestion (Zimmer, 2002). Previous work done on the microbes in isopods that inhabit these tissues has been limited in scope. None of these studies attempted to carry out a complete survey of the microbial community. Swiecicka and Mahillon (2006) focused on the diversity of *Bacillus cereus* strains isolated from the isopod *Porcellio scaber* to see how similar *B. cereus s.l.* isolates were and what their relationship with the *Porcellio scaber* host was. They found that strains extracted from the digestive tract of the isopods from three closely located sites had identical PFGE patterns, virulence gene content, and enterotoxicity. This indicated that the isolates had strong genetic and genomic relationships. Also, 70% of the isopods used in this experiment were found to contain *B. cereus s.l.* strains, which suggests that the digestive tract of the isopod may be one of the natural niches of this bacteria.

Kostanjsek et al. (2004) isolated and identified anaerobic bacteria from *Porcellio scaber* hindgut by amplifying 16sRNA gene segments and cloning them. They found the presence of resident anaerobic bacteria in the gut of *Porcellio scaber*. It has been suggested that anaerobic bacteria would not be present in the gut of isopods because of the short retention time of food in the tube-like hindgut. In addition, the analysis of the 16S rRNA gene sequences displayed a fair amount of them originating from organisms that have recognized anaerobic inhabitants of vertebrate gastrointestinal tracts such as affiliates of the genus *Bacteroides*.

Kostanjsek et al. (2004) and Wang et al. (2004) investigated the microflora of the hepatopancreas in *Porcellio scaber* isopods. The ecological role of these microflora and their relationship to isopod health is unclear. Electron microsopy of the hepatopancreas detected a number of rod-shaped, translucent oblong bacteria resident in the hepatopancreas. Kostanjsek et al. (2004) used 16S rRNA gene to characterize these species. Phylogenetic analysis placed these bacteria in order *Chlamydiales*, forming a distinctive lineage to the family *Simkaniacaea*. They proposed the name '*Candidatus* Rhabdochlamydia porcellionis' for these bacteria.

Wang et al. (2004) also investigated bacteria in the hepatopancreas of *Porcellio scaber* isopods. These bacteria are closely associated with the epithelial surface of the hepatopancreas. 16S rRNA analysis showed that the bacteria represent a lineage of the *Mollicutes* and are distantly related to members of the *Mycoplasmatales* and *Entomoplasmatales*. They proposed the name '*Candidatus* Hepatoplasma crinochetorum' for these bacteria.

PCR amplification of 16S rRNA variable sequences is a powerful tool to characterize the total number of species and strains in a mixed sample (for a review see Muyzer, 1999). The main objective of this study is to characterize the mircroflora of isopods using universal prokaryotic 16S rRNA primers. The extent of variation between males and females, between washed and unwashed gastrointestinal tracts, and the hepatopancreas will provide a more detailed basis for further study of the function and transmission of the microflora in isopods.

## Materials and Methods

**Isopod Collection and Identification**
Isopods were collected by hand during June and July 2007 from an established household compost pile in Grand Rapids, Michigan. Isopods were identified as either male or female *Oniscus asellus* under the dissecting microscope using a standard key (Van Name, 1936). Isopods were collected the same day as they were used for DNA extraction.

**Isopod Dissection and DNA Extraction**
Isopods were rinsed in distilled water and then placed in 70% ethanol for 2 minutes to surface sterilize them (Swiecicka and Mahillon, 2006). Isopods were then put into the freezer for five minutes prior to dissection. Isopods were dissected in a small amount of sterile water. The entire gastrointestinal tract and one of the two paired hepatopancreas glands were dissected out. For washed gastrointestinal tracts, the tract was split longitudinally and washed sequentially in three separate petri dishes in a small amount of sterile water. DNA was extracted using a DNeasy Blood and Tissue Extraction Kit from Qiagen. Tissues were pretreated with lysozyme by first adding 180 uL lysozyme buffer and then grinding with a sterile pestle for approximately 15 seconds. Solid lysozyme was then added to a concentration of 20 mg/mL and the sample incubated for 30 minutes at 37 °C. The sample was then treated with proteinase K following the DNeasy supplementary protocol for the total DNA extraction from ticks, and the DNA extracted following the spin column protocol. DNA samples were then stored at 4 °C until use.

**PCR Amplification**
16S rRNA DNA was amplified using primers for positions 518 and 338 with a 40 bp GC clamp (Nakatsu et al., 2000). 5 uL of DNA was amplified in a final volume of 25 uL using Ready-To-Go beads from GE Healthsystems. Amplification conditions were 94 °C for 4 minutes followed by 33 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 45 seconds. The final step was 72 °C for 8 minutes. Amplification products were checked on 4% E-Gels from Amersham. Commercially prepared *E. coli* B DNA (Sigma Aldrich) was used as a positive amplification control.

**DGGE Analysis**
Amplified samples were separated on a 6% DGGE gel with a 30% to 70% urea/formamide gradient. Normally gels were run for five hours at a constant 40 mA. The gels were stained for fifteen minutes using either Sybron Green or Sybron Gold and photographed under UV light. Banding patterns were analyzed using Gel2K software (Svein Norland, Department of Microbiology, University of Bergen, Norway) to produce a binary file of banding patterns. A Jaccard similarity tree based on these binary files was constructed using a CLUSTER program packaged with the Gel2K program.

**Results**
Figure One shows the results of CLUSTER analysis for washed gastrointestinal

samples from 5 males and 5 females. Males had an average of 3.4 ± 2.07 bands while females had an average of 3.2 ± 1.64 bands. The overall average was 3.30 ± 1.77. The number of bands detected per individual ranged from 6 to 2. The overall Jaccard similarity is low as indicated by deep branch points in the diagram. This indicates relatively low band sharing between individuals. Additionally, there is no sex-specific grouping between males and females.

Figure Two shows the results of CLUSTER analysis for unwashed gastrointestinal samples from 4 males and 4 females. Males had an average of 7.00 ± 2.94 bands while females had an average of 7.25 ± 2.63 bands. The overall average was 7.13 ± 2.59. The number of bands detected per individual ranged 11 to 4. The overall Jaccard similarity is again very low, indicating relatively low band sharing between individuals. Additionally, there is no sex-specific grouping between males and females. The average number of bands is significantly higher using a T-test for unwashed samples compared to washed samples for both males (1 sided T-test, $p = 0.034$), and females (2 sided T-test, $p = 0.025$), as well as for all samples pooled together (2 sided T-test, $p = 0.002$).

Figure Three displays the results of CLUSTER analysis for hepatopancreas samples from 7 males and 7 females. Males had an average of 3.57 ± 2.15 bands and females averaged 3.00 ± 1.29 bands. The overall average was 3.29 ± 1.73. The number of bands detected ranged from 2 to 7. The overall Jaccard similarity is higher, indicating band sharing between individuals but there is no sex-specific grouping. A prominent feature in many individuals is a doublet of closely migrating bands as seen in individuals F6, F2, M5, F4, M7, and M6. On the gel image these bands are the most prominent products. In individuals such as F7 and M1 another prominent doublet is present.

**Discussion**

The DGGE method presented here is successful in isolating and characterizing the microflora from both gastrointestinal tracks and the hepatopancreas. This should allow the characterization of the microbial communities and provides the opportunities for identification of specific species and strains by DNA sequencing. Sequencing of hepatopancreas bands in particular will allow matching with the results of Kostjansek et al. (2004b) and Wang et al. (2004) to determine if the same species are found in *Oniscus* as in *Porcellio*. The variation in banding patterns in the hepatopancreas should allow an investigation of the effect of different strains or communities on isopod health.

The diversity of banding patterns in washed and unwashed gastrointestinal samples also provides similar opportunities. While no highly common bands were found between individuals or between sexes, identification of specific species should provide insight into their possible effects on isopod health. The difference in banding patterns between washed and unwashed samples indicates that isopods may have a well established indigenous microflora (authochthonous) in contrast to the transient microflora (allochthonous).

The next step in using isopods will be to establish pure lines of isopods under laboratory conditions. The stability and composition of isopod microflora in these lines can then be checked using this same DGGE approach. Determination of the species composition of bands will allow specific isopod lines to be used for mating and mate choice experiments. This will allow insight into the dynamics of microflora transmission and colonization.

**Acknowledgments**

We would like to acknowledge the support of the McNair Post-baccalaureate Achievement Program and their staff in funding this project. We also would like to thank Brittany Benson for support in the laboratory. ■

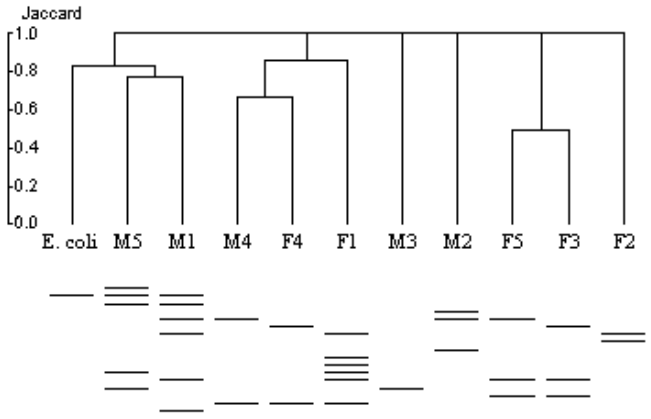


**Figure One.** CLUSTER diagram of DGGE of 16S rRNA from washed isopod *Oniscus asellus* gastrointestinal samples. 5 female (F) and 5 male (M) isopod samples were amplified and run on 6% DGGE gels and analyzed using Gel2K and CLUSTER software. Individuals had an average of 3.30 ± 1.77 bands. No sex-specific grouping is seen in these samples.

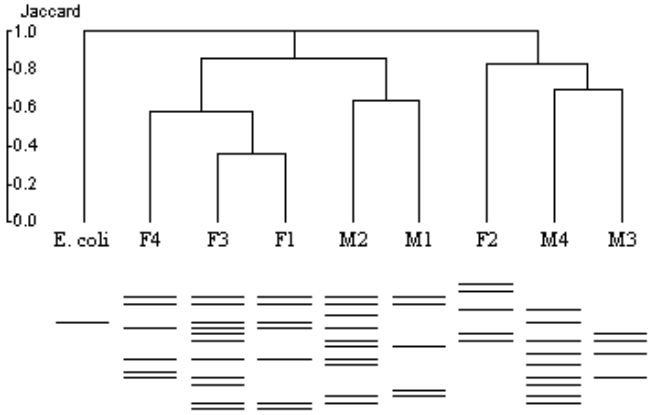


**Figure Two.** CLUSTER diagram of DGGE of 16S rRNA from unwashed isopod *Oniscus asellus* gastrointestinal samples. 4 female (F) and 4 male (M) isopod samples were amplified and run on 6% DGGE gels and analyzed using Gel2K and CLUSTER software. Individuals had an average of 7.13 ± 2.59 bands. No sex-specific grouping is seen in these samples.

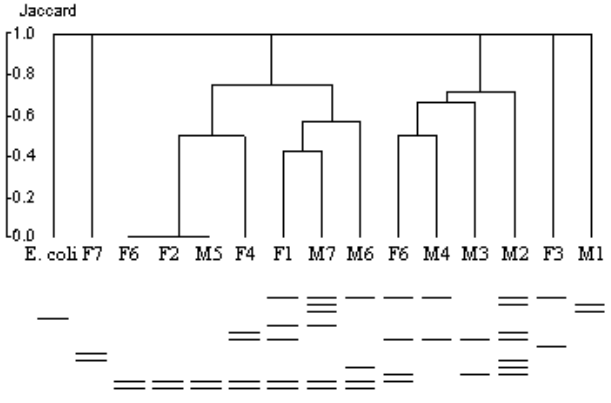


**Figure Three.** CLUSTER diagram of DGGE of 16S rRNA from isopod *Oniscus asellus* hepatopancreas samples. 7 female (F) and 7 male (M) isopod samples were amplified and run on 6% DGGE gels and analyzed using Gel2K and CLUSTER software. Individuals had an average of 3.29 ± 1.73 bands. No sex-specific grouping is seen in these samples. A common feature is the presence of two closely migrating bands in a majority of the samples. In some individuals, such as F7, these two bands are shifted, as compared to individuals such as F6, etc.

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