THE EFFECT OF NICKEL AND CADMIUM ON THE DIVERSITY OF THE *trfA2* GENE IN THE IncP PLASMID ORIGIN OF REPLICATION REGION

by

CATHERINE JEANNE KING

(Under the Direction of Charles H. Jagoe)

ABSTRACT

Sequences of the *trfA2* gene were compared to determine if exposure to cadmium or nickel increased IncP plasmid diversity. Community DNA was extracted from Savannah River sediments spiked with Cd, Ni, or unspiked controls, and the *trfA2* gene was amplified using established replicon-specific primers, cloned and sequenced. The majority of sequences were identified as *trfA2* genes from IncPβ plasmids, a few were identified as IncPα or IncPγ genes, but no IncPδ sequences were detected. However, research results also indicated the need for more selective primers for IncPδ and IncPγ subgroups. Considerable variability within the IncPβ *trfA2* sequences was observed, but AMOVA analysis did not indicate significant differences among treatments. Whittaker's Index of Association indicated that Cd and Ni plasmid communities were more similar to control communities than to each other.

INDEX WORDS: IncP plasmid, *trfA2* gene, Cadmium, Nickel, River sediment, Sequence diversity, Microcosms

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INTRODUCTION

While many heavy metals are toxic to bacteria, bacteria can develop resistance to toxic metals with sublethal exposures (7, 21, 22, 25, 31, 33). Smets *et al*. (37) noted that, in general, heavy metal concentrations greater than 1% have adverse effects on bacterial populations and concentrations between 1 and 10mM can be harmful to bacterial populations. However, bacteria can acquire increased resistance to metals by the acquisition and expression of heavy-metal-resistance genes (37). Silver (32), in a review of bacteria and metals, noted that most metal resistance genes are found on plasmids and that plasmids bearing metal-resistance mechanisms have been found in every Eubacteria group tested.

Recent studies (3, 41) have indicated that metal pollution may inadvertently increase antibiotic resistance because genes for both metal and antibiotic resistances can be carried on the same plasmid. McArthur and Tuckfield (20) observed a positive correlation between antibiotic resistance and mercury concentration in the sediments of a stream receiving effluents from an industrial/nuclear complex. Dhakephalkar and Chopade (12), in a study of *Acinetobacter* isolates from different environmental sources, found that 39 of 40 isolates were resistant to multiple metals and multiple antibiotics. One study looking at lead resistant isolates from soil found that all isolates had resistance to at least one antibiotic (26), and another study found that gram negative mercury resistant isolates from primate fecal material were also resistant with varying

frequencies to ampicillin, streptomycin, tetracycline, chloramphenicol, kanamycin, and sulfadiazine (51).

Even though the effects of metals on plasmid-born resistance genes are becoming better understood, the effects of metals on the movement of transferable genetic material in bacteria has not been as well studied, especially the movement and maintenance of plasmids within bacterial populations. One study by Saunders *et al*. (29) which looked at bacterial isolates from polluted and non-polluted locations found that plasmids were more numerous and larger at polluted sites. This study indicates that exposure of bacteria to pollutants may result in an increase in the quantity and variety of plasmids found in bacteria.

Bacterial plasmids are typically extra-chromosomal, double-stranded, covalently closed circular (CCC) pieces of DNA found in both gram positive and gram negative Eubacteria and some Archaea (38, 39). The two notable exceptions to the previous statement are linear plasmids and single-stranded plasmids found in some gram positive bacteria (38). Plasmids range in size from 2 kb to greater than 500 kb and reproduce autonomously (39). Plasmids carry non-essential genes that can be beneficial to the host cell under particular circumstances (38), such as resistance or degradation genes for antibiotic, metal, and xenobiotic compounds (39).

The number of copies of each plasmid per host cell can vary, and cells can contain more than one type of plasmid type. However, not all types of plasmids can coexist in the same host cell. Plasmids that cannot coexist are considered incompatible and are grouped into incompatibility (Inc) groups (38). Plasmids in the same Inc group share similar genetic material for replication and control (15).

IncP plasmids are part of a group of plasmids that are considered promiscuous, or broad-host-range, which means they can transfer to and be stable in most gram negative bacterial species (42). They also can be transferred, via conjugation, into some eukaryotic cells, yeast, and gram positive bacteria (1). They are divided into four subgroups, α, β, γ, and δ (50)). IncPα and β subgroups are classified based on sequence relatedness and have similar plasmid backbone regions containing genes essential for replication, stability, and transfer (1, 43). IncPα plasmids include many well-characterized plasmids such as RK2 and RP4, and IncPβ plasmids include well-characterized plasmids such as R751 (43).

RK2 and RK2-like plasmids have two regions involved in replication—an origin of vegetative replication (*oriV*) site and a *trans*-acting replication function *(trfA*) (42). The *trfA* gene, also referred to as the replication activating gene (1) and the replication initiation gene (16), is a promoter gene whose products are involved in the initiation of replication (14, 42) and in negative regulation of replication (1) at the *oriV* region.

We selected the *trfA2* gene to serve as an indicator of the diversity of IncP plasmids in this study based on the essential nature of the *trfA* gene in plasmid proliferation. Several papers have noted that the *trfA2* sequence can be used to identify IncPα or IncPβ plasmids (13, 14), and Smalla et al. (36) also noted that replicon-specific primers can be used to detect the presence of specific plasmids in community DNA extracts.

Because metal-resistance genes can be carried on IncP plasmids, we hypothesized that the diversity of IncP plasmids would be greater in bacterial communities exposed to metals. To explore the effects of metals on a natural

population of plasmids, we investigated the effects of two metals—cadmium and nickel—on the diversity of *trfA2* genes from IncP plasmids. Both of these metals are considered potentially polluting metals in the United States by the U.S. EPA (49). Cd is used in metal plating and coating, pigment manufacture, as a fungicide, and in Ni-Cd batteries. Cd is found in ores containing copper and lead and in fossil fuels such as coal. Cd can be released into the environment from naturally occurring sources or through anthropogenic activities such as smelting ore, burning of fossil fuels, and manufacturing. (48).

Ni is a natural component of the earth's crust, can be released by volcanic eruptions, and can be found in soils and sediment (4). It is a hard, malleable metal that is a good conductor of heat and electricity, and resists corrosion by strong alkalis. It is used in industry and manufacturing as a component of alloys and stainless steel, as a catalyst, and in plating, coloring, and battery manufacture (4). Ni can be released into the environment through anthropogenic activities including the combustion of fossil fuels, incineration, smelting operations, and other manufacturing activities.

We exposed bacterial communities to 100 μM Cd or 100 μM Ni to determine effects of the metals on the diversity of a natural population of the *trfA2* gene of IncP plasmids. These metals and concentrations were selected to correspond to conditions in a recent study by Stepanauskas *et al*. (in submission) (40), in which multiple resistances to metals and/or antibiotics were observed in cultured bacteria from microcosms of Savannah River water exposed to varying concentration of Cd, Ni, ampicillin, and tetracycline.

We hypothesized that the measurable diversity of IncP plasmids would increase when bacterial communities containing IncP plasmids were exposed to Cd or Ni. IncP plasmids that occurred in low frequency bacteria that contained Cd or Ni resistance mechanisms would be able to increase in frequency as more dominant non-resistant bacterial species were reduced due to the detrimental effects of Cd and Ni. For this hypothesis we assumed that the majority of the dominant bacterial species would not have Ni or Cd resistance mechanisms.

MATERIALS AND METHODS

Sediment and Water Collection and Preparation

The sediment and river water used for microcosms was collected on 12/6/04 from the Savannah River off Boat Ramp Dock, which is located on the Savannah River Site (SRS), Aiken County, SC, approximately 36 km south of Augusta, GA, USA. The dock is located near SRS road A-4.7. The sediment was collected approximately 9 m from shore and the water was collected approximately 11 m from shore. Four sediment samples were collected using an Ekman Bottom Grab Sampler and were transferred into sterile sampling bags (Fisherbrand #01-815-24) using a sterile beaker. River water was collected in a Nalgene Polycarbonate Square 1 L bottle (VWR #16121-220) that had been acid washed and autoclaved. The bottle was rinsed in river water 3 times prior to sample acquisition.

The sediment was transferred into an acid-washed, autoclaved 23 cm by 13 cm glass baking dish and was homogenized by manual manipulation. Coarse debris (leaves, sticks, stones) was removed from the sediment. Sediment was then aliquoted using autoclaved tongue depressors or pre-sterile specimen cups, and sediment was re-homogenized between aliquots. The bottle containing the river water was inverted several times to homogenize the water sample before each aliquot was removed.

Pre-Incubation Sampling

One 50 g wet weight aliquot of sediment was frozen for metal analysis and a 500 g wet weight aliquot was refrigerated for sediment characterization. Three

sediment samples, approximately 15 g each, were sonicated in 0.85% sterile saline in a Bransonic Ultrasonic Cleaner (Bransonic, B2200R-1), and the supernatant was preserved in 5% buffered formalin and used for bacterial enumeration. Six aliquots of sediment were put in sterile 2.0 mL Eppendorf tubes and frozen at -80^{o}C for later DNA extraction.

Three 5 mL water aliquots were preserved in 5% buffered formalin for bacterial enumeration and three 10 mL aliquots were filtered and preserved with 5% nitric acid for metal analysis. Three 50 g aliquots of sediment were placed in 50 mL conical tubes with enough de-ionized water to allow the sediment to settle. Upon settling, the average volume of the sediment in the conical tube was obtained. This average volume was used to determine the amount of metals to add to each microcosm.

Microcosms

Three microcosms each were set up for the Cd and Ni treatments and four microcosms were set up as controls. In random order, 50 g aliquots of sediment were measured into acid-washed, sterile, 400 mL beakers. Aliquots of 50 mL of Savannah River water for each microcosm were dispensed into 50 mL conical tubes water so that the metals could be added before the water was added to the microcosms. The microcosms were spiked by adding the appropriate volume of a filter-sterilized stock solution of $NiCl_2 \cdot 6H_2O$ or $CdCl_2$ to the river water to obtain a final concentration of 100 μM Ni (5.88 mg/L) or 100 μM Cd (11.25 mg/L) in the microcosm. The water/metal solution was mixed and gently poured over the sediment in the beaker. The control microcosms received an equivalent volume of filter-sterilized distilled water. After spiking, each beaker was covered with a sterile, breathable, adhesive film (Breathe

Easy Film, USA Scientific, #9123-6100). Microcosms were randomly placed in a shaker-incubator (New Brunswick, Innova 4080) and incubated at 23^{o}C and 75 rpm for 8 days. The position of each beaker in the shaker-incubator was randomly changed every day during the incubation period.

Mid-Incubation and Post-Incubation Sampling

An aliquot of water was taken from each microcosm on Day 4 and preserved in 5% buffered formalin for bacterial enumeration. After the aliquots were removed, each beaker was covered with new Breathe Easy Film. On Day 8, the water in each microcosm was decanted, homogenized by swirling, and one aliquot of the water from each microcosm was saved for bacterial enumeration and metal analysis as described above. Sediment from each microcosm was homogenized by mechanical manipulation, and from each microcosm 6 aliquots were saved for DNA extraction, 1 aliquot for bacterial enumeration, and 1 aliquot for metal analysis as described above.

Sediment Characterization

The sediment used in the microcosms was characterized from a 500 g sample (wet weight) for percent moisture content, electroconductivity (EC), pH, KCl pH, and particle size. All tests were performed in duplicate and the results averaged. Percent moisture was determined by weighing aliquots of sediment before and after drying overnight at 100^{o}C. Electroconductivity and pH were measured on a 10 g aliquot of air-dried sediment that had been resuspended in 20 mL nanopure water using a PHM95 pH/Ion Meter (Radiometer) with a combination electrode and a CDM210 Conductivity Meter (MeterLab, Radiometer). KCl pH was measured on an aliquot of sediment that

had been resuspended in a 1 M KCl solution. Particle size distribution was determined using the Bouyoucos hydrometer method (8).

Bacterial Abundance

Bacterial abundance was calculated from direct counts after staining with Acridine Orange (18) using 0.22 μm Poretics black polycarbonate 25 mm filters (Osmonics, Inc., K02BP02500). As mentioned earlier, aliquots of river sediment (pre-treatment) and sediment from each microcosm (post-treatment) were sonicated in sterile saline, and the supernatant was preserved in 5% buffered formalin. The sonicated sediment was dried in an Isotemp Programmable Oven (model 383F, Fisher Scientific) at 50^{o}C for at least a week to measure dry weight. Dilutions of the preserved sediment supernatant and water samples were made if necessary to obtain counts between approximately 5 and 40 bacteria/grid. Slides were counted using a Microphot-FXA epifluorescence microscope (Nikon), a Nikon PlanApo oil immersion (100x/1.40 oil) lens (Nikon), and a CFWN 10x/20 eyepiece. Ten randomly selected grids were counted per slide and the counts were averaged.

Metal Analysis

Sediment samples for metal analysis were stored frozen and were freeze-dried prior to analysis. Water samples were filtered using a 0.45 μm GHP membrane Acrodisc glass fiber filter (Pall Life Sciences, GF 25mm, PN 4559T) and were preserved in nitric acid (5% final concentration). Dried sediments were digested in a microwave oven (CEM Inc., Matthews, NC) in closed Teflon vessels containing concentrated nitric acid (USEPA Method 3052 (46)). Samples were diluted to appropriate volumes using high-purity deionized water. Metal concentrations were measured by Inductively

Coupled Plasma- Mass Spectrometry (USEPA Method 6020 (47)) on an ICP-MS Elan DRC Plus (Perkin-Elmer Sciex Instruments, Toronto, Canada). Samples of a soil (National Institute of Standards & Technology, Standard Reference Material 2711) with known, certified metal concentrations were digested along with sediments and analyzed for QA purposes.

DNA Extraction

Sixl aliquots of sediment were preserved at -80^{o}C in separate tubes for each microcosm. Three randomly selected aliquots of sediment were used per microcosm for separate DNA extractions. DNA was extracted from approximately 400mg of sediment using the FastPrep FP120 Instrument (Bio101, Savant, 6000-120) and the Bio101 FastPrep DNA SPIN Kit for Soil (Qbiogene, #6560-200) using the directions provided by the manufacturer with minor revisions (extra washes with 5.5 M guanidine thiocyanate to remove remaining humic material and air drying matrix for 30 minutes before elution). DNA was also extracted from a culture of *E. coli* HB101 containing plasmid RK2 (provided by Dr. Patricia Sobecky, Georgia Institute of Technology, Atlanta, GA) using the Bio101 FastPrep DNA Kit (Qbiogene, #6540-400) as per the manufacturer's instructions.

Extracted DNA was quantified by loading aliquots of extracts in Gel-Loading Buffer Type III (28) and running them on a 1% agarose (w/v) TBE-gel (28) containing 0.16-0.33 μg/mL ethidium bromide. DNA was electrophoresed using an Mini Sub CE, Model 17S/7645 electrophoresis system (Bio-Rad) and 80V applied with a Fisher Biotech Electrophoresis Systems FB105 (Fisher Scientific). Standards of Calf Thymus DNA (Sigma D4522) were also run on each gel and were used to determine the amount

of extracted DNA. Gels were imaged and quantified using an AlphaImager 3400 (Alpha Innotech, Cat. #IS-3400-5-110) using AlphaEaseFC (FluorChem 8800) Software for Windows, version 3.1.2.

Amplification of *trfA2* gene

PCR was performed using *trfA2* -1 5'-CGA AAT TCR TRT GGG AGA AGT A-3' forward primer and *trfA2* -2 5'-CGY TTG CAA TGC ACC AGG TC-3' reverse primer for the IncP plasmid *trfA2* gene (14). PCR reactions were set up with final concentrations of 0.5 μM each primer, 25 μg/mL BSA (bovine serum albumin, Sigma, B2518), 0.2 mM dNTPs (Promega Corp., U1240), 0.025 U/μL JumpStart Taq DNA Polymerase (Sigma D4184), 1X Reaction Buffer (Sigma D4184), 1.5 mM $MgCl_2$ (Sigma D4184), and 5 ng/μL template DNA. Reactions were brought up to 25 μL or 50 μL volumes using ultrapure water (Sigma, W4502). Reactions were run in an Eppendorf Mastercycler Gradient thermocycler for an initial cycle of 94^{o}C for 5 min, followed by 35 cycles of 94 ^{o}C for 30 sec, 57 ^{o}C for 30 sec, and 72 ^{o}C for 1 min. The program concluded with a 72 ^{o}C hold for 10 min. Water blanks were used as negative controls and genomic *E. coli* HB101 DNA containing plasmid RK2 DNA was used as the positive control. PCR product was imaged, sized, and quantified in the same manner described above except an Owl Model D3-14 Centipede Horizontal Electrophoresis System was used and the amplified inserts were sized by comparing with the Hi-Lo DNA Marker (Minnesota Molecular #1010) and were quantified by comparing with known concentrations of lambda DNA (New England BioLabs, N3011L).

Cloning of *trfA2* PCR product

The *trfA2* PCR product was ligated into the pCR 2.1-TOPO vector and the vector was used to transform One Shot TOP10 chemically competent cells using the TOPO TA Cloning Kit (Invitrogen, K4500-01) and the manufacturer's protocol. The transformed competent cells were plated on Luria Bertani (LB Agar, Miller) plates containing IPTG and S-gal (S-gal/LB Agar, Sigma C4478) and 50 μg/mL ampicillin, and the plates were incubated overnight at 37°C. Ninety-six individual white colonies were randomly removed from the plates using sterile (autoclaved) toothpicks and were incubated at 37°C and 150 rpm in Luria Bertani broth (Difco #0446-07-5) containing 50 μg/mL ampicillin. After approximately 24 hours of growth, the clones were refrigerated for several days and then were stored at -80°C in 17% (final concentration) glycerol.

The *trfA2* gene insert and about 200 bp flanking region of the pCR 2.1-TOPO vector was amplified from the clone cultures using M13f (5'-GTA AAA CGA CGG CCA GT-3') and M13r (5'-GGA AAC AGC TAT GAC CAT-3') primers. PCR reactions were set up with final concentrations of 0.5 μM each primer, 25 μg/mL BSA (bovine serum albumin, Sigma, B2518), 0.2 mM dNTPs (Promega Corp., U1240), 0.025 U/μL JumpStart Taq DNA Polymerase (Sigma D4184), 1X Reaction Buffer (Sigma D4184), 1.5 mM $MgCl_2$ (Sigma D4184), and 1-2 μL of clone culture. Reactions were brought up to 25 μL using ultrapure water (Sigma, W4502). Reactions were run in an Eppendorf Mastercycler Gradient thermocycler for an initial cycle of 95°C for 3-5 min, followed by 35 cycles of 95 °C for 20sec, 50 °C for 20sec, and 72 °C for 1min. Water blanks were used as negative controls. PCR product was imaged, sized, and quantified in the same manner as the *trfA2* gene described above.

Sequencing

For each of the 10 microcosms, 48 insert-containing transconjugants were sequenced using the following procedures. The M13 PCR product was cleaned using a 6 μL solution containing 5 μL of PCR product and 1 μL containing 5 U Exo I (New England BioLabs, #M0293S), and 0.75 U Shrimp Alkaline Phosphatase (USB, #70092Z). The reaction was incubated at 37°C for 15 min and then heated to 80°C for 15 min to denature the enzymes. Sequencing reactions were set up using 0.5 μL BigDye Terminator v3.1 (Applied Biosystem, #4337456), Dilution Buffer (120mM Tris-HCl, pH 9.0 and 3 mM $MgCl_2$), 0.33 μM M13f primer, and 50 ng of cleaned M13 PCR product in a 10 μL reaction. Reactions were run in an Eppendorf Mastercycler Gradient thermocycler or an GeneAmp PCR System 9700 (Applied Biosystems) for 50 cycles of 95 °C for 30 sec, 50 °C for 10 sec, and 60 °C for 4 min. Reactions were precipitated for 15 min at -20°C using 1 μL of a solution of 1.5 M sodium acetate/250 mM EDTA (pH 8.0) and 40 μL 95% ethanol. Precipitated reactions were pelleted, dried, and stored at -20°C until they were run.

Some of the sequences were run on an ABI Prism 377 DNA Sequencer (PE BioSystems) for 7 hours. Other sequences were run on a 3130*xl* Genetic Analyzer (Applied Biosystems) using either a 36 cm capillary or a 50 cm capillary. Samples run on the Prism 377-96 were resuspended in 1.5 μL of Dextran Blue/diFormamide Solution (40 μL of 10% Dextran Blue in deionized water and 200 μL of diFormamide). Every 6th sample loaded was resuspended in 1.5 μL of Dextran Blue/diFormamide Solution containing DET48 tracking dye (The Gel Company, DET48-FAM). The resuspended sequencing reactions products were denatured at 95°C for 5 min and kept on ice until

loaded. Sequences were run on an acrylamide sequencing gel containing a filtered and degassed solution of 10 mL 5X TBE (28), 5 mL Long Ranger Acrylamide (BioWhittaker Molecular Applications #50611), 18 g Urea (Amresco, #0568-25KG), 21 mL of distilled water, and a pinch of resin beads. The gel solution was polymerized with 35 μL TEMED (Amresco, #0761-50ML) and 250 μL of 10% APS (Ammonium Persulfate, Sigma, A3678). Samples were loaded onto the gel using 96-lane RapidLoad Membrane Combs (The Gel Company, CAM96) and 20% Ficoll Loading Solution (The Gel Company, DAL25). Data were collected using ABI Prism 377-96 Collection software version 2.6.

Samples run on the 3130*xl* were resuspended in 15 μL 50% Hi-Di Formamide (Applied Biosystems, 4311320) in an MicroAmp optical 96-well reaction plate (Applied Biosystem, N801-0560). The resuspended sequencing reactions products were denatured at 95^{o}C for 5 minutes and kept in the refrigerator until loaded. Some samples were run using a 36 cm array, POP-7 polymer, 35 min run time, 500 metric Length of Read (LOR), and Dye Set Z for BigDye version 3 (UltraSeq36_POP7). Other samples were run using a 50 cm array, POP-7 polymer, 1 hour run time, 700 LOR, and Dye Set Z for BigDye version 3 (3130POP7_BDTv3_KB_Denovo_v5.2).

Samples run on Prism 377-96 were post-processed using Analysis Software version 3.3.1 (PE BioSystems), which consisted of making lane-tracking corrections and extracting lanes. Samples run on the 3130*x*l were post-processed using Sequencing Analysis 5.2 (Applied Biosystems). All sequences were edited and arranged using Sequencher version 4.1.4 (Gene Codes Corporation).

Data Analysis

The estimated percent coverages of the obtained sequences versus the total number of sequences in the samples were calculated using the [1 – (*n*/*N*)] x 100 calculation from Dang and Lovell (11). The rarefaction curve was generated using FASTA formatted sequences exported from Sequencher and the FastGroupII online software (http://phage.sdsu.edu/research/projects/fastgroup/fg_tools.htm). Log transformed bacterial abundance data was analyzed using ANOVA and least square means analysis using PROC GLM in SAS (The SAS System for Windows, version 9.00, SAS Institute, Inc., Cary, NC).

DnaSP (27) was used to define sequence sets (microcosms) and identify haplotypes. The AMOVA (Analysis of Molecular Variance) was done using Arlequin (30). The "Haplotype Frequency Distribution Among the Samples" portion of the AMOVA output was used for the bar charts and stacked bar charts and the community analyses. Population similarity indexes were calculated using Whittaker's Index of Association and Jaccard coefficients. Formulas for the indexes were obtained from Hewson and Fuhrman (17).

Reference sequences for the phylogenetic tree were obtained from the GenBank database at NCBI (National Center for Biotechnology Information, National Institutes of Health, National Library of Medicine, http://www.ncbi.nih.gov/Genbank/index.html) on 8/24/05. The evolutionary model best fitting the haplotype data and reference sequences was determined using standard AIC analysis (not using branch lengths and including all models) in ModelTest, version 3.6 (24). The maximum likelihood model and haplotype data were imported into PAUP, version 4.b.10 to generate a distance

matrix and the phylogenetic tree, and TreeView, version 1.6.6 (23) was used for viewing and printing the tree.

The Network diagram was created using roehl-formatted haplotype file from DnaSP in Network, version 4.1.1.1 (www.fluxus-engineering.com). The network was calculated using Median Joining (5). Treatment frequencies for each haplotype were added into the Network diagram by both manually editing the Network input file and by editing the diagram with the Network editor.

Haplotypes were compared to GenBank sequences using MegaBLAST (http://www.ncbi.nih.gov/BLAST/, accessed 9/24/05) to determine the closest matching sequences and to obtain plasmid and IncP subgroup information for the matching sequences. Phylogenetic clusters were determined by visual examination of sequence similarity in the Network diagram and phylogenetic tree. Phylogenetic clusters and IncP plasmid subgroups information were added manually to the phylogenetic tree. Sequences for the 4 dominant haplotypes were compared with similar sequences in Genbank using MegaBLAST and BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/blast/, accessed 9/25/05).

RESULTS

The river sediment used in the microcosms was about 90% sand with the remainder silt and clay (Table 1). The sediment was analyzed for various metals (Table 1) and the metal concentrations were compared with USEPA Pollution Classification Limits [(45) In (6)] and maximum background levels for South Carolina stream sediment measured by Canova (10). All metal concentrations were below EPA levels and the stream sediment levels except barium and manganese. The concentration of barium in the sediment (80 ppm, Table 1) was classified as heavily polluted according to the EPA measurements, but was well below the maximum background level of 240 ppm measured by Canova. The concentration of manganese in the sediment (791 ppm, Table 1) was also classified as heavily polluted when compared with the EPA limits and was higher than the maximum background level of 620 ppm measured by Canova.

The concentrations of Ni and Cd were also measured in the microcosm sediment. Of the total amount of metal added to the microcosms, 86% of the Cd and 87% of the Ni (Figure 1) was extracted from the sediment at the end of the experiment. Thus, the majority of the introduced metal was bound to the sediment rather than dissolved in the water of the microcosms.

Bacterial abundances in water of the microcosms for all treatments were lowest on Day 0 (Figure 2A) and highest on Day 4. In the sediment, bacterial abundances were only measured at the beginning and end of the experiment to avoid disturbing the sediment during the incubation period. Bacterial abundance in the sediment (Figure 2B)

was highest on Day 0 and lowest on Day 8 for all treatments. The highest abundances in both water (Day 4 and Day 8) and sediment (Day 8) were found in the microcosms treated with cadmium. In sediments, ANOVA indicated that bacterial abundance varied significantly with time, but not among concentrations (Table 2). In water, bacterial abundance varied significantly with time, metal treatment, and their interaction (Table 2).

According to the coverage estimate calculations (Figure 3), the cloned and sequence samples accounted for 67-88% of the total population of *trfA2* gene haplotypes in the microcosms. The coverage estimates for the control microcosms ranged from 69-83%, the Ni microcosms ranged from 67-81% and the Cd microcosms ranged from 75-88%. The rarefaction curve (Figure 3) indicated that the rate of finding new haplotypes decreased as more samples were sequenced.

There were 65 unique haplotypes identified out of the 480 *trfA2* genes sequenced, and there was great variability in the occurrence of the haplotypes observed in each of the treatments (Figure 4). Haplotypes 1 and 2 were found in all microcosms and were dominant regardless of treatment regime (Figure 4). The four most dominant haplotypes were analyzed according to frequency by treatment type (Figure 5). Haplotype 4 was least dominant in the control than in the Cd and Ni treatments, Haplotype 6 was least dominant in the Cd treatment, and Haplotype 2 was least dominant in the Ni treatment. Of the remaining 61 haplotypes, 49 occurred in only one microcosm. Of these 49, 19 occurred in a control microcosm, 17 occurred in a Ni microcosm, and 13 occurred in a Cd microcosm.

The Analysis of Molecular Variance (AMOVA) results (Table 3) indicated that the percentage of total variance found within microcosms (96%) was much greater than the variance among microcosms within treatments (3%), and the variance among treatments (1%). The p-values for both the within-microcosms variance and the among-microcosms-within-treatments variance were significant ($p < 0.05$, Table 3).

Both Jaccard coefficients and Whittaker's Index of Association indicated similarities among treatments based on haplotype community composition (Table 4). The Jaccard coefficient is based on presence/absence of the haplotypes within each treatment and ranges from 0 (completely dissimilar) to 1 (identical). By this index, all treatments shared 16-17% of their haplotypes. The control and Cd treatments were most similar, with 17% of their haplotypes present in both treatments. The Whittaker's Index of Association is based on both occurrence and frequency of haplotypes in each treatment, and also ranges from 0 (completely dissimilar) to 1 (identical). By this index, the control and Ni treatments were 73% similar in haplotype makeup, as were the control and Cd treatments. The Ni and Cd treatments were the least similar (59%).

In addition to testing similarities among treatments, the haplotypes were also analyzed for phylogenetic relatedness. Several GenBank sequences, identified by Vedler *et al.* (50) as references sequences for the various IncP subgroups, were included in the phylogenetic tree as references (Table 5). The haplotype dataset and IncP *trfA2* gene reference sequences were first fit to the best fitting evolutionary model using ModelTest. The maximum likelihood model that best fit the data was the Transversion Model (TVM) which included rate variation among sites (G), referred to as TVM+G. The distance matrix used to create the phylogenetic tree (Figure 6) was

calculated using the TVM+G model and was analyzed using the neighbor joining method in PAUP.

The closest matches for each of the 65 haplotype were obtained from GenBank (Table 6). Often more than one GenBank sequence closesly matched a haplotype. The GenBank group I.D. and the associated GenBank sequences matching various haplotypes are given in Table 7. IncP subgroup information (α, β, γ, δ) was also obtained for the GenBank matches for each haplotype. If the majority of the GenBank sequences in a group belonged to a particular IncP subgroup and the remaining sequences were unidentified, the whole group was assigned the IncP subgroup of the majority. All haplotypes were related to either the IncPα, β, or γ subgroups (Table 6). No haplotypes matched the IncPδ subgroup.

Four clusters of sequences (Clusters A, B, C, and D) were identified from the Network Diagrams (Figures 7-12) and the Phylogenetic Tree (Figure 6). The Network cluster information for each of the haplotypes was also compared with the closest matches from GenBank (Table 6). Haplotypes 23 and 37 (Figures 6 and 7) were identified as IncPα sequences with GenBank matches of 100% and 95% respectively. Haplotypes of Cluster A, Cluster B, and Cluster C, as well as Haplotypes 9, 24, and 51 were all identified as IncPβ with matches $\geq$ 97%. Group D haplotypes all matched closest to the IncPγ sequence from pEMT3 (Table 7) with all matches $\leq$ 83%. Three of the outlying haplotypes (23, 37, and 51) occurred only in the control microcosms, and another isolated haplotype (9) only occurred in the microcosms treated with Ni or Cd. The remaining clusters contained haplotypes from all 3 treatments (Ni, Cd, or control) in varying proportions.

Primer sequences from the reference sequences and pEMT3 (IncPγ) were aligned and compared to the sequences published in Gotz *et al*. (Table 8). Identification of base pair mismatches were not made for sections of the primers that contained ambiguous bases. One mismatch occurred in the IncPδ sequence in the forward primer (trfA2-1) and 7 mismatches occurred in the reverse (trfA2-2). Four mismatches occurred in the IncPγ sequence in the forward primers (Table 8). The sequences for IncPα and IncPβ contained no mismatches when compared with the Gotz *et al*. sequences.

The four most dominant haplotypes (Haplotypes 1, 2, 4, and 6) were compared with the 10 closest-matching sequences in GenBank (Table 9). The GenBank sequences were identified by both plasmid type and associated resistance and/or degradation genes (Table 9). Genes carried on these plasmids included ones that conferred resistance to mercury and antibiotics such as amoxicillin, erythromycin, oxytetracycline, streptomycin, spectinomycin, tetracycline, and sulfonamides. Genes that degraded pesticides and other xenobiotic compounds such as 3-chloroaniline, chlorobenzenes, chlorocatechols, halogenated acetates, and substituted aromatic pollutants were also identified on these plasmids. A number of plasmids in GenBank were not specifically mentioned in the associated reference literature so we were unable to identify degradation or resistance genes associated with these plasmids.

DISCUSSION

The sediment from the Savannah River used in the microcosms was mostly sand with little organic matter or clay particles (Table 1). Except for manganese, no unusually high concentrations of metals were observed in the untreated river sediment (Table 1). The reason why the Mn concentration measured in the sediment was higher than the maximum background concentration measured by Canova (10) is unknown. Metal analysis of the spiked sediments in the microcosms showed that approximately 86% of the Cd added and 87% of the Ni added (Figure 1) was associated with the sediments. Cd and Ni tend to bind to organic and inorganic components of sediment, resulting in resulting in relatively low dissolved concentrations in the overlying water (2, 9, 44).

Bacterial abundances were measured pre- mid- and post-treatment in the overlying water (Figure 2A). However, the abundance in the sediment was only measured pre- and post-treatment so as not to disturb the sediment during the incubation period (Figure 2B). In the overlying water, there was a significant increase in the abundance of bacteria between Day 0 and Day 4 for the Ni and Cd treatments ($p<0.0001$, Table 2), but no significant difference between these days in the control microcosms ($p=0.2196$, Table 2). This increase in bacterial abundance following the addition of metals has also been observed by other researchers (40, 44). Stepanauskas *et al*. (40) observed increased bacterial abundance in Savannah River water spiked with 10-100 μM Cd and 100-1000 μM Ni as compared to controls. Titus

and Pfister (44) also noted an increase in bacterial abundance in a model aquatic system treated with a continuous input of Cd as compared to the control system. They theorized that this increase was due to the Cd-induced cell lysis and subsequent release of growth factors, and that these growth factors contributed to increased numbers of Cd-resistant bacteria. Another reason for this increase in bacterial abundance in the treatment microcosms could be that the metals had a detrimental affect on invertebrate predators of bacteria. Hoffman and Atlas (19) tested the effects of various concentrations of Cd (10 mg/L to 150 mg/L) on *Aspidisca costata*, a protozoan that grazes on bacteria, and found a slight decrease in number of protozoa over time following Cd exposure. They also observed that Cd exposure reduced particle ingestion within 1 hour of exposure. The concentration of Cd used in our experiment was 11 mg/L. By Day 8 of our experiment bacterial abundances in the treatment and control microcosms were similar (Figure 2).

Despite temporary effects on bacterial abundance in the treatment microcosms, there was no significant treatment effect on the diversity of the *trfA2* gene. The AMOVA indicated no significant treatment effect ($p=0.18$, Table 3) and showed that the majority of variation in haplotypes (96%) occurred within microcosms (Table 3). There was, however, considerable variability observed in *trfA2* gene sequences (Figures 3 and 4). Of the 480 sequences, 65 (14%) were unique haplotypes. The coverage estimates (Figure 3) indicated that more than half of the estimated genetic variability of the trfA2 gene in the microcosms (67-88%) had been sampled within the microcosms. The rarefaction curve (Figure 3) and the coverage estimates also indicated that a very large

number of samples would need to be sequenced to capture all the variability present within this gene in the microcosm systems.

Based on visual examination of Figure 4, no overall pattern of haplotype occurrence or frequency associated with the treatments was discernable. Some of the same haplotypes occurred in all treatments, but at different frequencies, and others only occurred in one or two treatments at very low frequencies. The addition of metals to the microcosms did not appear to affect the number of individual haplotypes within each treatment or the frequency of haplotypes within each treatment (Figure 4). In addition, the AMOVA indicated that there was no significant effect of treatment on haplotype diversity (Table 3). There are many possible reasons for this result. The most obvious is, of course, that the addition of Cd or Ni to the microcosms did not affect the diversity of the plasmid community (as measured by the *trfA2* gene) present in the microcosms. Another possibility is that other factors such as the transition from an open system to a closed system may have masked any effects the metals may have had.

Some of the most dominant haplotypes exhibited individual responses that may indicate possible haplotype-specific treatment effects (Figure 5). Exposures to Cd and Ni appear to have resulted in an increase in the frequency of Haplotype 4 (Figure 5). Also, Haplotypes 1 and 6 appear to have been negatively effected by exposure to Cd but not to Ni. Further research on characterizing IncP plasmids with these *trfA2* haplotype is needed to determine the reasons for the responses observed.

Haplotype presence and relative abundance were analyzed Whittaker's Index of Association and Jaccard coefficients to examine the similarity of the haplotype communities among treatments (Table 4). The Jaccard coefficients (16-17% similarity)

suggested considerable variability in the IncP plasmid communities among treatments. The Whittaker's Index ranged from 73% similarity (control compared to Ni and control compared to Cd) to 65% (Cd compared to Ni). Thus when the abundance of each haplotype is taken into account, the communities appear more alike. The Whittaker's indexes also suggest that the Cd and Ni microcosm plasmid communities are more similar to the control microcosm communities than they are to each other. This may indicate that the Cd and Ni may have affected plasmid populations in different ways. Some further ways to investigate this possibility would be to study the effects of different concentrations of Ni or Cd on *trfA2* diversity over a longer time period (i.e. several weeks) using an open system.

The next portion of our research was to investigate the phylogenetic relationship of the haplotypes to each other and to standard *trfA2* sequences from the different IncP subgroups in Genbank. The majority of the haplotype sequences matched closest to IncPβ sequences. Even though the primers used in this study (14) were developed from the IncPα plasmid RK2 sequence, Gotz *et al*. (14) noted that they amplified *trfA2* sequences from both IncPα and IncPβ plasmids. Droge *et al*. (13) noted that of 10 IncP plasmids isolated from activated sewage sludge, 2 were IncPα and 8 were IncPβ. They also noted that the IncPβ *trfA2* sequences were more diverse than the IncPα. Smalla [(34) In (13)] also observed more diversity in IncPβ sequences than in IncPα. Our findings, based on the use of the Gotz *et al*. primers, certainly confirm that there is considerable variability within the IncPβ *trfA2* sequences. Our results are also similar to Droge et al. in that fewer sequences were identified as IncPα (2 sequences) than IncPβ (55 sequences).

None of the *trfA2* sequences we sampled from the microcosms were a close match to the IncPδ reference sequence. This may have been because no IncPδ plasmids were present in the microcosms or were present in quantities that were below detection. Another possible reason for the lack of sequences from IncPδ plasmids could be because the sequence was not amplifiable with the primers used. Although not tested empirically, it seems unlikely that IncPδ plasmid *trfA2* DNA would have been amplified when using the reverse primer (7 out of 20 bases mismatched, Table 8), suggesting that any IncPδ plasmids present in the microcosms may have gone undetected.

The comparison of primers with the corresponding sequences from the reference GenBank IncP sequences also showed an 18% mismatch in the forward primers sequence for the IncPγ sequence from pEMT3 (Table 8). Since IncPγ sequences were amplified from the microcosms, the mismatched bases did not completely inhibit IncPγ *trfA2* gene amplification. Of course, the best way to determine the actual extent of the presence and diversity of IncPδ and IncPγ plasmids in the microcosms would be to develop primers more specific for the *trfA2* gene from these two plasmid types.

Visual inspection of the Network Diagrams (Figures 7-12) indicated 4 distinct clusters of haplotypes and 4 individual haplotypes lying outside of the clusters. Clusters A, B, and C contained haplotypes that matched closest with IncPβ GenBank sequences (Tables 6 and 7 and Figure 7). Two of the "isolated" haplotypes (Haplotypes 23 and 37) were closely matched to IncPα GenBank sequences.

Cluster D contained haplotypes that were more similar to the IncPγ sequence from pEMT3 (Tables 6 and 7 and Figure 7). All Cluster D haplotypes matched $\leq$83%

with GenBank sequences, whereas the percent match for all other clusters ranged from 97-100%. This may indicate that the Cluster D *trfA2* genes could belong to an unidentified IncP subgroup, although more in-depth research on the backbone organization of plasmids containing these *trfA2* sequences would be necessary to determine this. It should be noted that Gstalder *et al*., placed pEMT3 into a separate IncP subgroup, despite the similarity of the *rep* gene sequence to IncPβ *rep* sequences, because of structural differences they observed in plasmid backbone (15).

All four Network clusters contained haplotypes from the each of the Ni and Cd treatment microcosms as well as the control microcosms (Figure 8-12), indicating that there was no treatment selection for these particular haplotype clusters. However, most of the haplotypes that were phenotypically located outside of the clusters (Haplotypes 23, 37, 51, 24) occurred in only the control microcosms. Also, two of these haplotypes (Haplotypes 23 and 37) belonged to the IncPα subgroup. This may indicate that the metals had more of a detrimental effect on the IncPα subgroup and on other plasmids containing *trfA2* haplotypes that were phylogenetically "distant" from the clusters observed in this study. Since the quantity of IncPα sequences and other "isolated" haplotypes detected in this study was low, more research needs to be done on the effects of metals on IncPα plasmids and on genetically distant IncPβ *trfA2* genes to determine whether selection occurred. However, a study by Smalla *et al*. (35) noted that IncPβ sequences were found in the rhizosphere of copper-treated soil but not in the rhizosphere of untreated soil. They also found that IncPβ sequences were found in PCB contaminated soil but were not found in uncontaminated soil from the same location. The Smalla *et al*. study raises the question as to whether pollutants are

selecting for certain IncP plasmid subgroups. Obviously more research needs to be done on the effects of pollutants on IncP subgroups to determine this.

Resistance and degradation genes found on the 10 closest matching sequences in GenBank to the four dominant haplotypes give an indication as to the variety of degradation and resistance genes that may have been located on the IncP plasmids in the microcosms (Table 8). The genes identified from GenBank sequences include ones that degrade problematic environment pollutants such as substituted aromatic compounds, chloroorganic compounds, and halogenated acetates. Some plasmids also carried resistance determinants for mercury and a variety of antibiotics. Adamczyk and Jagura-Burdzy (1) noted that IncPα plasmids often carry genes that confer a wide spectrum of antibiotic resistances while IncPβ plasmids often carry genes conferring antibiotic resistance and/or mercury as well as degradation capabilities.

IncP plasmids and IncP plasmid backbone genes have been found in a variety of environments. The IncPβ sequences that were the most similar to the sequences obtained in this research were obtained from bacteria isolated from a variety of environments, including a ditch, contaminated soil, waste-water treatment plants, and activated sludge. The IncPα *trfA2* closest-matching sequences were isolated from bacteria found in activated sludge and from a clinical setting.

CONCLUSIONS

The purpose of this study was to determine if the diversity of the *trfA2* gene as a representative of the IncP plasmid population in a sediment bacterial community would increase as a result of metal exposure. A significant change in *trfA2* gene diversity was not observed. However considerable variability was observed within the *trfA2* genes sequenced as has been noted in previous studies. A greater quantity of IncPβ-type *trfA2* sequences were found than IncPα-type sequences which is also consistent with previous studies.

This research indicates the need for more selective primers for the IncPδ subgroup and possibly the IncPγ subgroup. Also more information needs to be obtained on plasmids containing the Cluster D haplotype sequences to determine their relationship to the other IncP subgroups. The results indicate that the primers used in this study can be used to screen potential isolates for plasmids containing Cluster D-like haplotypes and these plasmids could then be isolated and characterized to determine IncP relatedness. Further research is needed to determine if exposure of bacterial communities to pollutants results in increased selection for IncPβ plasmids versus other IncP subgroups.

Table 1. Pre-Treatment Sediment Characterization and Metal Analysis.

Sediment Characterization:

Percent Moisture	pH	KCl pH	EC*	Percent Silt	Percent Clay	Percent Sand
28.74	6.68	5.30	52.7	6.02	3.81	90.17

Metal	Savannah River Sediment	
	average ppm (mg/kg)	*standard deviation*
Al	6761.64	395.40
As	1.15	0.06
Ba	80.37[B]	3.63
Be	0.45	0.02
Cd	0.04	0.00
Co	5.96	0.17
Cr	13.32	0.35
Cs	0.92	0.05
Cu	7.44	0.53
Fe	11826.38	852.04
Hg	0.07[A]	0.02
Mn	791.13[B,C]	63.92
Mo	0.26	0.01
Ni	9.34	0.32
Pb	5.59	0.10
Rb	15.32	0.99
Sb	0.07[A]	0.01
Se	0.43	0.02
Sr	5.19	0.13
Tl	0.15	0.01
U	0.95	0.04
V	21.39	0.64
Zn	27.27	0.79

*electroconductivity
[A] value below the detection limit
[B] value above USEPA Pollution Classification limit [(45) ln (6)]
[C] value above maximum background concentrations for South Carolina stream sediment (10)

Table 2. ANOVA and Least Square Means Analysis of Log_{10} Transformed Bacterial Abundances.

ANOVA and LSMEANS Analysis of Log Abundance in Sediment by Treatment and Day

Source	*DF*	*Type III SS*	*Mean Square*	*F Value*	*Pr > F*
treatment	2	0.044687	0.0223434	1.36	0.2901
day	1	2.815601	2.8156014	171.76	<.0001
treatment*day	2	0.044687	0.0223434	1.36	0.2901

Least Square Means Comparisons	Pr > |t|*
Control Day 0 vs Control Day 8	<.0001
Cadmium Day 0 vs Cadmium Day 8	<.0001
Nickel Day 0 vs Nickel Day 8	<.0001

ANOVA and LSMEANS Analysis of Log Abundance in Water by Treatment and Day

Source	*DF*	*Type III SS*	*Mean Square*	*F value*	*Pr > F*
treatment	2	0.356589	0.1782944	13.51	0.0002
day	2	1.507396	0.7536979	57.12	<.0001
treatment*day	4	0.429134	0.1072834	8.13	0.0005

Least Square Means Comparisons	Pr > |t| *
Cadmium Day 0 vs Cadmium Day 4	<.0001
Cadmium Day 4 vs Cadmium Day 8	<.0001
Control Day 0 vs Control Day 4	0.2196(ns)
Control Day 4 vs Control Day 8	0.0622(ns)
Nickel Day 0 vs Nickel Day 4	<.0001
Nickel Day 4 vs Nickel Day 8	<.0001

*for H0: LSMean(i)=LSMean(j)
Analysis done using SAS Proc GLM procedure, alpha=0.05

Table 3. AMOVA (Analysis of Molecular Variance) results.

Source of Variation	d.f.	Sum of squares	Variance Components	Percentage of variation	P(rand. value >= obs. value)
Among treatments	2	2.669	0.00381	1.21	0.17693 + -0.01226
Among microcosms within treatments	7	5.118	0.00895	2.84	0.00000 + -0.00000
Within microcosms	470	141.812	0.30173	95.94	0.00000 + -0.00000
Totals	479	149.6	0.31449		

Significance tests (1023 permutations)

Table 4. Similarity Indexes: Jaccard and Whittaker's.

Jaccard Index

$S_j = W / (a_1 + a_2 - W)$

Control vs Nickel	0.16
Control vs Cadmium	0.17
Cadmium vs Nickel	0.16

Whittaker's Index of Association

$S_w = 1 - \sum (|b_{i1} - b_{i2}| / 2)$

Control vs Nickel	0.73
Control vs Cadmium	0.73
Cadmium vs Nickel	0.65

Table 5. Sequences from GenBank used as references for the phylogenetic tree.

Plasmid Name	Plasmid Type	GenBank Acc. No.	Sequence Title
pEST4011	IncPδ	NC_005793	Achromobacter denitrificans plasmid **pEST4011**, complete sequence.
R751	IncPβ	EAU67194	Enterobacter aerogenes plasmid **R751**, complete sequence.
RK2	IncPα	L27758	Birmingham IncP-alpha plasmid (R18, R68, **RK2**, RP1, RP4) complete

Table 6. Haplotypes and associated phylogenetic cluster, GenBank match, and percent identity of GenBank match.

Haplo-type	Phylo-genetic Cluster[A]	GenBank Group[B]	Closest Matching IncP Group[B]	Percent Identity[C]
2	A	Y	beta	100.00
3	A	Y	beta	97.99
4	A	Y	beta	99.50
7	A	Y	beta	99.50
8	A	Y	beta	99.49
12	A	Y	beta	99.50
15	A	Y	beta	98.99
17	A	Y	beta	99.50
18	A	Y	beta	99.50
19	A	Y	beta	99.50
20	A	Y	beta	99.50
21	A	Y	beta	99.50
22	A	Y	beta	99.50
25	A	Y	beta	97.49
27	A	Y	beta	99.50
28	A	Y	beta	99.50
29	A	Y	beta	98.49
30	A	U	beta	100.00
31	A	Y	beta	98.99
32	A	Y	beta	98.99
36	A	Y	beta	99.50
39	A	Y	beta	99.50
41	A	Y	beta	96.98
43	A	Y	beta	99.50
44	A	Y	beta	97.49
45	A	Y	beta	99.50
47	A	Y	beta	96.98
52	A	S	beta	100.00
54	A	Y	beta	99.50
55	A	Y	beta	99.50
56	A	Y	beta	98.99
60	A	Y	beta	99.50
64	A	Y	beta	99.50
1	B	Z	beta	100
5	B	Z	beta	99.5
13	B	Z	beta	98.49
16	B	Z	beta	99.5
34	B	Z	beta	99.5
42	B	Z	beta	99.5
46	B	Z	beta	99.5
48	B	Z	beta	99.5
50	B	Z	beta	99.5
53	B	Z	beta	97.49
61	B	Z	beta	97.99
63	B	Z	beta	99.5
65	B	Z	beta	99.5
10	C	W	beta	98.49
11	C	W	beta	97.5
33	C	W	beta	97.49
35	C	W	beta	97.49
58	C	W	beta	98.99
59	C	W	beta	98.99
6	D	R	gamma	82.65
14	D	R	gamma	82.14
26	D	R	gamma	81.63
38	D	R	gamma	80.61
40	D	R	gamma	83.16
49	D	R	gamma	82.14
57	D	R	gamma	82.14
62	D	R	gamma	83.16
23		T	alpha	100
37		T	alpha	94.47
9		X	beta	100
51		Y	beta	88.6
24		Z	beta	97.89

[A] Phylogenetic clustering of haplotypes from Figures 6 and 7.

[B] GenBank Group information from Table 7.

[C] Percent Identity information from GenBank MegaBLAST results.

Table 7. Information on sequences from GenBank that were the closest matches to the sequenced haplotypes.

Group	Plasmid	IncP Type	NCBI Sequence Information	NCBI Acc. #
R	pEMT3	gamma	Uncultured bacteria ssb gene and trfA gene	AJ414161
S	pB3	beta	Plasmid pB3 complete genome	AJ639924
S	pSMA196	beta	IncP1 beta plasmid pSMA196 partial trfA gene	AJ493455
T	pTB11	alpha	uncultured bacterium pTB11 plasmid complete genome	AJ744860
T	RK2	alpha	Plasmid RK2 transcriptional repressor TrfA (trfA) gene, complete cds	U05774
U	pSMA205	beta	IncP1 beta plasmid pSMA205 partial trfA gene	AJ493457
W	pA1	beta	Sphingomonas sp. A1 plasmid pA1 DNA, complete sequence	AB231906
X	pB12	beta	Pseudomonas sp. B13 IncP-beta plasmid pB12 TrfA2 (trfA2) gene, partial cds	AF148974
Y	p1	?	Ralstonia eutropha JMP134 plasmid 1, complete sequence	CP000093
Y	pB10	beta	Uncultured bacterium IncP-1beta multiresistance plasmid pB10	AJ564903
Y	pJP4	beta	Wautersia eutropha strain JMP134 plasmid pJP4, complete sequence	AY365053
Z	pA81	?	Achromobacter xylosoxidans plasmid pA81, strain A8	AJ515144
Z	pPS12-1		Burkholderia sp. PS12 plasmid pPS12-1 transcriptional repressor protein TrfA2 (trfA2)	AF073901
Z	pTA8	beta	Pseudomonas sp. TA8 IncP-beta plasmid pTA8 TrfA2 gene, partial cds	AF467935

? = plasmid type information not provided

Table 8. Primer Alignment of IncP Reference Sequences from GenBank.

Primer trfA2-1

Gotz *et al*., 1996	C	G	A	A	A	T	T	C	R	T	R	T	G	G	G	A	G	A	A	G	T	A
R751 IncP1β	C	G	A	A	A	T	T	C	G	T	G	T	G	G	G	A	G	A	A	G	T	A
pEST4011 IncPδ	C	G	A	A	A	T	T	C	G	T	G	T	G	G	G	A	A	A	A	G	T	A
RK2 IncPα	C	G	A	A	A	T	T	C	A	T	A	T	G	G	G	A	G	A	A	G	T	A
pEMT3 IncPγ	:	C	A	A	G	T	T	C	A	T	T	T	G	G	G	A	G	A	A	G	T	A

Primer trfA2-2 (reverse compliment)

Gotz *et al*., 1996	G	A	C	C	T	G	G	T	G	C	A	T	T	G	C	A	A	R	C	G	
R751 IncP1β	G	A	C	C	T	G	G	T	G	C	A	T	T	G	C	A	A	G	C	G	
pEST4011 IncPδ	G	A	C	T	C	G	A	T	C	T	A	C	T	G	C	C	A	G	C	G	
RK2 IncPα	G	A	C	C	T	G	G	T	G	C	A	T	T	G	C	A	A	A	C	G	
pEMT3 IncPγ	G	A	C	C	T	G	G	T	G	C	A	T	T	G	C	A	A	G	C	G	

*pEMT3 Sequence obtained from GenBank (Acc. No.: AJ414161)

Table 9. Closest GenBank trfA2 Sequence Matches to Haplotypes 1,2, 4, and 6: Plasmid Identifications and Associated Resistance and/or Degradation Genes.

Haplo-type	Genebank Number	Bacteria or Plasmid	Reference	Plasmid	Resistance and Degradation Genes Identified on Plasmid	Resistances	Compounds Degraded
2,4,6	AB063332	Delftia acidovorans plasmid pUO1 DNA, complete sequence	Sota, 2002; Sota, 2003	pUO1	mercury resistance; *dehH1, dehH2*	Hg	fluoroacetate, chloroacetate, bromoacetate, iodoacetate
1	AF073901	Burkholderia sp. PS12 plasmid pPS12-1 transcriptional repressor protein TrfA2 (trfA2) gene, partial cds	Beil, 1999	pPS12-1	*tecA*		1,2,4,5 tetrachlorobenzene
6	AF148974	Pseudomonas sp. B13 IncP-beta plasmid pB12 TrfA2 (trfA2) gene, partial cds	Droge, 2000	pB12		Ery, Str, Spc, Sla, Otc	
6	AF148975	Pseudomonas sp. B13 IncP-beta plasmid pB10 TrfA2 (trfA2) gene, partial cds	Droge, 2000	pB10		Amx, Hg, Str, Sla, Otc	
6	AF148976	Pseudomonas sp. B13 IncP-beta plasmid pB8 TrfA2 (trfA2) gene, partial cds	Droge, 2000	pB8		Amx, Str, Spc, Sla	
1	AF467929	Achromobacter sp. PB31 IncP-beta plasmid pPB31 TrfA2 gene, partial cds.	Dejonghe,2002	pPB31			3-CA
1	AF467930	Comamonas testosteroni TB18 IncP-beta plasmid pTB18 TrfA2 gene, partial cds.	Dejonghe,2002	pTB18			3-CA and 3,4-DCA
1	AF467931	Acidovorax sp. TA35 IncP-beta plasmid pTA35 TrfA2 gene, partial cds.	Dejonghe,2002	pTA35			3-CA
1	AF467933	Delftia sp. MA34 IncP-beta plasmid pMA34 TrfA2 gene, partial cds.	Dejonghe,2002	pMA34			3-CA
1	AF467934	Delftia sp. MA22 IncP-beta plasmid pMA22 TrfA2 gene, partial cds.	Dejonghe,2002	pMA22			3-CA
1	AF467935	Pseudomonas sp. TA8 IncP-beta plasmid pTA8 TrfA2 gene, partial cds.	Dejonghe,2002	pTA8			3-CA
1	AF467936	Comamonas testosteroni TB30 IncP-beta plasmid pTB30 TrfA2 gene, partial cds.	Dejonghe,2002	pTB30			3-CA
1	AF467939	Achromobacter sp. PA4 IncP-beta plasmid pPA4 TrfA2 gene, partial cds.	Dejonghe,2002	pPA4			3-CA
6	AJ414161	Uncultured bacteria ssb gene and trfA gene	Gstalder, 2003	pEMT3	*tfd* genes		2,4-D
2,4	AJ493452	IncP1 beta plasmid pSMA168	Heuer, 2002	plasmid not mentioned specifically in paper			
2,4	AJ493453	IncP1 beta plasmid pSMA178	Heuer, 2002	plasmid not mentioned specifically in paper			
2,4	AJ493454	IncP1 beta plasmid pSMA179	Heuer, 2002	plasmid not mentioned specifically in paper			
2,4	AJ493456	IncP1 beta plasmid pSMA289	Heuer, 2002	plasmid not mentioned specifically in paper			
2,4	AJ493458	IncP1 beta plasmid pSMA200	Heuer, 2002	plasmid not mentioned specifically in paper			
2,4	AJ493459	IncP1 beta plasmid pSMA214	Heuer, 2002	plasmid not mentioned specifically in paper			
1	AJ515144	Achromobacter xylosoxidans strain A8	Jencova, 2004	pA81	chlorocatechol degradation genes		chlorocatechols
2,4,6	AJ564903	Uncultured bacterium IncP-1beta multiresistance plasmid pB10	Schluter, 2003	pB10	*tetA,tetR; strA,strB; sul1; oxa2* cassette; *merRTPADE*	Amx, Hg, Str, Tet, Sul	
6	AJ639924	Plasmid pB3 complete genome	Heuer, 2004	pB3	*bla* $_{NPS-2}$, *tetA(C)-tetR(C), cmlA1, aadA2, qacE* Δ*1, sul1*		
2,4,6	AY365053	Wautersia eutropha strain JMP134 plasmid pJP4, complete sequence.	Trefault, 2004	pJP4	*tfd*-I, *tfd*-II; mercury resistance determinants	Hg?	substituted aromatic pollutants (e.g 3-CB, 2,4-D)
2,4,6	CP000093	Ralstonia eutropha JMP134 plasmid 1, complete sequence	unpublished	p1	no information given	no information given	no information given
6	U66917	Pseudomonas sp. ADP atrazine catabolic plasmid pADP-1, complete sequence.	Martinez, 2001	pADP-1	*atzA, atzB, atzC, atzD, atzE, atzF*; mercury resistance operon	Hg	atrazine

2,4-D = 2,4-dichlorophenoxyacetic acid
3-CA = 3-chloroaniline
3-CB = 3-chlorobenzoic acid
3,4-DCA = 3,4-dichloroanaline
aadA2 = Str/Spc adenyltransferase
Amx = amoxicillin
atzABCDEF = produce enzymes that degrade atrazine
bla $_{NPS-1}$ = a Class D β-lactamase (confers resistance to amoxicillin, azlocillin, piperacillin)
chlorocatecols = pollutents produced by chlorination of wood pulp and other processes
cmlA1 = chloramphenicol exporter protein
dehH1 = monohaloacetate dehalogenases acting predominantly on fluoroacetate
dehH2 = monohaloacetate dehalogenases acting on chloro-, bromo-, and iodoacetate
Ery = erythromycin
Hg = mercury
merRTPADE = confers resistance to mercury
Otc = oxytetracycline
oxa2 cassette = oxacillin hydrolysing type β-lactamase, confers resistance to amoxacillin
qacE Δ*1* = multidrug exporter
Sla = sulfanilimide
Spc = spectinomycin
Str = streptomycin
strA = aminoglycoside-3"-phosphotransferase, confers streptomycin resistance
strB = aminoglycoside-6-phosphotransferase, confers streptomycin resistance
Sul = sulfonamide
sul1 = dihydropteroate synthetase, confers sulfonamide resistance
tecA = chlorobenzene dioxygenase
Tet = tetracycline
tetA = tetracycline-resistance repressor
tetR = tetracycline efflux protein
tetA(C)-tetR(C) = tetracycline resistance module
tfd-I, *trd*-II = chlorocatechol-degrading gene clusters required for degradation of substituted aromatic pollutants such as 3-CB and 2,4-D

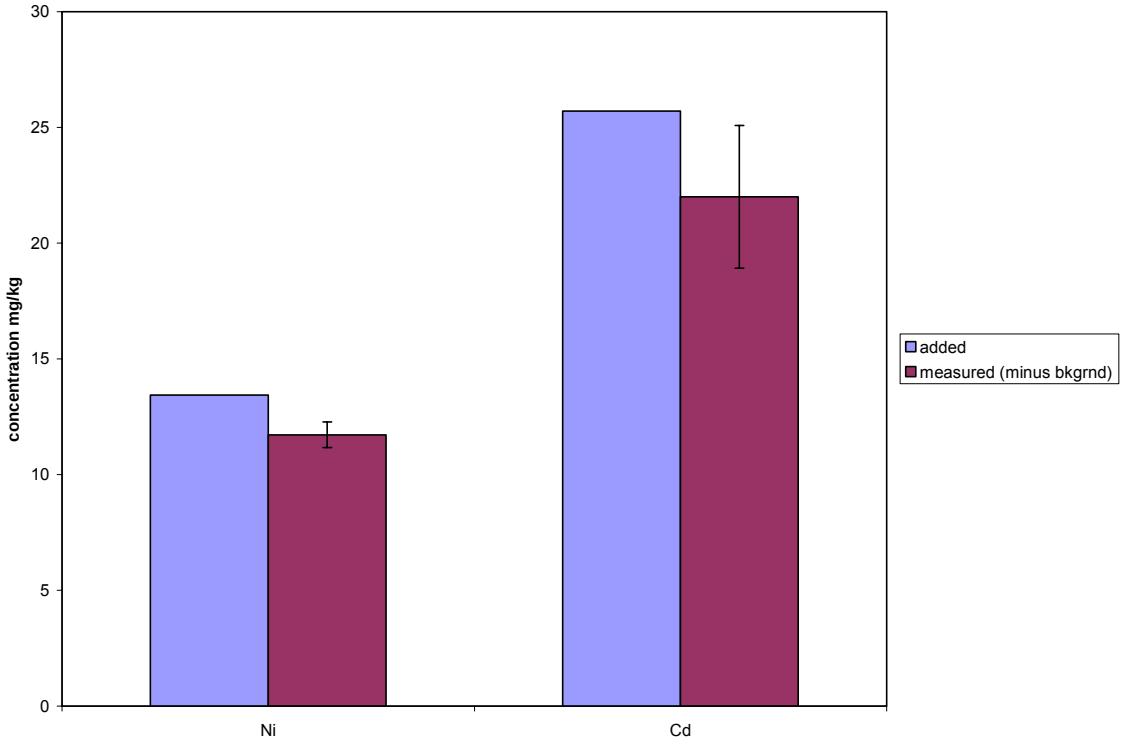


Figure 1. Comparison of Nickel and Cadmium Amounts Added To and Extracted from the Sediments in the Microcosms After 8 Days.

Figure 2A. Bacterial Abundance in Water

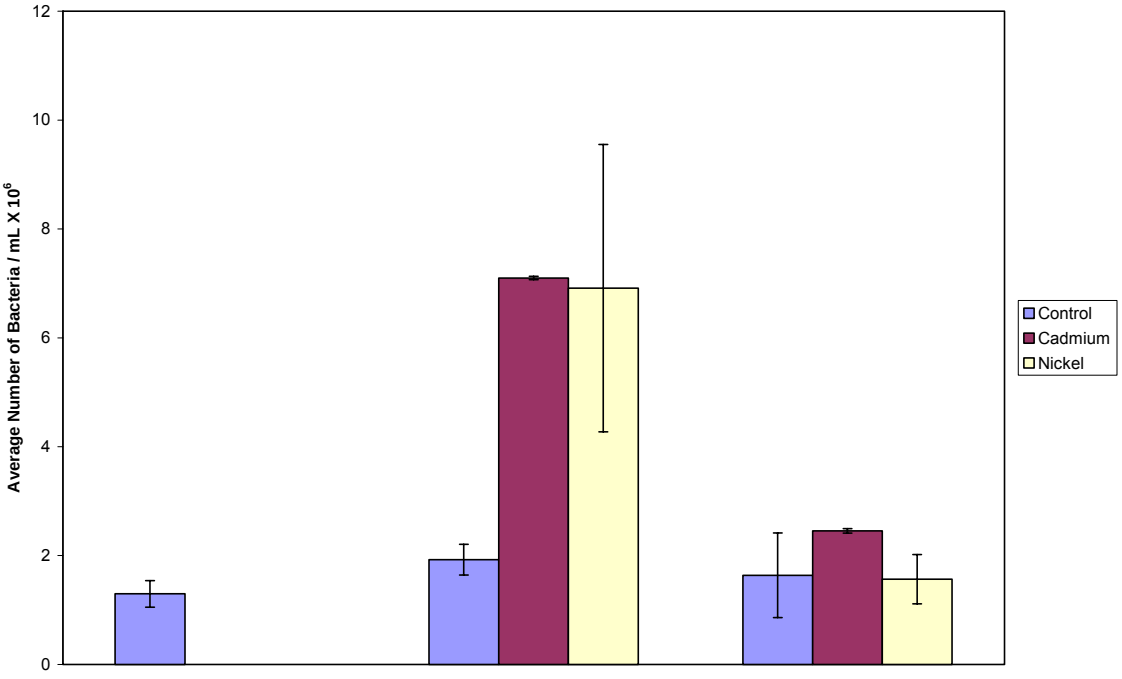


Figure 2B. Bacterial Abundance in Sediment

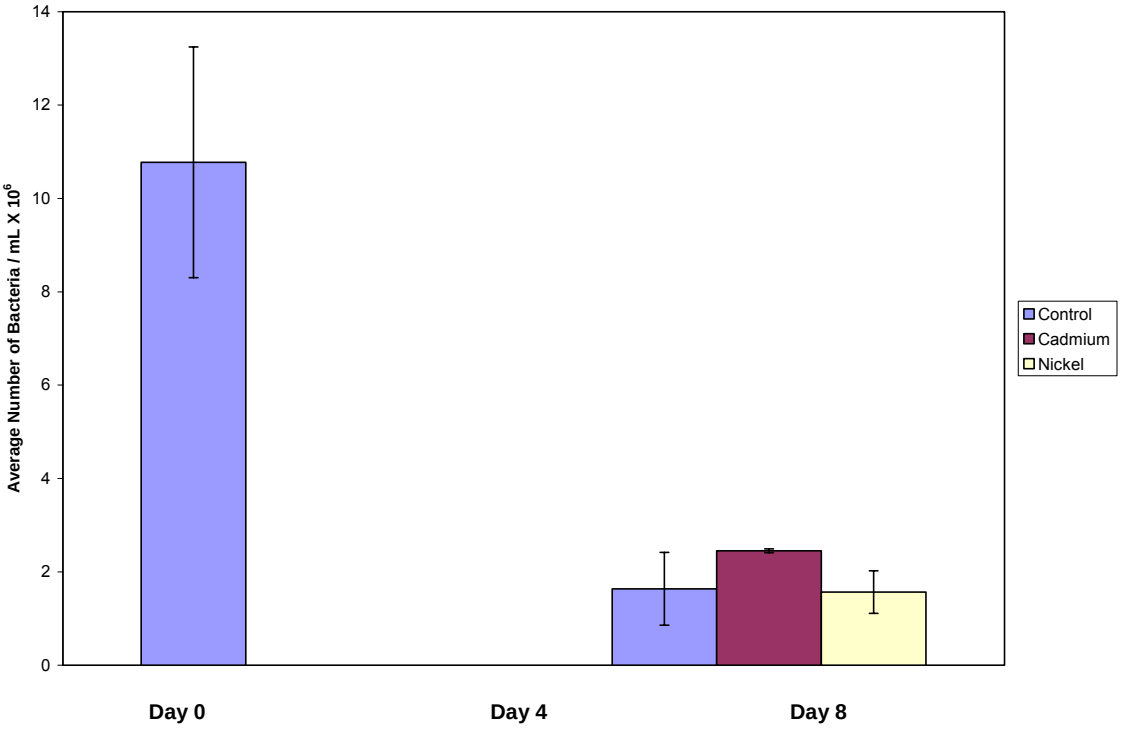
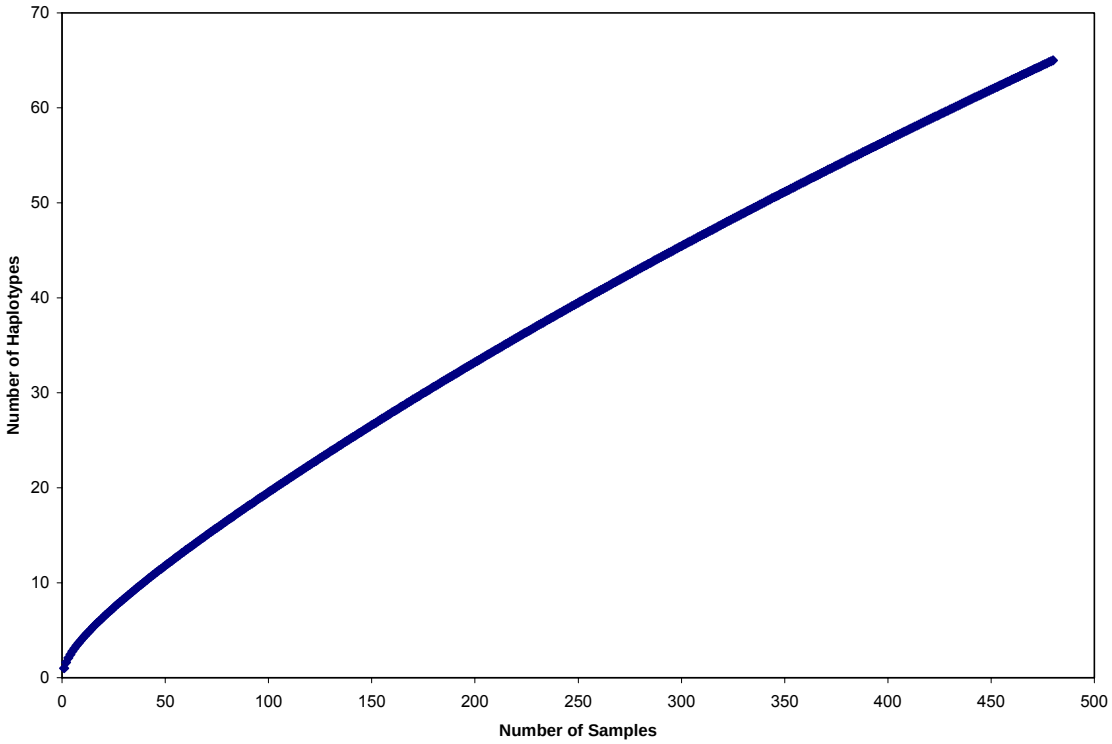


Figure 2. Bacterial Abundances Before, During, and After Treatment in Water and Sediment.



Coverage Estimate from Dang and Lovell (11)

Coverage = [1-(n/N)]*100 where N=number of samples sequences and n=number of haplotypes.

	Number of Sequences	Number of Haplotypes	Percent Variability Measured
Treatment	**N**	**n**	**[1-(n/N)]*100**
Ctrl1	48	8	83
Ctrl2	48	8	83
Ctrl4	48	13	73
Ctrl7	48	15	69
Ni3	48	11	77
Ni6	48	9	81
Ni10	48	16	67
Cd5	48	6	88
Cd8	48	12	75
Cd9	48	12	75

Figure 3. Rarefaction Curve and Coverage Estimate.

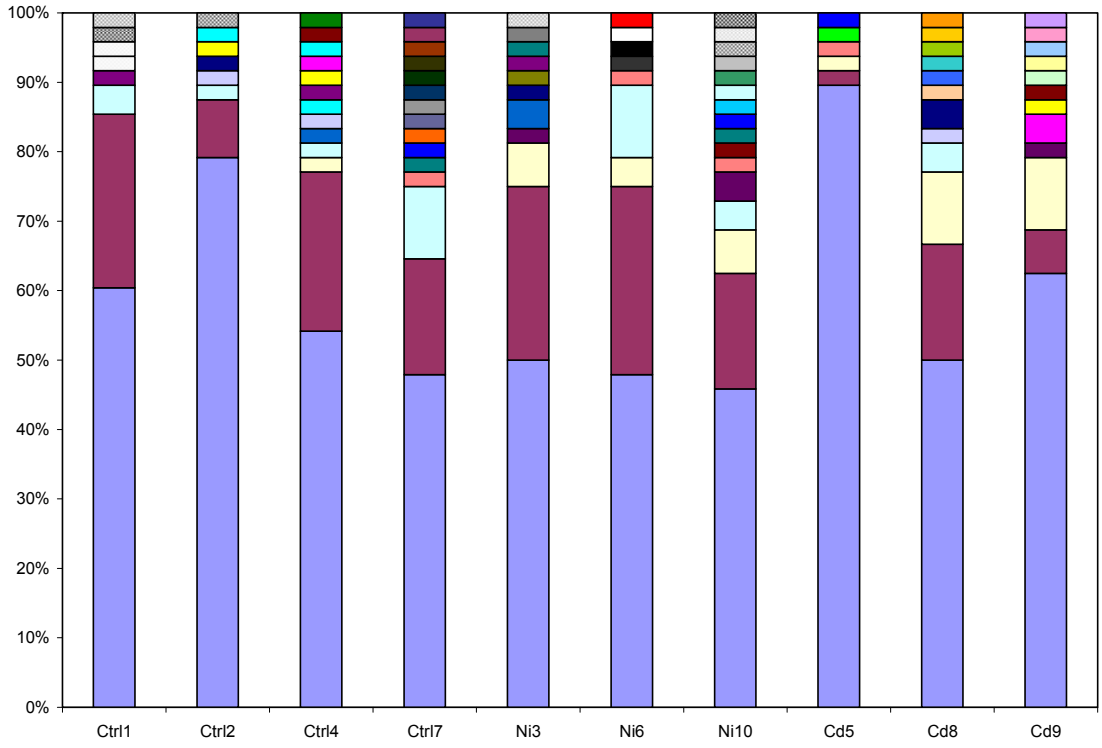


Figure 4. Relative Frequency of Haplotypes by Treatment.

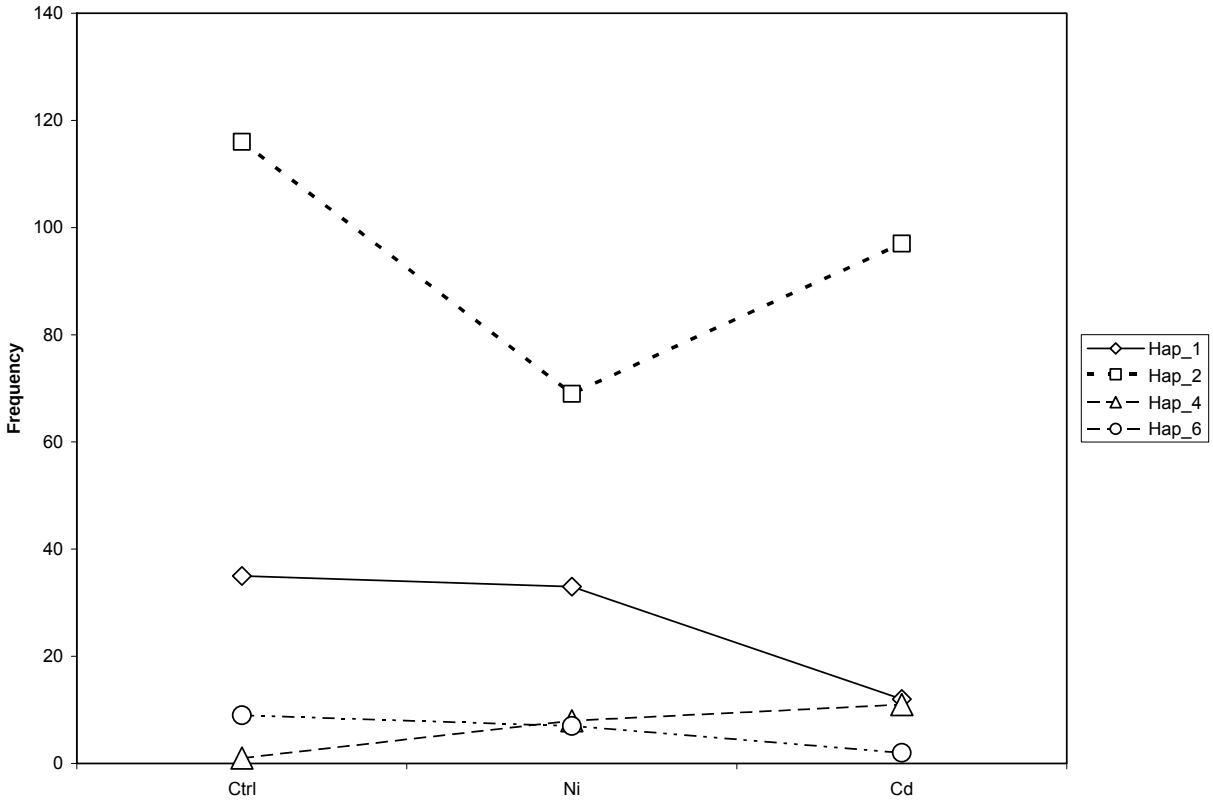


Figure 5. Frequency of the Four Most Dominant Haplotypes.

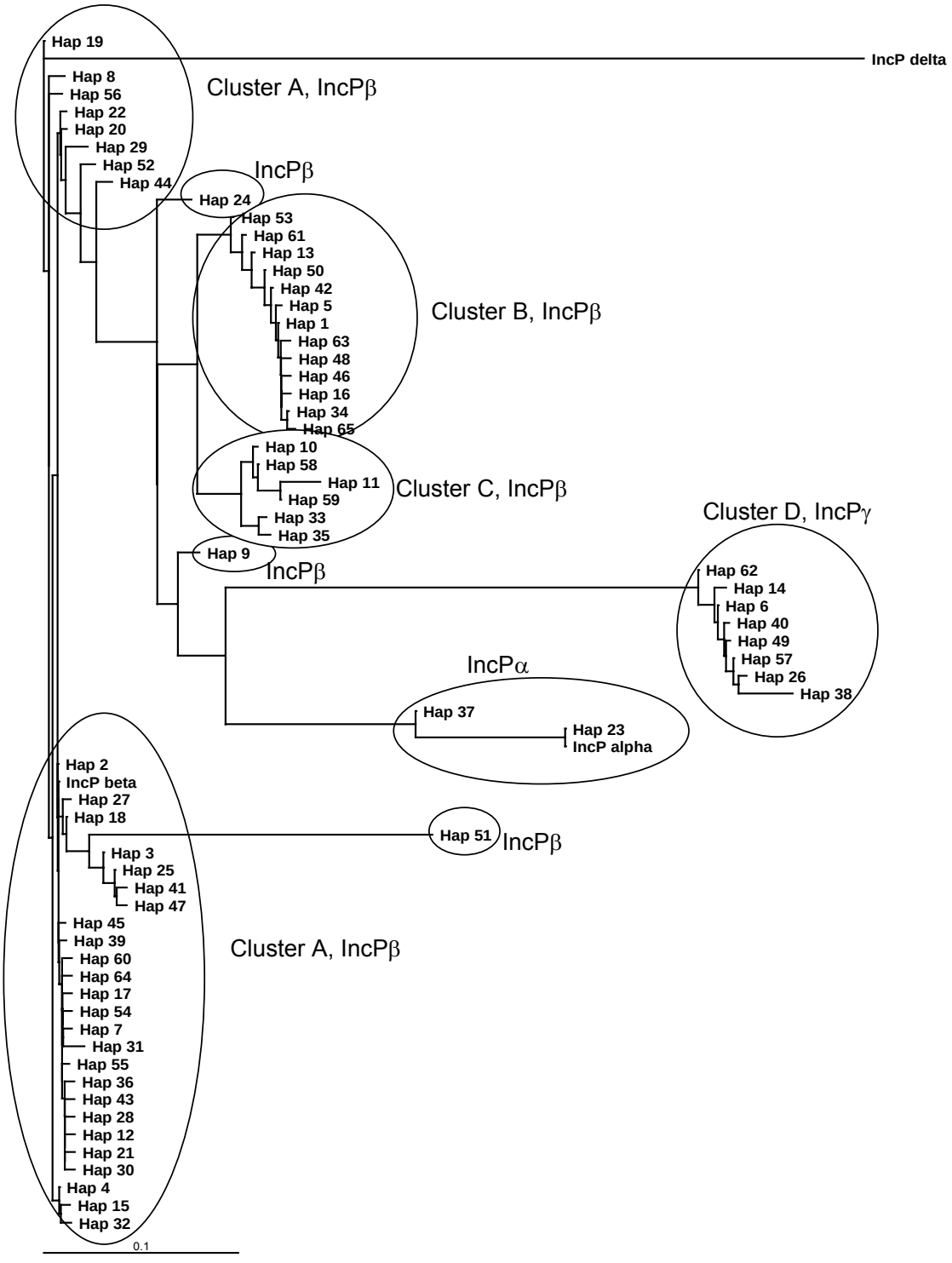


Figure 6. Phylogenetic Tree of *trfA2* gene haplotypes.

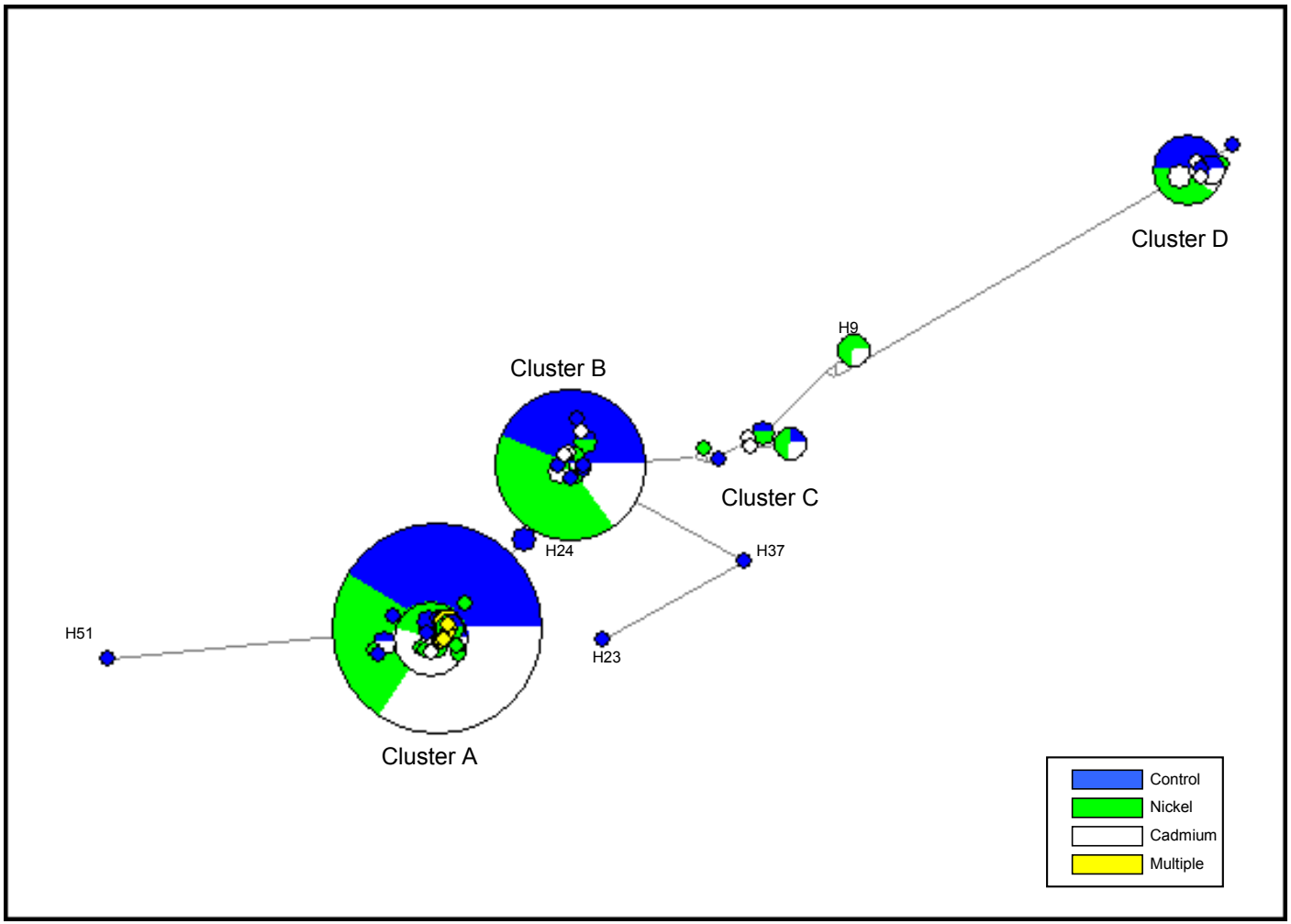


Figure 7. Network diagram of haplotypes and haplotype frequencies by treatment showing haplotype I.D. Ball size corresponds to haplotype frequency. Clusters are magnified in Figure 8-12.

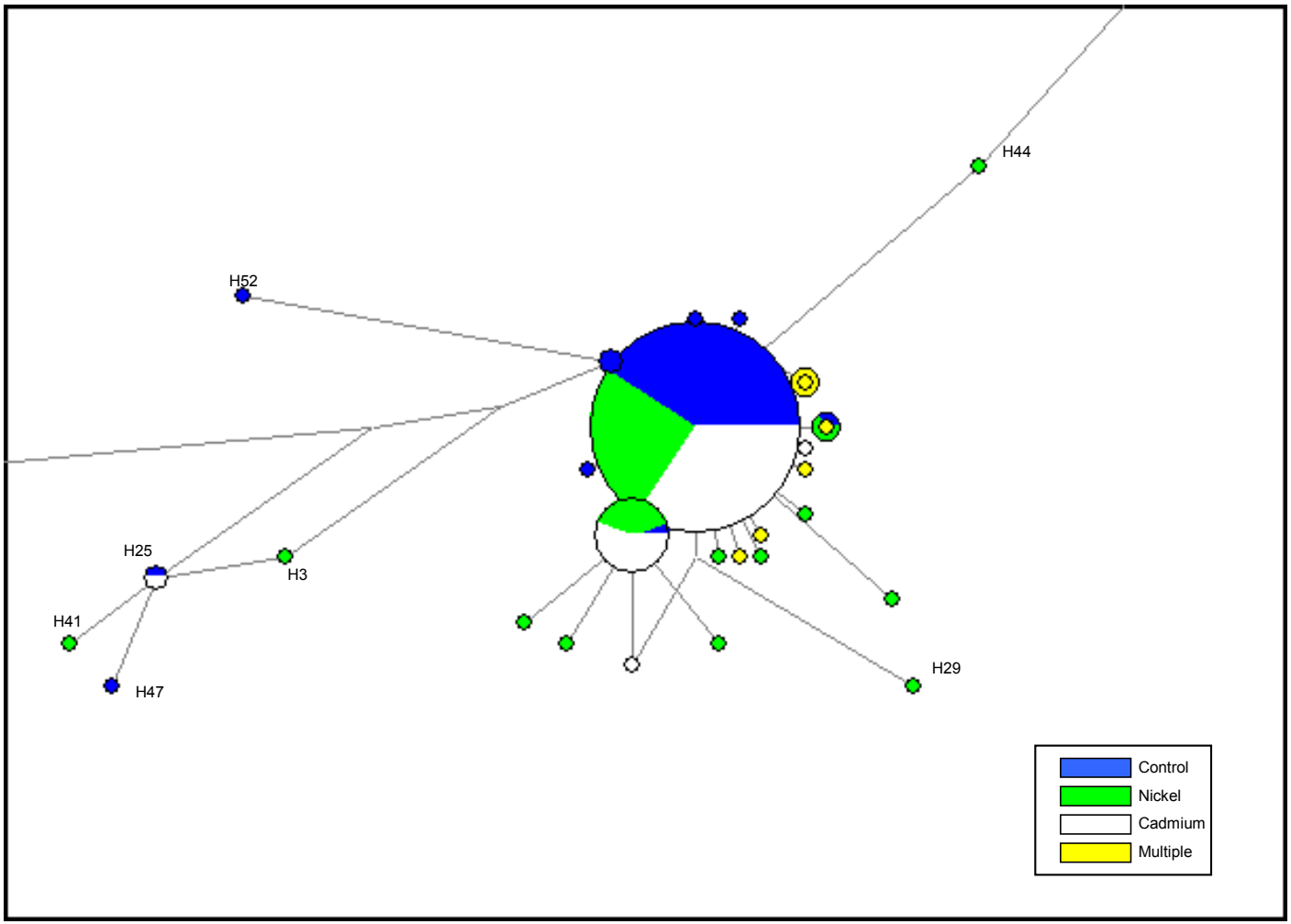


Figure 8. Enlargement of Figure 7, Cluster A, outer portion, showing haplotype I.D. Ball size corresponds to haplotype frequency.

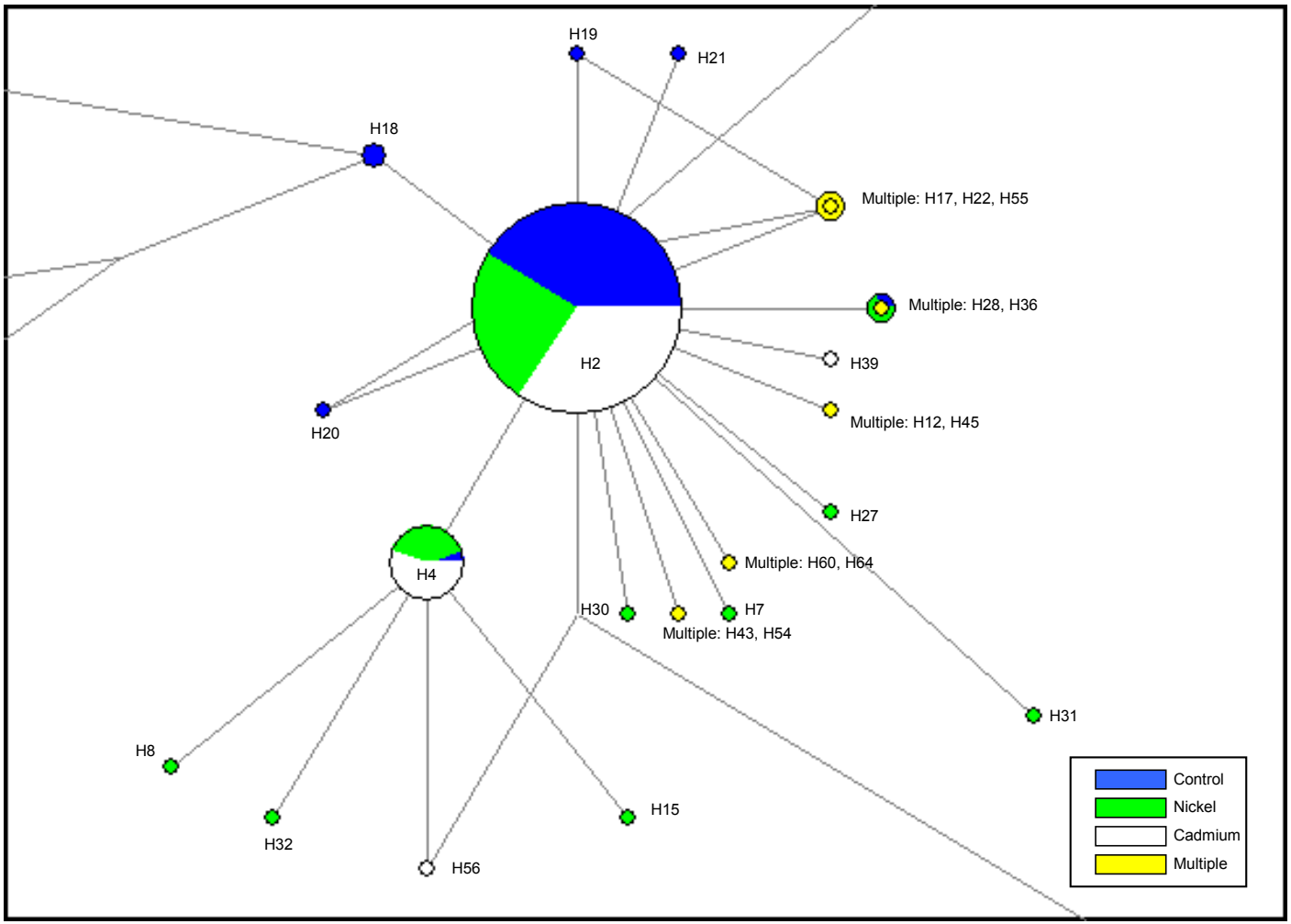


Figure 9. Enlargement of Figure 7, Cluster A, center portion, showing haplotype I.D. Ball size corresponds to haplotype frequency.

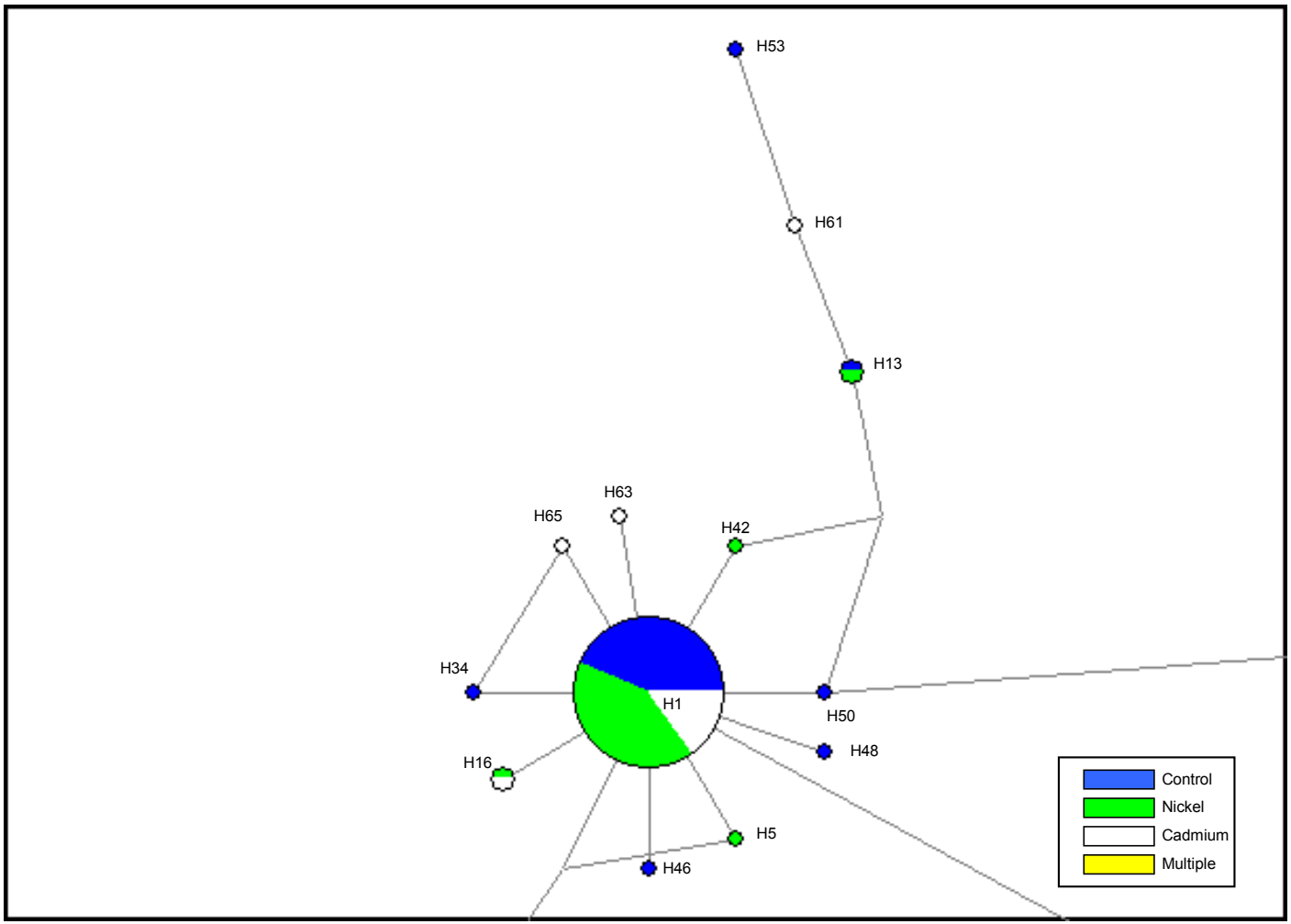


Figure 10. Enlargement of Figure 7, Cluster B, showing haplotype I.D. Ball size corresponds to haplotype frequency.

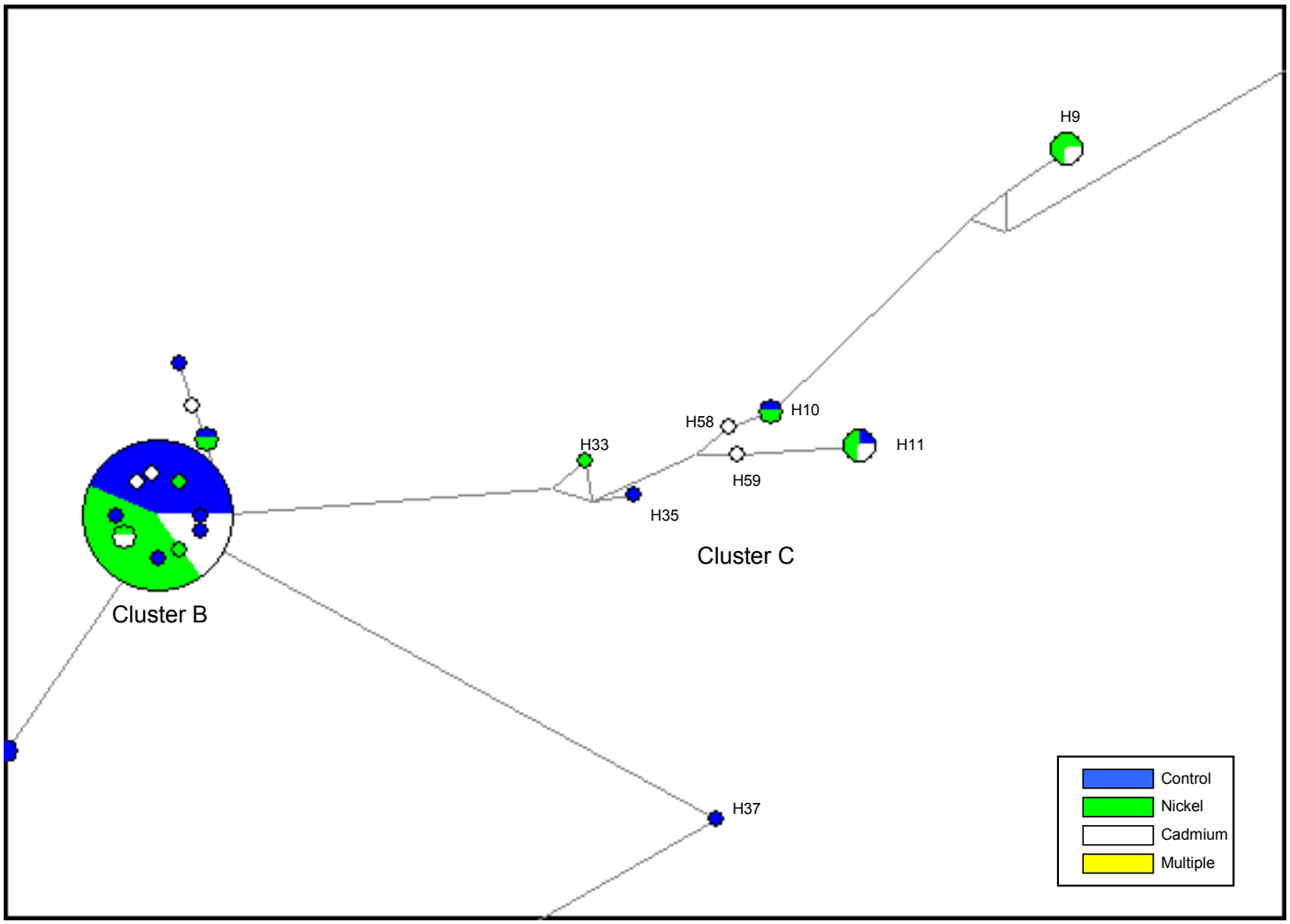


Figure 11. Enlargement of Figure 7, Cluster C, showing haplotype I.D. Ball size corresponds to haplotype frequency.

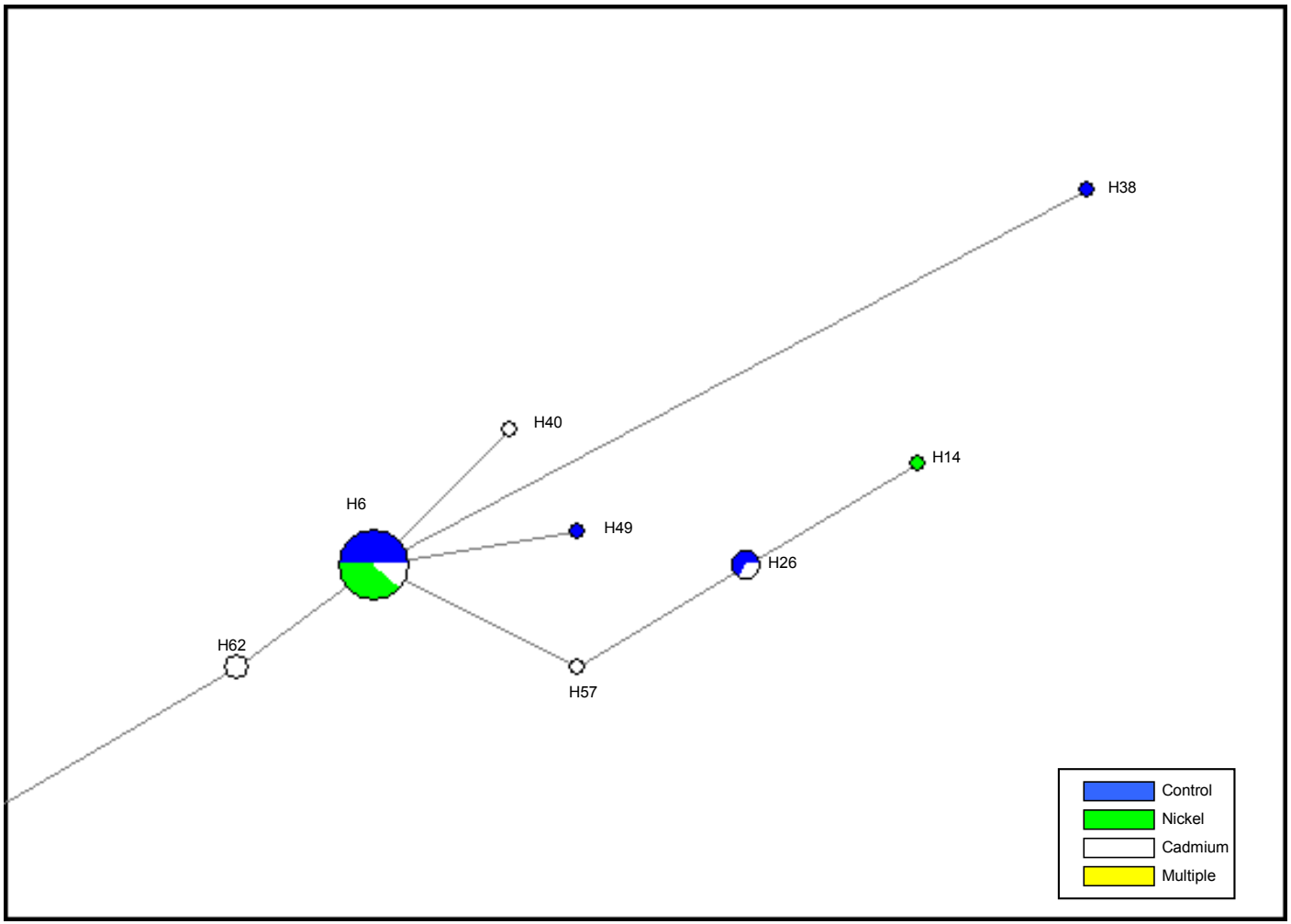


Figure 12. Enlargement of Figure 7, Cluster D, showing haplotype I.D. Ball size corresponds to haplotype frequency.

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APPENDIX A

Products Used in Master's Research

Chemicals

Acetic Acid, Glacial, JT Baker 9508-00
Acridine Orange, Fisher, S101
Agarose. Genetic Technology Grade, Fisher Scientific ICN800669
Ampicillin, sodium salt, Sigma A0166
Bacto Agar, BD 214010
Boric Acid, anhydrous, Sigma B6768
Bromphenol Blue (Sigma, B5525)
Calf Thymus DNA, Activated, Sigma D4522
Chloramphenicol (Chloromycetin), US Biological C4350
EDTA (Ethylenediaminetetraacetic acid), disodium salt, dehydrate, Sigma E5134 or JT Baker 8993-01
Ethidium bromide
Ethyl Alcohol, denatured, FisherChemicals A407-20
Exonuclease I, 20,000U/mL, New England Biolabs, M0293S
Fast DNA SPIN Kit For Soil (Q-Biogene, Bio101 Systems, 6560-200)
Fast DNA Kit (Q-Biogene, Bio101 Systems, 6540-400)
Formaldehyde, J.T. Baker, 2106-01
Gentamycin Sulfate USP (Micromonospora purpurea), US Biological G2030
Hi-Lo DNA Marker (50-10,000 bp), Minnesota Molecular, Inc. 1010
Hydrochloric Acid, Sigma H1758 or FisherChemical A144-212
Kanamycin (Kanamycin A) Monosulfate, Sigma K4000
Lambda DNA, 500μg/mL (New England BioLabs, N3011L)
Lauryl Sulfate (SDS), sodium salt, Sigma L4390
LB (Luria-Bertani) Agar, Miller, Difco 244520
Lysozyme (Muramidase; Mucopeptide N-acetylmuramoylhydrolase), Sigma L7651
Mineral Oil, Extra Heavy, Good Health XXXX?
Nutrient Broth, Difco 234000
Pellet Paint Co-Precipitant, Novagen 69049-3
pH: Freshwater pH Test Kit, Aquarium Pharmaceuticals, Inc.
Phenol:Chloroform:Isoamyl Alcohol (25:24:1), Sigma P3803
Potassium Acetate, Sigma P1190
Ribonuclease A, from Bovine Pancreas, Sigma R6513
S-gal/LB Agar Blend (contains S-Gal and IPTG), Sigma C4478
Shrimp Alkaline Phosphatase, 1000U/mL (USB Corporation, 70092Y)
Sodium Acetate Buffer Solution (3M, pH 5.2), Sigma S7899 or Sodium Acetate (3M, pH 5.2), Novagen 69049-3

Sodium Chloride, Sigma S3014
Sodium Hydroxide, anhydrous pellets, Sigma S8045
Streptomycin Sulfate, Sigma S9137
Sucrose, anhydrous, Sigma S0389
Tetracycline Hydrochloride, Sigma T3383
Trizma Base (Tris[hydroxymethyl]aminomethane), Sigma T1503 or Sigma T6066
Trizma-HCl, 1M, pH 8.0, Biotechnology Grade (Sigma T2694)

PCR

IncP trfA2-1 5'-CGA AAT TCR TRT GGG AGA AGT A-3' (manufactured by Integrated DNA Technologies)

IncP trfA2-2 5'-CGY TTG CAA TGC ACC AGG TC-3' (manufactured by Integrated DNA Technologies)

M13f 5'-GTA AAA CGA CGG CCA GT-3' (manufactured by Integrated DNA Technologies)

M13r 5'-GGA AAC AGC TAT GAC CAT-3' (manufactured by Integrated DNA Technologies)

Bovine Serum Albumin (Sigma, B2518)

Deoxynucleotide Triphosphates, set of dATP, dCTP, dGTP, dTTP, 40μmol each (Promega Corporation, U1240)

Taq: JumpStart Taq DNA Polymerase, 2.5U/μL (with 10X Reaction Buffer without $MgCl_2$ and 25mM $MgCl_2$) (Sigma D4184)

Ultrapure Water: Water, Molecular Biology Grade (Sigma, W4502)

Sequencing

ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, 4336875)

APS (Ammonium Persulfate) (Sigma, A3678)

Blue Dextran/diFormamide Solution (40μL of 10% Blue Dextran in deionized water and 200μL of diFormamide)

Blue Dextran (Sigma, D5751)

DET48 tracking dye (The Gel Company, DET48-FAM)

Blue Dextran/diFormamide Solution (40μL of 10% Blue Dextran in deionized water and 200μL of diFormamide)

Dilution Buffer (Sequencing) = home-made: 400mM Tris-HCl, pH 9.0 and 10mM $MgCl_2$

20% Ficoll Loading Solution (The Gel Company, DAL25).

50% Hi-Di Formamide (Applied Biosystems, 4311320)

Long Ranger Acrylamide (BioWhittaker Molecular Applications #50611)

Resin Beads: AG® 501-X8(D) Resin, Molecular Biology Grade, Hydroxide + Hydrogren Form, 20-50 Mesh (Bio-Rad, #143-6425)

TEMED (Amresco, #0761-50ML)

Urea (Amresco, #0568-25KG)

MicroAmp optical 96-well reaction plate (Applied Biosystem, N801-0560)

RapidLoad Membrane Combs (The Gel Company, CAM96)

ABI Prism 377 DNA Sequencer (PE BioSystems), using ABI Prism 377-96 Collection software version 2.6; post-processed using Analysis Software version 3.3.1 (PE BioSystems)

3130*xl* Genetic Analyzer (Applied Biosystems) with Foundation Data Collection version 3.0, using either a 36cm capillary-UltraSeq36_POP7 (36cm array, POP-7 polymer, 35min run time, kilobyte base caller, and Dye Set Z for BigDye version 3) or a 50cm capillary-3130POP7_BDTv3_KB_Denovo_v5.2 (50cm array, POP-7 polymer, 1hr run time, kilobyte base caller, and Dye Set Z for BigDye version 3); post-processed using Sequencing Analysis 5.2 (Applied Biosystems)

Sequencher version 4.1.4 (Gene Codes Corporation)

Sequences run on the 3130*xl* that were classified as “good” or “very good” were used in the analysis in preference to other sequences. For an individual sample, if there were discrepancies between the sequence run on the 3130*xl* and sequences run on the Prism 377-96, the 3130*xl* sequence was assumed correct because it was not subject to the potential error in lane identification that the previous sequences were subject to.

If the choice of which sequence to use was between sequences run on the old sequencer and the sequences matched perfectly, the better “looking” (higher, more well defined peaks) was used. If the sequences didn’t match and if there was an error in the lane identification (i.e. lane was empty, comb tooth was bent, etc.), then that sequence was discarded and the other sequence was used. If both sequences had no loading problems noted, then the sample was re-amplified, re-sequenced, and run on the 3130*xl*. If a redo was not possible, then the sample was excluded from analysis. Samples with sequences with multiple overlapping bases—indicating that the sample was 2 or more colonies—were excluded from analysis.

Supplies

Breathe-Easy Film (sterile polyurethane film) (USA Scientific, 9123-6100)

Cover Slips: 25mm square cover glasses (No. 1, Corning Lab Equipment)

Film: Fisherbrand PCR Plate Thermal Adhesive Sealing Film (Fisher Scientific, 05-500-33)

Filters (acridine orange slides): 24mm circular glass microfibre filters (Whatmann, 1827-024); Poretics black polycarbonate, 0.22μm, 25mm filter (Osmonics, Inc., K02BP02500)

Mats: Sealing Mat for 96-Well PCR Plates (silicone rubber) (USA Scientific, 1400-9605); Mat: FisherBrand Mat Cover for 96-Deep Well BioBlock Titre Plates (EVA) (Fisher Scientific, 05-501-02)

Nalgene Polycarbonate Square 1L bottle (VWR #16121-220)

Plates: Fisherbrand Assay Blocks, 1.2mL (Fisher Scientific, 05-501-00)

Plates: Fisherbrand Thin-Wall PCR Plates, 0.2mL (Fisher Scientific, 05-500-48)

Slides: 25x75mm Ultra Frost Rite-On Micro Slides (0.93-1.05mm thick, approx. ½ GR., Gold Seal, #3056)

Sterile Sampling Bags (Fisherbrand, 01-815-24)

Syringes (metal analysis): Norm-Ject Luer syringe (Henke Sass Wolf GMPH, Germany, 10mL, CE-0123)

Syringes (filter sterilization): BD 10mL Syringe, Luer-Lok Tip (Becton, Dickinson & Company, NJ, #309604)

Syringe Filters (metal analysis): Acrodisc glass fiber filter (Pall Life Sciences, GF 25mm, PN 4559T)

Syringe Filters (filter sterilization): FisherBrand 25mm Syringe Filter, 0.2μm Nylon, sterile, #09-719C

Equipment

AlphaImager 3400 (Alpha Innotech IS-3400-5-110) using AlphaEaseFC (FluorChem 8800) Software for Windows, version 3.1.2, on a Dell Optiplex GX260 Computer

Autoclave: Market Forge Sterilmatic Autoclave

Bottom Grab Sampler (Eckman)

Balance: Mettler PM200, Mettler AJ100

Centrifuge: IEC Centra-4B Centrifuge, 244 MicroPlate Rotor (Thermo IEC, International Equipment Company)

Distriman Repetitive Pipet, Rainin #D-Man; with Presterile DistriTip Micro (125uL), DistriTip Mini (1250uL) and DistriTip Maxi (12.5mL), Rainin DT-125S, DT-1250S, DT-12MLS.

Dri-Bath (Thermolyne, Type 16500)

Electrophoresis: Owl Model D3-14 Centipede Horizontal Electrophoresis System; Mini Sub CE, Model 17S/7645 electrophoresis system (Bio-Rad)

FastPrep FP120 Instrument (Bio101, Savant, 6000-120)

Fisherbrand Finnpipette, 1-10uL (Fisher Scientific #4173370)

Fisherbrand Finnpipette, 5-50uL (Fisher Scientific #4173300)

ICP-MS Elan DRC Plus (Perkin-Elmer Sciex Instruments, Toronto, Canada)

Incubator-Shaker: Digital Incubator Shaker, Model I-2400 (New Brunswick Scientific Co #I-2400) and an Innova 4080 (New Brunswick Scientific)

Isotemp Programmable Oven (model 383F, Fisher Scientific)

LabConco PCR Enclosure (LabConco, 5102000)

Laminar Flow Hood: BBL Class II Bio-Hazard Cabinet, NSF Std. No. 49 (BBL Microbiology System, Becton Dickinson)

Meters: Corning pH Meter 220 with a general purpose electrode; PHM95 pH/Ion Meter (Radiometer) with a combination electrode; and a CDM210 Conductivity Meter (MeterLab, Radiometer); Radiometer pH probe.

Micro Centrifuge: Eppendorf Centrifuge 5415C (Brinkmann Instruments, Inc.)

Microscope: Microphot-FXA epifluorescence microscope (Nikon), a Nikon PlanApo oil immersion (100x/1.40 oil) lense (Nikon), and a CFWN 10x/20 eyepiece

Pipetman: P2, P20, P100, P200, P1000, P5000. Rainin Pipet-Plus: R2, R100 (Rainin Instrument Company)

Power Pack: Fisher Biotech Electrophoresis Systems FB105 (Fisher Scientific)

Sonicator: Bransonic Ultrasonic Cleaner (Bransonic, B2200R-1)

Stirrer/HotPlates (Corning)

Stirrer: Nuova II Stirrer (Sybron, Thermolyne)

Thermocycler: Eppendorf Mastercycler Gradient

Vacuum Manifold (10 piece) (no manufacturer information on manifold)

Vacuum Pump: Gast Non-Lubricated Vacuum Pump (Gast Manufacturing, Inc., a Unit of IDEX Corporation, MI, #DOA-P704-AA)

Vortex Genie 2 (Fisherbrand)

Waterbath: All Stainless Steel Water Bath, Model 183 (Precision Scientific, 66551)

APPENDIX B

PAUP Data File

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#NEXUS

[Sequences from Cathy King; trfA sequence with primers removed;
analyzed September 28, 2005]

begin data;
dimensions nchar=199 ntax=68;
format datatype=dna missing='?' matchchar='.' gap='-';
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GAGCTGCGCGAAAATGGCCTGGTGGACAGCGCCTGGATCAATGAC
Hap_64
CCGCAAGCTGTCGCCCACCGCGCGGCGCATGTTCGACTACTTCGCCACCCACAAGGAGCCGTACCCGCTCAAGCTGG
AGACGTTCCGGCTGATGTGCGGCTCGGATTCCACCCGGCCGAAGAAATGGCGCGAGCAGGTAGGCGAAGCGTGCGAC
GAGCTGCGCGAAAACGGCCTGGTCGAAAGTGCGTGGGTGAACGAC
Hap_65
CCGCAAGCTGTCGCCCACCGCGCGGCGGATGTTCGACTACTTTGCCACGCACAAGGAGCCGTACCCGCTCAAGCTGG
AGACGTTCCGGCTCATGTGCGGTTCGGATTCCACCCGCGTGAAGAAGTGGCGCGAGCAGGGCAGCGAGGCATGCGAC
GAGCTGCGCGAAAACGGCCTGGTGGACAGCGCCTGGATCAATGAC

IncP_beta
CCGCAAGCTGTCGCCCACCGCGCGGCGCATGTTCGACTACTTCGCCACCCACAAGGAGCCGTACCCGCTCAAGCTGG
AGACGTTCCGGCTGATGTGCGGCTCGGATTCCACCCGGCCGAAGAAGTGGCGCGAGCAGGTAGGCGAAGCGTGCGAC
GAGCTGCGCGAAAACGGCCTGGTCGAAAGTGCGTGGGTGAACGAC
IncP_alpha
CCGCAAGCTGTCGCCGACGGCCCGACGGATGTTCGACTATTTCAGCTCGCACCGGGAGCCGTACCCGCTCAAGCTGG
AAACCTTCCGCCTCATGTGCGGATCGGATTCCACCCGCGTGAAGAAGTGGCGCGAGCAGGTCGGCGAAGCCTGCGAA
GAGTTGCGAGGCAGCGGCCTGGTGGAACACGCCTGGGTCAATGAT
IncP_delta
CCGCGAGCTGTCCCCTACTGCCCGGCGCATGTTCGATTACTTCGTCTCTCACAAGGAACCGTTCCCCTTGAAGCTGG
AGACGTTCAGGCTGATGTGCGGGTCCGATTCCGCCAGGCTCAAGAAGTGGCGCGAGCAGACCGGCCAGGCGTGCGAA
GAGTTGAGCCAGAGCGGGCTTATCAAGCTGTCATGGGTGCAGGGT

;

ENDBLOCK;

[!Likelihood settings from best-fit model (TVM+G) selected by hLRT or
AIC in Modeltest 3.6]

BEGIN PAUP;
Lset  Base=(0.1773 0.3232 0.3209)  Nst=6  Rmat=(2.6196 8.3597 0.6178
3.1750 8.3597)  Rates=gamma  Shape=0.5725  Pinvar=0;
END;
```