AND SUB-SPECIES STRUCTURING IN THE ENTOMOPATHOGENIC NEMATODE *HETERORHABDITIS*

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ABSTRACT

We used Random amplified polymorphic DNA (RAPDs), partial ribosomal DNA (rDNA), the mitochondrial gene cytochrome oxidase subunit I (*cox1*) and major sperm protein (*msp*) sequence analysis to investigate genetic diversity and population structure in *Heterorhabditis bacteriophora, H. indica, H. marelata, H. megidis, H. downesi* and *H. zealandica* comprising 18 isolates collected from different parts of the world. Blastn similarity search performed for the partial rDNA sequences confirmed the identity of *Heterorhabditis* species and suggested that all the unknown isolates belonged to *H. bacteriophora*. Blastn e-values for the species and isolates ranged between 0 to 9e-145. Genetic diversity and phylogenetic analysis produced dendrograms that showed high degrees of genetic variations among *Heterorhabditis* species with the overall average pairwise distance values of 0.3217, 0.6391, 0.7963 and 0.0572 for RAPD, partial rDNA, *cox1* and *msp*, respectively. Although we expected low genetic diversity among *H. bacteriophora* isolates due to alternate automictic and amphimictic lifecycle and lack of long distance movement capability restricting gene flow, our results demonstrate highly structured genetic variation among the isolates. Phylogenetic analysis showed that *H. bacteriophora* isolates is a species complex that contain at least two two new species KMD10 and GPS5. Further *H. bacteriophora* sensu Pionar populations can be divided into two major groups: "HP88" and "Oswego". We conclude that strictly relying on ITS sequences based blastn for *Heterorhabditis* species identification is misleading.

INTRODUCTION

- **Entomopathogenic nematodes *Heterorhabditis* and *Steinernema* posses tremendous potential for biological control of insect pests (Grewal et al., 2005).**
- **Although entomopathogenic nematodes have worldwide distribution only 10 species of *Heterorhabditis* have been described (Stock & Hunt, 2005).**
- **A major problem in the recognition of *Heterorhabditis* species is the high morphological conservation and difficulties in performing cross breeding tests due its complex life cycle.**
- ***Heterorhabditis* life cycle can be initiated by a single infective juvenile that matures into a self reproducing hermaphrodite. However, inside the insect cadaver both males and females can occur in the subsequent generation. This life cycle can lead to continuous change in allele frequency and population genetic structure.**
- **As *Heterorhabditis* species specialize on buried insects and lack long distance self movement, their life cycle must result in low intra-specific genetic diversity.**
- **However, numerous studies indicate high genetic diversity among populations of the cosmopolitan *H. bacteriophora* (Grewal et al., 2005).**

HYPOTHESIS

***Heterorhabditis bacteriophora* is a species complex containing several species.**

OBJECTIVES

- **1) Investigate the inter- and intra-specific genetic variation in *Heterorhabditis*.**
- **2) Investigate the presence of subspecies structuring within *H. bacteriophora* populations.**
- **3) Compare between the resulting phylogenetic relationships inferred from RAPD patterns and partial rDNA, *cox1*, and *msp* sequences.**

METHODS

- **Genomic DNA was extracted using Qiagen® Genomic Tip 100/G according to the manufacturer's recommendations.**
- **PCR conditions were adjusted and products were amplified, purified and sequenced.**

Nematode Species	*Strain/Isolate*	*Original locality*
H. indica	EG2	Egypt
H. marelata	Oregon	Oregon, USA
H. megidis	UK	Site 76, UK
H. zealandica	X1	Australia
H. downesi	K122	UK
H. bacteriophora	HP88	Logan, Utah, USA
Heterorhabditis sp.	NC1	Clayton, North Carolina, USA
Heterorhabditis sp.	Riwaka	Riwaka, New Zealand
Heterorhabditis sp.	Oswego	Oswego, New York, USA
Heterorhabditis sp.	OH25	Hermiston, Oregon, USA
Heterorhabditis sp.	Acows	Ogallala, Nebraska, USA
Heterorhabditis sp.	KMD10	Akron, Ohio, USA
Heterorhabditis sp.	KMD19	Union City, Ohio, USA
Heterorhabditis sp.	GPS1	Jeromesville, Ohio, USA
Heterorhabditis sp.	GPS2	Jeromesville, Ohio, USA
Heterorhabditis sp.	GPS3	Jeromesville, Ohio, USA
Heterorhabditis sp.	GPS5	Jeromesville, Ohio, USA
Heterorhabditis sp.	GPS11	Dellroy, Ohio, USA

Table I. List of entomopathogenic nematode species and strains used in this study and their original localities.

- **Data were collection and processed for RAPD analysis.**
- **DNA sequences were edited using Bioedit and BLAST (Basic Local Alignment Search Tool) sequence similarity searches were performed.**
- **DNA Sequences were aligned using clustal W, MAFFT and PRRN programs.**
- **Amino acid translations of *cox1* and *msp* sequences were obtained and analyzed by MEGA 3.1 software.**
- **Resulting alignments compared, the final alignments improved manually and prepared in FASTA, MEGA and NEXUS formats.**
- **The best base-substitution models for distance analysis and reconstructing phylogeny were obtained using Find-Model software.**
- **Phylogenetic trees were constructed using neighbor joining (NJ), minimum evolution (ME), Bayesian analysis of phylogeny (BAP), maximum parsimony (MP) and maximum likelihood (ML) methods using PAUP V4B10, MEGA 3.1, MrBayes v3.1 and Tree Puzzle softwares.**

RESULTS

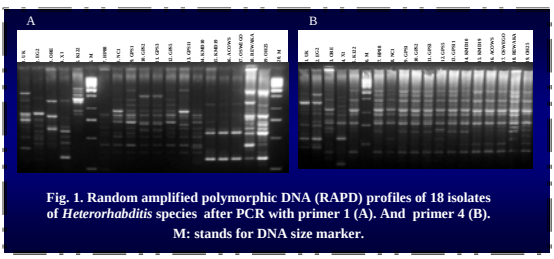

Fig. 1. Random amplified polymorphic DNA (RAPD) profiles of 18 isolates of *Heterorhabditis* species after PCR with primer 1 (A). And primer 4 (B). M: stands for DNA size marker.

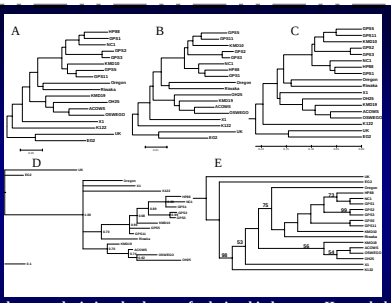

Fig. 2. Phylograms depicting the degree of relationship between *Heterorhabditis* species and isolates produced from the RAPD analyses data generated using Neighbor joining (A), Minimum evolution (B), UPGMA (C) Bayesian analysis (The number of generations used is 1000000. Number in front of the nodes are the credibility values) (D) and Maximum parsimony (Bootstrap replicates = 1000) (E) methods.

- **RAPD analysis showed relatively high values of pairwise among all species. The overall pairwise distance for the 18 isolates was 0. 3217.**

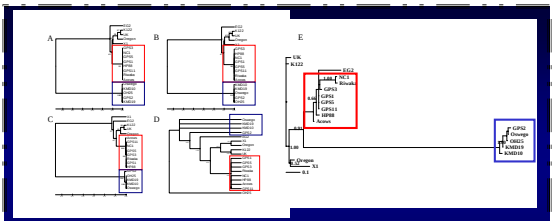

Fig. 3. Phylograms depicting the degree of relationship between *Heterorhabditis* species and isolates produced from partial rDNA generated using Neighbor joining (A), Minimum evolution (B), UPGMA (C) and Maximum parsimony (Bootstrap replicates = 1000) 75 % consensus tree (D), and Bayesian analysis methods (E), the number of generations used is 1000000. Number in front of the nodes are the bootstrap and credibility values.

- **The phylogenetic analysis of the partial rDNA sequences revealed strong sub-structuring among *H. bacteriophora* isolates.**
- **All partial rDNA based phylogenetic trees agreed for division of the species into two major phylogenetic groups.**

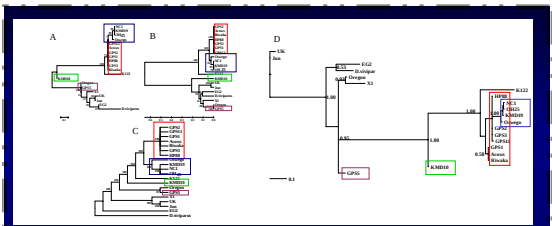

Fig. 4. Phylograms depicting the degree of relationship between *Heterorhabditis* species and isolates produced from partial cox1 DNA generated using Neighbor joining (A), UPGMA (B), and Maximum parsimony (Bootstrap replicates = 1000) 75 % consensus tree (C), and Bayesian analysis methods (D), the number of generations used is 1000000. Number in front of the nodes are the bootstrap and credibility values.

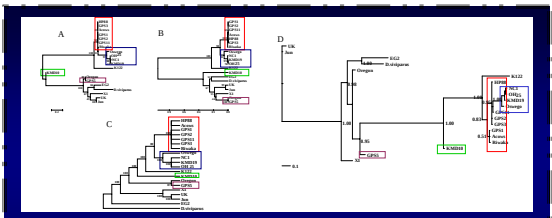

Fig. 5. Phylograms depicting the degree of relationship between *Heterorhabditis* species and isolates produced from partial cox1 amino acid sequence generated using Neighbor joining (A), UPGMA (B), and Maximum parsimony (Bootstrap replicates = 1000) 75 % consensus tree (C), and Bayesian analysis methods (D), the number of generations used is 1000000. Number in front of the nodes are the bootstrap and credibility values.

- **The *cox 1* also showed high values of genetic diversity among *Heterorhabditis* species. The overall average pairwise difference is 0.6534. The overall value of pairwise distance among *H. bacteriophora* isolates is 0.4273, when all the haplotypes were included.**
- **All the *cox 1* (DNA and amino acid sequences) based phylogenetic trees showed high degree of sub-species structuring within *H. bacteriophora*.**
- **All phylogenetic trees agreed for division of the species into two phylogenetic groups:**
- **HP88 group containing isolates HP88, Acows, Riwaka, GPS1, GPS3 and GPS11.**
- **Oswego group containing Oswego, NC1, KMD19 and OH25 with high bootstrap and clade credibility values.**
- **Both KMD10 and GPS5 are representing new phylogenetic species.**

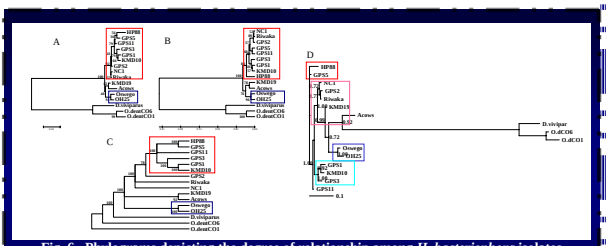

Fig. 6. Phylograms depicting the degree of relationship among *H. bacteriophora* isolates produced from partial *msp* DNA sequence generated using Neighbor joining (A), UPGMA (B), and Maximum parsimony (Bootstrap replicates = 1000) 75 % consensus tree (C), and Bayesian analysis methods (D), the number of generations used is 1000000. Number in front of the nodes are the bootstrap and credibility values. *D.viviparus* and O. *dentatum* were used as out groups.

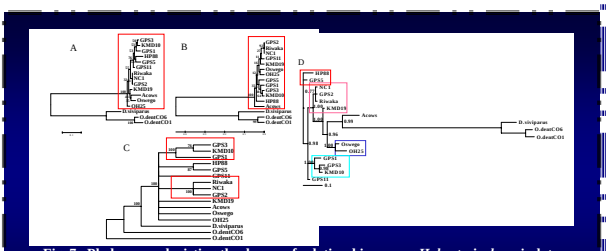

Fig. 7. Phylograms depicting the degree of relationship among *H. bacteriophora* isolates produced from *msp* amino acid sequence generated using Neighbor joining (A), UPGMA (B), and Maximum parsimony (Bootstrap replicates = 1000) 75 % consensus tree (C), and Bayesian analysis methods (D), the number of generations used is 1000000. Number in front of the nodes are the bootstrap and credibility values. *D.viviparus* and O. *dentatum* were used as out groups.

- **As expected, major sperm protein gene *msp* is highly conserved among *Heterorhabditis* isolates as the overall value of pairwise distance among *H. bacteriophora* isolates was only 0.0572.**
- **Although highly conserved genes do not usually show sub-species structuring, *msp* revealed the presence of sub-structuring among *H. bacteriophora* isolates.**

DISCUSSION

- **The ITS-rDNA sequence based blastn searches are commonly used for identification of *Heterorhabditis* species (Adams et al., 1988; Stock and Hunt, 2005).**
- **However our results indicate that ITS regions in *Heterorhabditis* does not show high genetic diversity and may hid species differentiation.**
- **We found that *cox1* is a more reliable target for species identification in *Heterorhabditis.***

CONCLUSION

- ***cox1 is* suitale for species identification in *Heterorhabditis* than ITS-rDNA.**
- ***H. bacteriophora* is a species complex that contain two new species "KMD10" and "GPS5".**
- ***H. bacteriophora* sensu Poinar also showed distinct sub-structring.**
- **To test the biological aspect of speciation, crosses between the new proposed species and the other known *Heterorhabditis* species will be conducted.**

ACKNOWLEDGMENTS

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