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Phylogenetic and cophylogenetic relationships of entomopathogenic nematodes (*Heterorhabditis*: Rhabditida) and their symbiotic bacteria (*Photorhabdus*: Enterobacteriaceae)

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ABSTRACT

Mutualistic association between entomopathogenic *Photorhabdus* bacteria and *Heterorhabditis* nematodes represents one of the emerging model systems in symbiosis studies, yet little is known about this partnership from a coevolutionary perspective. Herein, we investigated phylogenetic and cophylogenetic relationships of *Heterorhabditis* and *Photorhabdus* strains using molecular markers Internal Transcribed Spacer and *gyrase B* gene sequences, respectively. The phylogenies presented consistent, well supported, monophyletic groups in the parsimonious and likelihood analyses for both the nematode and bacterial strains and supported the placement of currently recognized taxa, from which a potentially new *Heterorhabditis* species represented by a Thailand strain MP68 was identified. While the nematode strains with distant geographic distributions showed no detectable phylogenetic divergence within *H. bacteriophora* or *H. georgiana* monophyletic groups, their respective symbiotic bacteria speciated into two *Photorhabdus* species: *P. luminescens* and *P. temperata*, indicating the occurrence of duplication. Although such evolutionary process reduces the phylogenetic congruence between *Heterorhabditis* nematodes and *Photorhabdus* bacteria, global cophylogenetic tests using ParaFit detected a highly significant correlation between the two phylogenies (ParaFitGlobal = 0.001). Further, the associations between *H. zealandica*, *H. indica* and *H. megidis* strains and their symbiotic bacteria exhibited significant contribution to the overall cophylogenetic structure. Overall, this study reveals evidence of coevolution between *Photorhabdus* bacteria and *Heterorhabditis* nematodes and provides a framework for further examination of the evolution of these associations.

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1. Introduction

Microbial symbioses are ubiquitous and are often hypothesized to have undergone coevolution with their animal or plant hosts, which can eventually lead to parallel speciation or cospeciation in both partners (Page and Charleston, 1998). In strict cospeciation, the symbiotic partners have coordinated speciation and mirrored phylogenetic relationships. However, a number of processes such as host switching, duplication, sorting events and failure of one lineage to speciate in response to speciation of the other lineage can create incongruence between phylogenies of symbiotic partners (Page and Charleston, 1998). Accordingly, coevolution does not necessarily culminate in species-specificity, and the knowledge on the degree of cospeciation can provide an essential step toward understanding the historical role of coevolution in shaping speciation and diversification in symbiotic partners (Kawakita et al., 2004).

As one of the most diverse animals on earth, nematodes represent an attractive system for evolutionary studies. They have adapted to nearly every ecological niche from marine to fresh water and from soils to animal tissues. In fact, nematodes parasitize all known animal groups including themselves (Andrássy and Zombori, 1976), and association with bacteria has certainly played a part in this evolutionary feat. One of the best-known associations between nematodes and bacteria is between entomopathogenic nematodes *Heterorhabditis* and their symbiotic bacteria *Photorhabdus* (Ruby, 2008). Taxonomic studies have demonstrated that almost every entomopathogenic nematode species possesses a specific symbiotic bacteria species (Liu et al., 1997). In nature,

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the bacteria colonize the intestine of the nematode infective juveniles which invade a susceptible insect host (Boemare, 2002). After release into the insect hemolymph by the infective juveniles, the bacteria multiply, killing the insect and converting the cadaver into a food source suitable for nematode growth and reproduction (Poinar, 1990). The nematodes reproduce for one to three generations depending upon host size and emerge as infective juveniles when food resources are depleted. The bacteria recolonize the emerging infective juveniles and persist by lowering their metabolism in the enduring infective juveniles ensuring their transmission to a new insect host (An and Grewal, 2010a). This nematode–bacteria symbiotic partnership is extremely effective in killing a diverse species of insects and has been successfully implemented in biological control and integrated pest management programs worldwide (Grewal et al., 2005).

Heterorhabditis nematodes have been found widely distributed throughout North and South America, Australia, Europe, Asia and Africa. Fourteen species under this genus are currently recognized and various strains have been found distributed in different parts of six continents except Antarctica including some islands (Hominick, 2002; Malan et al., 2008; Nguyen and Hunt, 2007; Nguyen et al., 2008). The bacterial partners have only been characterized from seven *Heterorhabditis* species, *H. indica*, *H. bacteriophora*, *H. megidis*, *H. zealandica*, *H. downesi*, *H. marelatus*, and *H. gerrardi* and three distinct bacterial species *Photorhabdus luminescens*, *Photorhabdus temperata* and *Photorhabdus asymbiotica* have been recognized (Adams et al., 2006; Nguyen and Hunt, 2007; Plichta et al., 2009). Although mutualistic association between *Heterorhabditis* and *Photorhabdus* species was originally thought to be strictly one-to-one in terms of cospeciation, with the increased number of nematode and bacterial isolates, it has been observed that some bacterial species colonize more than one recognized nematode species. For example, while all the bacterial partners of *H. megidis* are identified to be *P. temperata* (Kuwata et al., 2007), this bacterial species has been found associated with *H. bacteriophora*, *H. zealandica*, and *H. downesi* (Adams et al., 2006; An and Grewal, 2010b, 2011; Boemare, 2002; Tóth and Lakatos, 2008). While this calls into question the strict specificity of *Heterorhabditis–Photorhabdus* species associations, it has been assumed that the apparent exceptions do not fundamentally modify the one-to-one concept just because they are essentially the result of taxonomic classification based on phenotypic and genotypic discriminations (Boemare and Akhurst, 2006). However, with the direct use of genetic data, cospeciation between *Heterorhabditis* and *Photorhabdus* may be analyzed (Liu et al., 1998). Herein, we determined the molecular phylogeny of *Heterorhabditis* strains and their symbiotic bacteria based on sequences of the nematode ribosomal Internal Transcribed Spacer (ITS) region and the bacterial *gyrase B* (*gyrB*) gene fragment. We hypothesized that there is a degree of correlation or cospeciation rather than a random association between the phylogenies of *Heterorhabditis* nematodes and their symbiotic *Photorhabdus* bacteria. Using the sequence data for *Heterorhabditis–Photorhabdus* pairs from the same strain, we tested this hypothesis by statistically comparing the phylogenetic trees between these two evolutionary partners.

2. Materials and methods

2.1. Isolates of nematodes and bacteria

We used a total of 67 nematode strains in this study, of which 57 were from the continental USA, two from the Midway Islands (USA), three from Thailand, one each from New Zealand, UK, Argentina, Hungary and Trinidad and Tobago. The original localities and sources of all the strains are listed in Table 1. The nematodes were maintained in the laboratory by recycling through the last instar wax moth *Galleria mellonella* once a month. The symbiotic bacteria from the nematode strains were isolated using the following procedure. Within 48 h post nematode infection,

Table 1
The origin of *Heterorhabditis* and their corresponding *Photorhabdus* strains used in this study. The nematode strains were obtained from different sources and the corresponding bacterial strains were isolated as described in Section 2.

Strain	Origin	Source
ACOWS	Nebraska, USA	Byron J. Adams
Mar	Oregon, USA	Byron J. Adams
OH25	Oregon, USA	Byron J. Adams
Riwaka	New Zealand	Byron J. Adams
Arg	Argentina	S. Patricia Stock
BF2	California, USA	Edwin E. Lewis
GPS1	Ohio, USA	Parwinder S. Grewal
GPS11	Ohio, USA	Parwinder S. Grewal
GPS12	Ohio, USA	Parwinder S. Grewal
GPS13	Ohio, USA	Parwinder S. Grewal
GPS14	Ohio, USA	Parwinder S. Grewal
GPS15	Ohio, USA	Parwinder S. Grewal
GPS16	Ohio, USA	Parwinder S. Grewal
GPS17	Ohio, USA	Parwinder S. Grewal
GPS18	Ohio, USA	Parwinder S. Grewal
GPS19	Ohio, USA	Parwinder S. Grewal
GPS20	Ohio, USA	Parwinder S. Grewal
GPS21	Ohio, USA	Parwinder S. Grewal
GPS22	Ohio, USA	Parwinder S. Grewal
GPS23	Ohio, USA	Parwinder S. Grewal
GPS24	Ohio, USA	Parwinder S. Grewal
GPS25	Ohio, USA	Parwinder S. Grewal
GPS26	Ohio, USA	Parwinder S. Grewal
GPS27	Ohio, USA	Parwinder S. Grewal
GPS28	Ohio, USA	Parwinder S. Grewal
GPS29	Ohio, USA	Parwinder S. Grewal
GPS30	Ohio, USA	Parwinder S. Grewal
GPS31	Ohio, USA	Parwinder S. Grewal
GPS32	Ohio, USA	Parwinder S. Grewal
GPS33	Ohio, USA	Parwinder S. Grewal
GPS34	Ohio, USA	Parwinder S. Grewal
GPS35	Ohio, USA	Parwinder S. Grewal
KMD1	Ohio, USA	Michael D. Klein
KMD10	Ohio, USA	Michael D. Klein
KMD19	Ohio, USA	Michael D. Klein
KMD24	Virginia, USA	Michael D. Klein
KMD37	Ohio, USA	Michael D. Klein
KMD41	Ohio, USA	Michael D. Klein
KMD42	Ohio, USA	Michael D. Klein
KMD60	Ohio, USA	Michael D. Klein
KMD61	Ohio, USA	Michael D. Klein
KMD62	Ohio, USA	Michael D. Klein
KMD63	Ohio, USA	Michael D. Klein
KMD64	Ohio, USA	Michael D. Klein
KMD65	Ohio, USA	Michael D. Klein
KMD69	Ohio, USA	Michael D. Klein
KMD70	Ohio, USA	Michael D. Klein
KMD74	Hungary	Michael D. Klein
KMD81	Ohio, USA	Michael D. Klein
KMD82	Ohio, USA	Michael D. Klein
KMD83	Tennessee, USA	Michael D. Klein
KMD84	Ohio, USA	Michael D. Klein
MID09	Midway Island, USA	Michael D. Klein
MID10	Midway Island, USA	Michael D. Klein
OB1	Ohio, USA	Michael D. Klein
OB2	Ohio, USA	Michael D. Klein
TN2	Tennessee, USA	Michael D. Klein
UK76	Site of 76, UK	Jeremy D. Pearce
MP17	Khon Kaen, Thailand	Patchareewan Maneesakorn
MP68	Kanchanaburi, Thailand	Patchareewan Maneesakorn
MP111	Krabi, Thailand	Patchareewan Maneesakorn
NC1	North Carolina, USA	Raymond J. Akhurst
RDS96	Indiana, USA	Douglas S. Richmond
RDS109	Indiana, USA	Douglas S. Richmond
RDS123	Indiana, USA	Douglas S. Richmond
SPCM3	Indiana, USA	James A. Cate
TTO1	Trinidad and Tobago	Todd A. Ciche

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G. mellonella cadaver was surface sterilized by dipping into 95% ethanol, igniting and plunging into sterile distilled water. A drop of *G. mellonella* hemolymph was collected by bleeding a foreleg of the surface sterilized *G. mellonella* cadaver with sterile scissors, and was spread on NBTA indicator plates (5 g/L peptone, 3 g/L beef extract, 15 g/L agar, 0.025 g/L bromothymol blue, and 0.04 g/L triphenyl tetrazolium chloride) (Akhurst, 1980). After overnight incubation at 25 °C, the bacterial colonies were tested for bioluminescence (Boemare, 2002) and colonies showing positive luminescent activity were selected for further studies.

2.2. Genomic DNA extraction

To extract nematode genomic DNA, *G. mellonella* larvae were infected with 100 infective juveniles of each nematode strain. After 96 h a single female adult nematode was dissected out from *G. mellonella* cadaver using a platinum needle and placed into a PCR tube with 20 μL lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% [w/v] gelatin and 60 μg/mL proteinase K). The mixture was incubated at −80 °C for 15 min and then incubated at 60 °C for 1 h and 95 °C for 15 min in a BIO-RAD DNA Engine® thermocycler (Lissemore et al., 2005). The resulting DNA samples were examined by agarose gel electrophoresis. For bacterial genomic DNA extraction, the bacterial cells were collected by centrifugation and homogenized using a Polytron Tissue Homogenizer for 10–15 s in 400 μL sterile salt homogenizer buffer (0.4 M NaCl, 10 mM Tris–HCl pH 8.0, and 2 mM EDTA pH 8.0). Then, 40 μL of 20% SDS (2% final concentration) and 8 μL of 20 mg/mL proteinase K (400 μg/mL final concentration) were added and mixed well. The bacterial samples were incubated at 60 °C for at least 1 h or overnight, after which 300 μL of 6 M NaCl (NaCl saturated H_2O) was added to each isolate. Each bacterial sample was vortexed for 30 s at maximum speed, and tubes were spun for 30 min at 10,000*g*. The supernatant was transferred to fresh tubes and an equal volume of isopropanol was added to each tube, mixed well, and incubated at −20 °C for 1 h. Bacterial samples were then centrifuged at 10,000*g* for 20 min at 4 °C. The pellets were washed with 70% ethanol, air-dried and finally resuspended in 200 μL elution buffer. The DNA samples were examined by agarose gel electrophoresis.

2.3. PCR amplification and cloning

Previous studies have suggested that the ITS sequences provide detailed information about variation within and among species of *Heterorhabditis* (Malan et al., 2008; Nguyen et al., 2008), and *gyrB* gene sequences are more useful in investigating intrageneric phylogenies of *Photorhabdus* in contrast to the normally used 16S ribosomal DNA sequences (Akhurst et al., 2004; Tóth and Lakatos, 2008). For phylogenetic analyses in this study, ITS region and *gyrB* gene were amplified from the genomic DNA of the nematodes and the bacteria, respectively. PCR amplification was carried out in 50 μL reactions containing 10 μL 5× GoTaq® buffer (Promega), 1 μL 10 μM dNTP (Promega), 1.25 U GoTaq® DNA Polymerase (Promega), 2 μL each of 10 μM primers, 32.75 μL sterilized water and 2 μL of template DNA. The primers used for ITS amplification were ITS-F (5-TTG AAC CGG GTA AAA GTC G) and ITS-R (5-TTA GTT TCT TTT CCT CCG CT) as described previously (Stock et al., 2001). The primers used for amplification of 16S ribosomal DNA (16S rDNA) sequences were 16S-F2 (5-CAG ACT CCT ACG GGA GGC AGC A) and 16S-R2 (5-CTC ACG GTT CCC GAA GGC ACT). The primers used for amplification of *gyrB* gene were gyrB-F (5-GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AAR TTY GA) and gyrB-R (5-AGC AGG GTA CGG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT) according to Yamamoto and Harayama (1995). The PCR reactions were incubated at 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min in a BIO-RAD DNAEngine® thermocycler. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). The purified ITS and 16S rDNA PCR product for each strain was separately cloned into pGEM®-T Easy vector (Promega) according to the manufacturer's instructions. The plasmids containing ITS and 16S rDNA PCR products were isolated using the QIAprep Spin Miniprep Kit (Qiagen) for sequencing.

2.4. Gene sequence and phylogeny analysis

The plasmids were sequenced from both ends using the universal primers T7 and SP6 with an Applied Biosystems 3730 DNA Analyzer. The purified *gyrB* gene PCR products were sequenced from both ends using the oligonucleotides with the same sequences as underlined in primers gyrB-F and gyrB-R as described by Yamamoto and Harayama (1995). Site polymorphisms in the sequenced products were recorded as described previously (Nadler et al., 2006). After removing low quality and vector sequences, the resulting high quality sequences were used to produce alignments of ITS or *gyrB* with other known nematodes or bacteria (Appendix A) for comparative purposes using ClustalW algorithm (Thompson et al., 1994). For phylogenetic analysis, the unaligned sequence ends were deleted, gaps in the aligned domains were treated as either missing data or a fifth base, and *Caenorhabditis elegans* and *Xenorhabdus nematophila* were used as outgroup taxa and to root the trees for *Heterorhabditis* and *Photorhabdus*, respectively according to previous studies (Malan et al., 2008; Tóth and Lakatos, 2008). Initially, all our 67 strains were included in phylogenetic analyses using both maximum parsimony (MP) and maximum likelihood (ML) methods. MP analysis was performed with bootstrap (500 replications) using Mega software (Tamura et al., 2007). ML reconstruction was carried out in PHYML (Guindon and Gasquel, 2003) using the model of nucleotide substitution estimated from log-likelihood parameters following the Akaike Information Criterion (AIC) 1 with 4 gamma categories in ModelGenerator (Keane et al., 2006). ML trees were inferred using a neighbor-joining starting tree with NNI tree search algorithm, and support for the topology was tested using 100 bootstrap pseudoreplicates. However, as many new strains formed a big monophyletic group, the datasets were pruned in each phylogenetic analysis to include just representatives for each reciprocally monophyletic group in order to simplify searches of tree space.

2.5. Cophylogenetic analysis

We evaluated whether there is evidence for cophylogeny between *Photorhabdus* and *Heterorhabditis*. Initially, all 67 strains from this study plus five pairs of *Heterorhabditis*–*Photorhabdus* associations available from the NCBI database (see Supplementary Table 1) were included in the cophylogenetic test using ParaFit analysis (Legendre et al., 2002) as incorporated in the CopyCat platform (Meier-Kolthoff et al., 2007). The nematode and bacterium phylogenies were then pruned so that only one representative from each locality was selected in order to avoid possible overrepresentation in the cophylogeny tests. As most strains in this study were identified to be *H. bacteriophora* and considering that genetic divergence among populations of this species is relatively independent of geographic distance (Jagdale et al., 2006), we defined one locality at both the state and country levels. The strains included in this test are listed in Table 2. In ParaFit analysis, the distance matrices among the nematodes or the bacteria were computed from the ML tree using the best model as described above (Keane et al., 2006). Probabilities were based on 999 permutations and the correlation was considered significant at $p < 0.02$ (Meier-Kolthoff et al., 2007). The null hypothesis of the global test is that

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Table 2
The *Heterorhabditis* and their corresponding *Photorhabdus* strains used for cophylogeny evaluation. Data for nematode and bacterium strains D1, HF85, K122, Iran1 and NZH3 were obtained from the NCBI database while the rest were from this study.

Isolates	Origin	Nematode		Bacteria	
		Species	Accession	Species	Accession
ACOWS	NE, USA	*H. georgiana*	HQ225842	*P. luminescens*	HQ225956
D1	Guangdong, China	*H. indica*	AY170329	*P. luminescens*	AY278499
GPS11	OH, USA	*H. bacteriophora*	HQ225862	*P. temperata*	GU249303
HF85	The Netherlands	*H. megidis*	EF043439	*P. temperata*	AY278502
K122	Irish	*H. downesi*	EF043442	*P. temperata*	EU930355
Iran1	Iran	*H. bacteriophora*	EU163272	*P. luminescens*	FJ653915
KMD24	VI, USA	*H. bacteriophora*	HQ225866	*P. luminescens*	HQ225919
KMD74	Hungary	*H. bacteriophora*	HQ225877	*P. luminescens*	HQ072280
NC1	NC, USA	*H. bacteriophora*	HQ225882	*P. temperata*	HQ225953
KMD83	TE, USA	*H. bacteriophora*	HQ225880	*P. luminescens*	HQ225958
Arg	Argentina	*H. bacteriophora*	HQ225906	*P. temperata*	HQ225922
UK76	UK	*H. megidis*	HQ225905	*P. temperata*	HM072279
TT01	Trinidad and Tobago	*H. bacteriophora*	HQ225894	*P. luminescens*	BX571859
MID10	Midway Island, USA	*H. indica*	HQ225902	*P. luminescens*	HM072278
MP17	Khon Kaen, Thailand	*H. indica*	HQ225859	*P. luminescens*	HQ225942
MP68	Kanchanaburi, Thailand	*Heterorhabditis* sp	HQ225860	*P. luminescens*	HQ225941
MP111	Krabi, Thailand	*H. indica*	HQ225858	*P. luminescens*	HQ225961
NZH3	New Zealand	*H. zealandica*	EF530041	*P. temperata*	AY278513
BF2	CA, USA	*H. bacteriophora*	HQ225900	*P. luminescens*	HQ225943
OH25	OR, USA	*H. georgiana*	HQ225885	*P. luminescens*	HM072282
RDS96	IN, USA	*H. bacteriophora*	HQ225891	*P. luminescens*	HQ225947

the associations between *Heterorhabditis* and *Photorhabdus* are randomly distributed on the phylogeny. In the test of individual links, the null hypothesis is that a given *Heterorhabditis*–*Photorhabdus* association is established at random. The congruence between the ML trees of nematodes and bacteria was also visualized using TreeMap 1.0, a tree topology based program (Page, 1994).

3. Results

3.1. Nematode phylogenetic analyses

The nematode ITS sequences for all 67 strains showed high similarity to that of *Heterorhabditis* species in the NCBI GenBank database. Out of the 67 strains, 52 exhibited high similarity (99% or higher) to deposited sequences of *H. bacteriophora*. Nine (GPS29, ACOWS, GPS30, KMD82, KMD1, OH25, SPCM3, KMD37 and KMD64) were highly similar (99% or higher) to that of *H. georgiana*, one (UK76) showed high similarity (99%) to *H. megidis*, four (MID09, MID10, MP17 and MP111) had high similarity (99% or higher) to *H. indica*, and one (MP68) was 98% similar to *H. amazonensis*. The ITS sequences of the nematode strains varied in the length of the aligned region. For strains with similarity to *H. bacteriophora*, *H. georgiana*, *H. megidis*, *H. indica* and *H. amazonesis*, the length of the aligned ITS region excluding the gap was 901, 901, 885, 869 and 890 base pairs, respectively. The topology of the trees based upon the ITS sequences showed high similarity between ML and MP analyses. The loose consensus trees (Supplementary Figs. 1 and 2) indicated that the strains were divided into five species, *H. bacteriophora*, *H. georgiana*, *H. megidis*, *H. indica* and a potentially new *Heterorhabditis* species comprising the MP68 strain collected from Thailand. The MP tree was well resolved for strains UK76, MP68, MP17, MP111, MID09, MID10, KMD1, KMD37, KMD64, KMD82, GPS29, GPS30, ACOWS, SPCM3 and OH25 with high bootstrap support. The MP17, MP111, MID09 and MID10 strains were sister to *H. indica*, forming a monophyletic group with strong (99%) bootstrap support, UK76 was part of the *H. megidis* clade (99% bootstrap support), and MP68 was sister to *H. amazonensis* and a paraphyletic group formed by *H. mexicana*, *H. tayearae* and *H. floridensis*. This suggests that MP68 may represent a new *Heterorhabditis* species. Strains KMD1, KMD37, KMD64, KMD82, GPS29, GPS30, ACOWS, SPCM3 and OH25 formed a *H. georgiana* monophyletic group with high bootstrap support (96%). For the rest of the strains forming *H. bacteriophora* group, bootstrap MP analysis received weak clade support (<70%), which may be due to the overwhelming number of strains in this clade. By reducing the number of strains that were part of *H. bacteriophora* clade to perform the MP and ML analyses, this clade received high bootstrap support (>90%) as shown in one of the representative examples (Fig. 1).

3.2. Bacteria phylogenetic analyses

The symbiotic bacteria of all 67 nematode strains were confirmed to be *Photorhabdus* species based on the analysis of their 16S rDNA sequences (data not shown) which have been typically used to identify bacterial species. Their *gyrB* gene sequences also showed high similarity to that of *Photorhabdus* in the NCBI GenBank. The loose consensus trees determined by 766 base pairs of the *gyrB* gene sequences using both MP and ML analyses showed that all strains were divided into two clades, *P. temperata* and *P. luminescens* (Supplementary Figs. 3 and 4). Within *P. temperata* clade, five monophyletic groups were identified with 97% bootstrap support, which are *P. temperata* subsp. *temperata* including UK76 strain, *P. temperata* subsp. *cinerea*, *P. temperata* subsp. *thracensis*, *P. temperata* subsp. *stackebrandtii* and *P. temperata* subsp. *tasmaniensis*. Within *P. luminescens* clade, the monophyletic group comprising the symbiotic bacteria of KMD81, KMD37, KMD82, ACOWS and OH25 represents a recently recognized *P. luminescens* subspecies, *P. luminescens* subsp. *kleinii*. Also while *gyrB* gene sequences of these five bacterial strains were highly similar (>99%) to each other, they showed only 90–93% similarity to other currently recognized *Photorhabdus* species. The bacterial strains MP17, MP68, MP111, MID09 and MID10 were part of *P. luminescens* subsp. *akhurstii* clade. The strain KMD74 belonged to the clade represented by *P. luminescens* subsp. *kayaii* with 100% MP bootstrap support. The remaining strains belonged to *P. luminescens* subsp. *laumondii* clade within which two monophyletic groups were identified. As most of our bacterial strains belonged to *P. luminescens* subsp. *laumondii* clade, we reduced the number of bacterial strains to perform the MP and ML analyses. The resulting trees are

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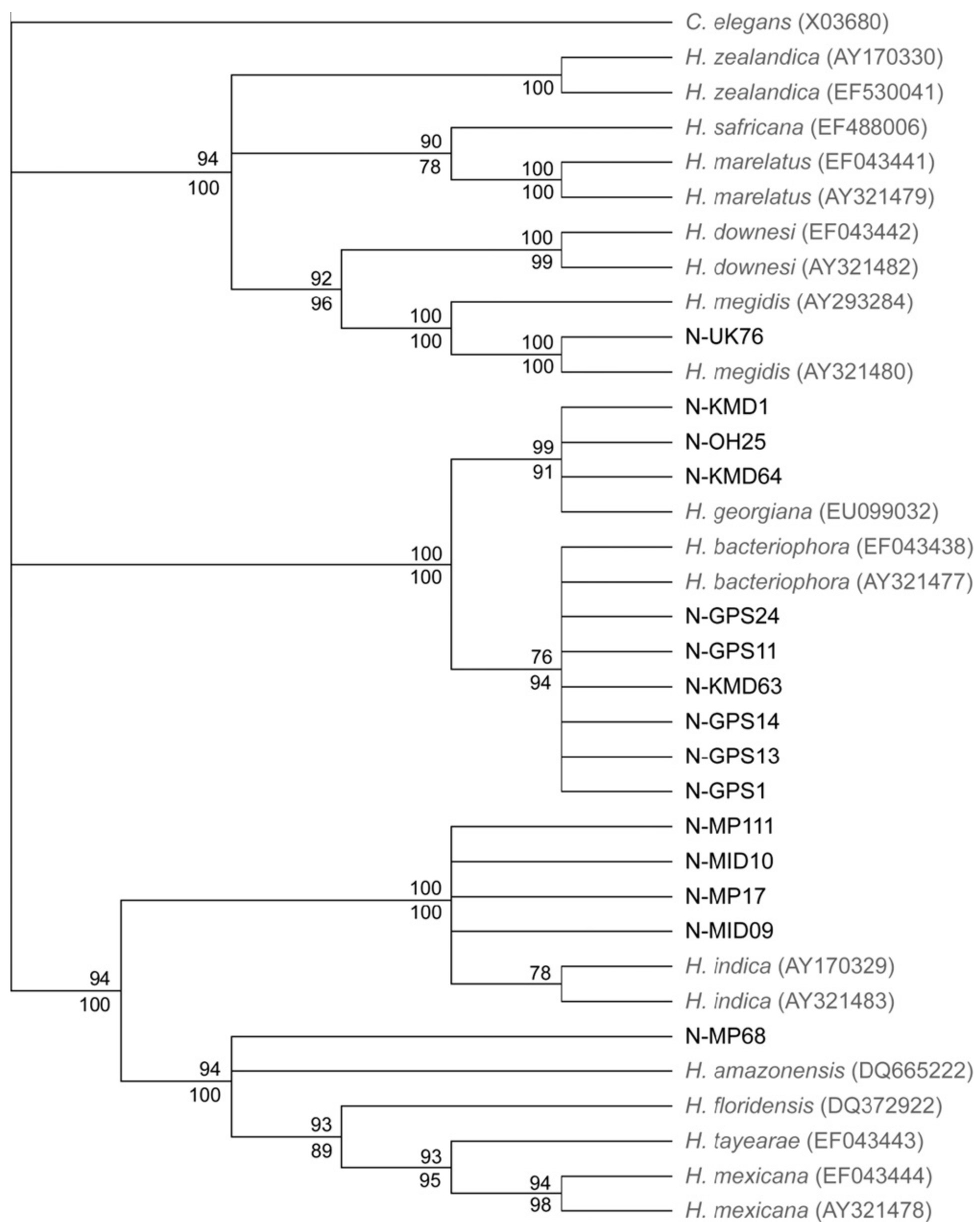

Fig. 1. Maximum likelihood tree inferred from sequences of Internal Transcribed Spacer (ITS) region in *Heterorhabditis* species and 15 of the 67 strains used in this study. Maximum likelihood bootstrap values ⩾70% shown above the internal nodes. Maximum parsimony bootstrap clade frequency values ⩾70% mapped below the internal nodes.

consistent with the ones generated using all the strains. The topology of the ML tree was highly similar to the MP tree, with the exception of differences in *P. luminescens* subsp. *kleinii* and *P. temperata* subsp. *cinera* clades due to differences in bootstrap support for these two clades. In ML tree (Fig. 2), the *P. luminescens* subsp. *akhurstii* monophyletic group was sister to *P. luminescens* subsp. *luminescens* first, forming a sister group to *P. luminescens* subsp. *kayaii*, *laumondii* and *kleinii*. In MP tree, clades representing four of the currently recognized *P. luminescens* subspecies, *akhurstii*, *kayaii*, *laumondii* and *luminescens*, are sister to each other, forming a big sister group to the *P. luminescens* subsp. *kleinii* clade. Such difference between MP and ML analyses is just the reverse for *P. temperata* subsp. *cinerea* clade. Both ML and MP trees support the classification of currently recognized bacterial species and subspecies and the recent effort in proposing *P. luminescens* subsp. *kleinii* as a new *P. luminescens* subspecies.

3.3. Bacteria and nematode cophylogenetic analyses

Global tests of cophylogeny using ParaFit detected a highly significant correlation between the nematode and bacteria trees (ParaFitGlobal < 0.02). Not all *Heterorhabditis*–*Photorhabdus* associations equally contributed to the global fit between the two phylogenies (Supplementary Table 1). The individual associations including those between *H. zealandica, H. indica* and *H. megidis* strains and their symbiotic bacteria significantly ($p < 0.02$) contributed to the overall cophylogenetic structure. Other individual associations including those between the symbiotic bacteria and their respective nematode hosts *H. bacteriophora* and *H. georgiana* strains appear not to contribute significantly to the overall cophylogenetic structure. Further, regardless the number of *Photorhabdus* – *H. bacteriophora* associations included in the ParaFit analyses, the observed significance of cophylogenetic relationship

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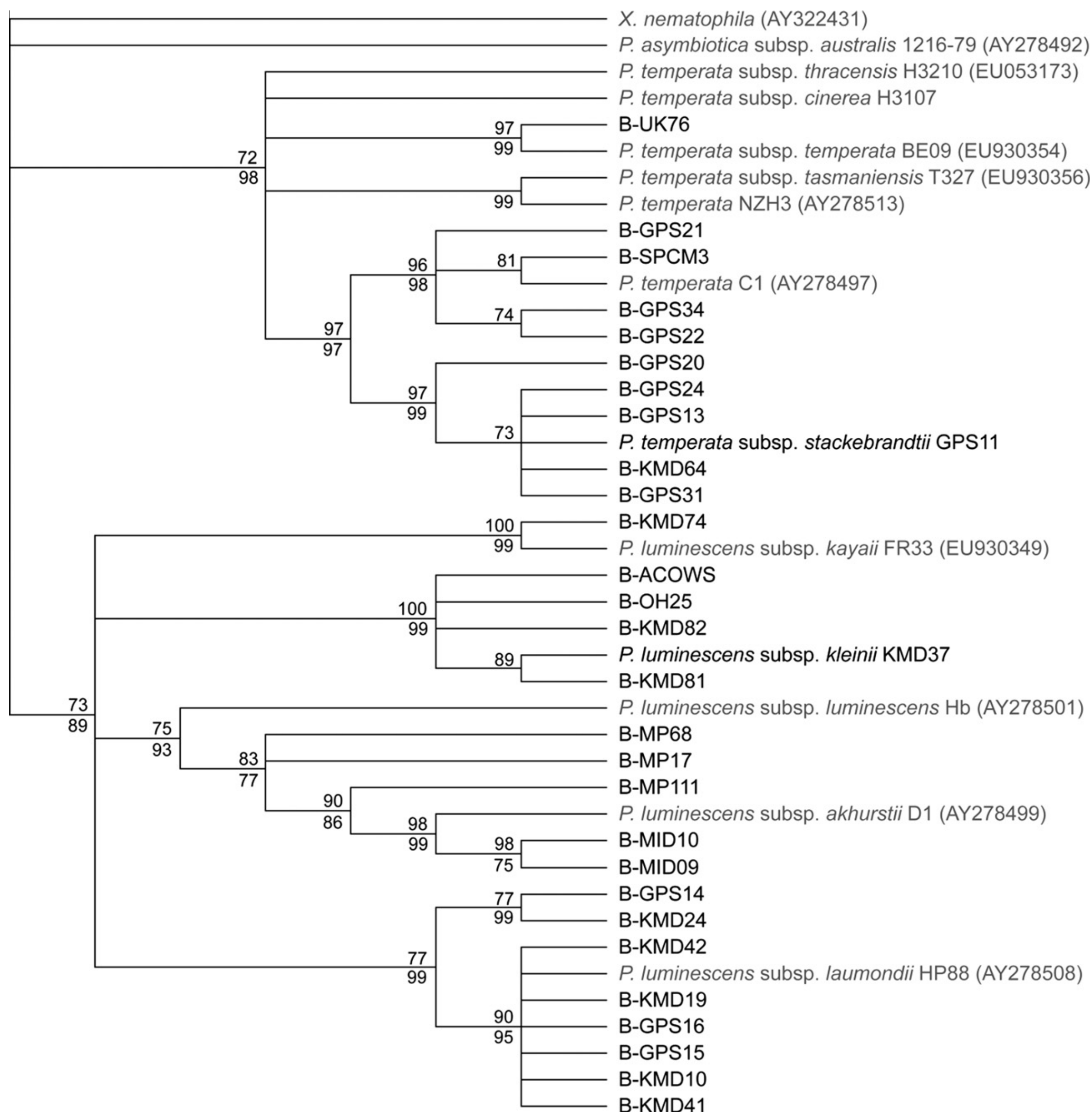

Fig. 2. Maximum likelihood tree inferred from *gyrase* B gene sequences in *Photorhabdus* species and subspecies and 30 of the 67 strains used in this study. Maximum likelihood bootstrap values ⩾70% shown above the internal nodes. Maximum parsimony bootstrap clade frequency values ⩾70% mapped below the internal nodes.

(Supplementary Table 2, Fig. 3c) was consistent and independent of sampling scenarios. The tanglegram built in TreeMap 1 to compare symbiotic partners ML topologies demonstrated congruence between *Photorhabdus* and *Heterorhabditis* phylogenies. Reconstruction analyses with heuristic search for 21 nematode–bacterium associations which are geographically distant at state level (Table 2, Supplementary Fig. 5) detected constant events for cospeciation (9), duplication (11) and host switches (1). Randomization test with one thousand randomly generated bacterial trees using the proportional-to-distinguishable model suggested that the number of observed coevolution events was significantly greater than could be obtained by chance ($p < 0.001$). When reducing the number of associations between *Photorhabdus* bacteria and *H. bacteriophora* so that only one representative from each country was included in the reconstruction analysis, the duplication event dropped dramatically from 11 to 1 (Figs. 3a–b), which is consistent to the observations from ParaFit analysis since associations between *H. bacteriophora* and its symbiotic bacteria do not significantly contribute the cophylogenetic congruence.

4. Discussion

This study thoroughly examines the phylogenies of *Heterorhabdutis* nematodes and their symbionts *Photorhabdus* bacteria and evaluates the extent of cophylogeny between these two evolutionary partners. The global phylogeny presented supports the placement of currently recognized nematode species (Adams et al., 1998; Nguyen and Hunt, 2007; Smits et al., 1991), identifies a potentially new *Heterorhabditis* species represented by the strain MP68, and affirms recent efforts in proposing *P. luminescens* subsp. *kleinii* (An and Grewal, 2011) and classifying *P. temperata* subsp.

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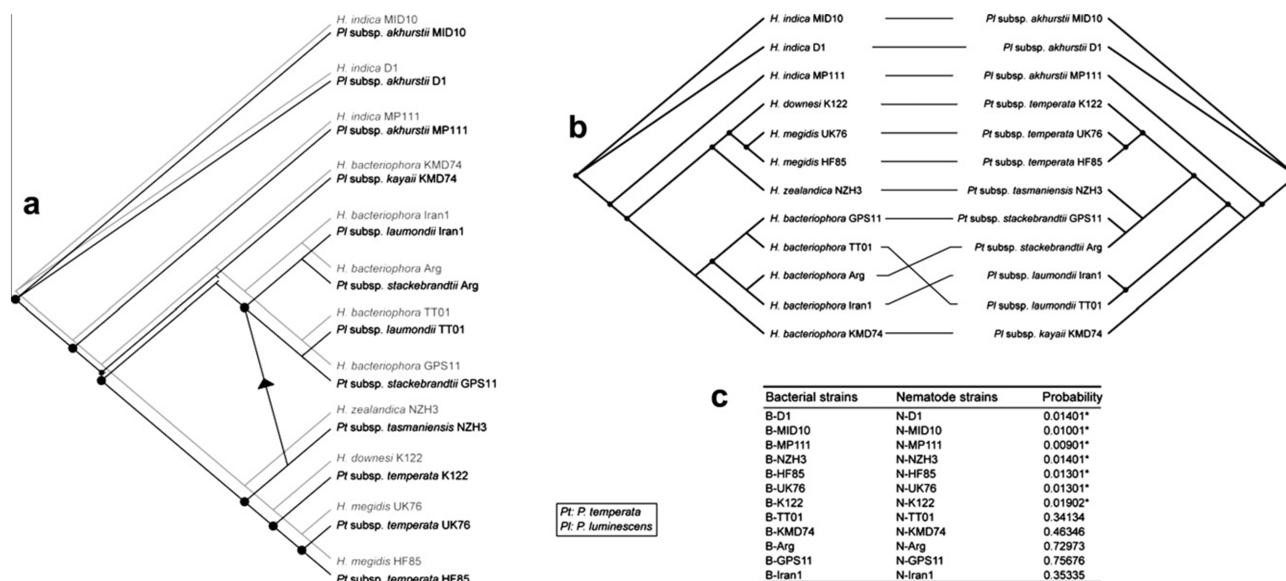

Bacterial strains	Nematode strains	Probability
B-D1	N-D1	0.01401*
B-MID10	N-MID10	0.01001*
B-MP111	N-MP111	0.00901*
B-NZH3	N-NZH3	0.01401*
B-HF85	N-HF85	0.01301*
B-UK76	N-UK76	0.01301*
B-K122	N-K122	0.01902*
B-TT01	N-TT01	0.34134
B-KMD74	N-KMD74	0.46346
B-Arg	N-Arg	0.72973
B-GPS11	N-GPS11	0.75676
B-Iran1	N-Iran1	0.35335

Fig. 3. Cophylogenetic relationships between *Heterorhabditis* nematodes and *Photorhabdus* bacteria estimated using TreeMap and ParaFit programs. The nematode and bacterial phylogenies are inferred from the internal transcribed spacer (ITS) and *gyrase* B gene sequences, respectively. (a) Stacked TreeMap reconciliations of nematode and bacterial maximum likelihood trees, indicating cospeciation (closed circle), duplication (closed square) and host switching (closed arrow) evolutionary events. (b) Tanglegram comparing nematode and bacterial maximum likelihood trees, with indicated cospeciating nodes (closed circle). (c) The ParaFit analysis results showing the significant contribution (asterisk) of nematode-bacterium individual associations to their overall cophylogenetic relationship.

thracensis (An and Grewal, 2011; Peat et al., 2010; Tailliez et al., 2010) which was originally designated as *P. luminescens* subsp. *thracensis* (Hazir et al., 2004). Based on the phylogenetic analyses, geographic distributions of the nematodes seem to be of little link to the phylogenetic distances. For example, the strains Mar (Oregon, USA), Riwaka (New Zealand), Arg (Argentina), BF2 (California, USA), KMD24 (Virginia) and KMD74 (Hungary) within the clade of *H. bacteriophora* are from geographically very distant regions, whereas they are on the same placement in the phylogenetic trees and cannot be separated from each other. This is in agreement with a previous observation suggesting that genetic divergence among populations of *H. bacteriophora* is relatively independent of geographic distance (Jagdale et al., 2006). The nematode strains from Ohio belong to either *H. bacteriophora* or *H. georgiana* species, suggesting that these two species occur in sympatry. Further, speciation between these two *Heterorhabditis* species may be cryptic as they are morphologically highly similar (Nguyen et al., 2008). In contrast to the nematode species, the bacterial symbionts tend to be phylogenetically more distant from each other over the geographic range. For example, *P. luminescens* subsp. *kleinii* and *P. temperata* subsp. *stackebrandtii* are composed of strains isolated from American nematodes and the strains of *P. temperata* subsp. *temperata* are all from the European nematodes. Together, our results suggest that both the sympatric and allopatric speciation for the bacterial partners of *H. bacteriophora* species have occurred across the geographic range, resulting in host duplication.

Photorhabdus luminescens is currently divided into six subspecies, four of which, *P. luminescens* subsp. luminescens, *P. luminescens* subsp. caribbeanensis *P. luminescens* subsp. *laumondii* and *P. luminescens* subsp. kayaii were isolated from *H. bacteriophora,* one *P. luminescens akhurstii* from *H. indica,* and one *P. luminescens* subsp. *kleinii* from both *H. bacteriophora* and *H. georgiana* (Table 3). Association of *P. temperata* appears to be even more diverse and complex. This species has been found associated with *H. bacteriophora, H. marelatus, H. megidis, H. zealandica,* and *H. downesi.* Our results further showed that both *P. luminescens* and *P. temperata* can also associate with *H. georgiana.* While such outcomes may challenge the well established concept of one-to-one species association between entomopathogenic nematodes and their symbiotic bacteria, cophylogenetic tests using ParaFit rejected the null hypothesis that Photorhabdus bacteria and Heterorhabditis nematodes have evolved independently. While these results indicate that Heterorhabditis and Photorhabdus phylogenies are consistent with a global cophylogenetic pattern, there are also cases of apparent mismatch between the two trees, especially the associations between the symbiotic bacteria and their respective nematode hosts *H. bacteriophora* and *H. georgiana* strains.

One of the possible explanations for the mismatch of associations between *H. bacteriophora* strains and the symbiotic bacteria may be the wide distribution of this nematode species (Boemare and Akhurst, 2006), which may present more duplication opportunities to *Phtorhabdus* bacteria. By reducing the number of strains of *H. bacteriophora* in cophylogenetic analysis, we found that the duplication events dramatically dropped from eleven to one, indicating that duplication has occurred when *Photorhabdus* bacteria speciate in absence of the speciation of this nematode species. In this study, we observed that while nematode strains KMD1, SPCM3, KMD64, GPS29, GPS30, KMD37, ACOWS, KMD82 and OH25 are at the same phylogenetic placement, their bacterial partners diverge into two *Photorhabdus* species. This suggests that the *Heterorhabditis* nematodes in America have the ability to carry diverging species of *Photorhabdus*. Alternatively, the converse of duplication, which occurs when the bacteria fail to speciate in response to the nematode speciation, may also exist. For example, although the bacterial partners of KMD81 and KMD82 are at the same phylogenetic placement, their nematode hosts diverge into *H. georgiana* and *H. bacteriophora*, two possible cryptic species occurring in sympatry. The occurrence of such situations is possibly due to the closer phylogenetic relationship between *H. georgiana* and *H. bacteriophora* than with other species (Supplementary Fig. 1) as it has been previously suggested that association with closely related host species by the same symbiont may be parsimonious in the cophylogenetic history (Hugot et al., 2001). Similarly, this indicates that *Photorhabdus* bacteria in North America retain

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Table 3
The presently recognized symbiotic associations between *Heterorhabditis* nematodes and *Photorhabdus* bacteria.

Photorhabdus sp. and subsp.		*Heterorhabditis* sp.						
		bacteriophora	*downesi*	*georgiana*	*indica*	*marelatus*	*megidis*	*zealandica*
P. luminescens subsp.	akhurstii	×			×			
	caribbeanensis	×						
	kayaii	×						
	kleinii	×		×				
	laumondii	×						
	luminescens	×						
P. temperata subsp.	cinerea		×				×	
	khaniii	×					×	
	stackebrandtii	×		×			×	
	tasmaniensis					×		×
	temperata		×					

the potential to disperse between the diverging species of *Heterorhabditis*. Yet another factor reducing the phylogenetic congruence may be host switching. Previous studies indicate that although *H. bacteriophora* nematodes grown on the symbiotic bacteria of *H. megidis* typically produce germfree infective juveniles, occasionally some *H. bacteriophora* infective juveniles can retain the bacterium (Gerritsen et al., 1998), implying the possibility of host switching in *Heterorhabditis*–*Photorhabdus* association. However, this host switching is incomplete since the bacteria can colonize additional species of the nematode and thus expand their host range (Johnson and Clayton, 2004). As a result, incomplete host switching reduces the level of host specificity and increases the phylogenetic incongruence. Occurrence of host switching is in part attributed to the widespread distribution of phylogenetically conservative traits which is also called ecological fitting (Brooks et al., 2006). In our case, ecological fitting may play a role driving the bacteria to shift the host between *H. georgiana* and *H. bacteriophora* since these two morphologically similar nematode species are also phylogenetically very close.

The pattern of cophylogeny between *Heterorhabditis* and *Photorhabdus* appears to be quite different from that of another well-known evolutionary partnership between entomopathogenic nematodes *Steinernema* and their symbiotic bacteria *Xenorhabdus*. In comparison of phylogenies between *Steinernema* and *Xenorhabdus*, Lee and Stock (2010) found no evidence for cospeciation, even though mutualistic relationship between is specialized (Goodrich-Blair and Clarke, 2007). We assume that this discrepancy occurs, in part, because *Heterorhabditis* nematodes have an obligate requirement for their cognate *Photorhabdus* symbiont for reproduction while *Steinernema* nematodes exhibit optimal reproduction on *Xenorhabdus* species (Goodrich-Blair and Clarke, 2007; Grewal et al., 1997).

Finally, we should point out that some factors may have limited the power of our analyses on cophylogeny. First, we did not cover all the currently recognized *Heteorhabditis* species and *Photorhabdus* subspecies in the cophylogenetic analysis since the partners of most newly recognized nematode species or bacterial subspecies have not been described, such as the undefined nematode host for recently proposed *P. luminescens* subsp. *caribbeanensis* and *P. temperata* subsp. *tasmaniensis* (Tailliez et al., 2010). We have no idea whether these additional associations may show more evidence of cospeciation than those we have observed in this study. Second, although ITS and *gyrB* loci are currently recognized as most useful sequences in phylogenetic analysis of *Heterorhabditis* and *Photorhabdus* populations (Akhurst et al., 2004; Malan et al., 2008), respectively they are heterogeneous molecular markers, which means they are probably evolving at different rates of molecular evolution. Given this fact, this study is limited in estimates of the relative timing of speciation events to make a compelling case for a history of cospeciation. Therefore, future research exploring new powerful homogeneous molecular markers is needed.

5. Conclusions

In summary, our analyses of entomopathogenic nematode strains and their symbiotic bacteria provide insights into the diversity and cophylogeny of the nematode–bacterium partnership. The cophylogenetic analysis revealed a significant coevolutionary relationship between *Photorhabdus*–*Heterorhabditis* association despite few mismatches. Considering that the symbiotic bacteria of most currently recognized species and strains of *Heterorhabditis* have not been described, we recommend that future research on the descriptions of entomopathogenic nematode species should also include descriptions of the bacterial partners from the same strain and vice versa to facilitate robust coevolutionary studies and to discover novel nematode and bacteria associations for use in biological control.

The present phylogenetic and cophylogenetic study also provides a framework for further investigations of nematode–bacterium symbiotic associations and prompt some intriguing questions. For example, whether mismatches between *Heterorhabditis* and *Photorhabdus* phylogenies can be resolved by cophylogenetic analysis using less conservative genes which are discovered to be involved in symbiotic interactions. In addition, with *P. asymbiotica* recognized as an insect and human pathogen, it will be interesting to determine whether "missing the boat" and "ecological fitting" has happened for this *Photorhabdus* species. Subsequently, what will be the shared traits among nematode, insect and human tracked by the bacteria in terms of ecological fitting. It is hoped that comparative analysis of available genomic sequences will ultimately enable us to gain a better understanding regarding these symbiotic associations.

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Appendix A

The ITS and *gyrB* sequences determined in this study have been deposited at GenBank under the accession numbers HQ225842–

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HQ225969. In addition to the strains from this study, the other nematodes and bacteria included in the phylogenetic analyses were *H. amazonensis* (DQ665222), *H. bacteriophora* (AY321477; EF043438), *H. downesi* (AY321482; EF043442), *H. floridensis* (DQ372922), *H. georgiana* (EU099032), *H. indica* (AY321483; AY170329), *H. marelatus* (AY321479; EF043441), *H. megidis* (AY293284; AY321480), *H. mexicana* (AY321478; EF043444), *H. safricana* (EF488006), *H. tayearae* (EF043443), *H. zealandica* (EF530041; AY170330) and *Caenorhabditis elegans* (X03680); *P. temperata* strains C1 (AY278497), Habana (AY278503), Meg1 (AY278512), NZH3 (AY278513), T327 (EU930356), *P. temperata* subsp. *cinerea* strain H3107 (EU053168), *P. temperata* subsp. *temperata* strain BE09 (EU930354), *P. asymbiotica* subsp. *australis* strain 1216-79 (AY278492), *P. luminescens* subsp. *thracensis* strain H3210 (EU053173), *P. luminescens* subsp. *kayaii* strain FR33 (EU930349), *P. luminescens* subsp. *akhurstii* strain D1 (AY278499), *P. luminescens* subsp. *luminescens* strain Hb (AY278501), *P. luminescens* subsp. *laumondii* strain HP88 (AY278508) and *X. nematophila* (AY322431).

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.02.012.

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