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Molecular identification, phylogeny and phylogeography of the entomopathogenic nematodes of the genus *Heterorhabditis* Poinar, 1976: a multigene approach

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Summary – Presently, the genus *Heterorhabditis* contains 16 valid entomopathogenic nematode species. In this study we used samples from 11 species: *H. amazonensis*, *H. bacteriophora*, *H. baujardi*, *H. beicherriana*, *H. downesi*, *H. floridensis*, *H. georgiana*, *H. indica*, *H. megidis*, *H. noenieputensis*, and *H. zealandica* to amplify and sequence five gene fragments: the D2-D3 expansion segments of 28S rRNA, ITS rRNA, *COI* mtDNA genes and *unc*-87 and *cmd-1* genes encoding thin filament (F-actin)-associated protein and calmodulin, respectively. Fifty new sequences for 11 species were generated. More than 980 sequences of five genes were analysed. Phylogenetic and sequence analysis of these genes using Bayesian inference, maximum likelihood and statistical parsimony confirmed a division of the genus into three clades (groups): '*Indica*', '*Bacteriophora*' and '*Megidis*'. The analysis of gene sequences downloaded from GenBank and identified as *Heterorhabditis* revealed many cases of species misidentifications and presence of reading mistakes in some sequences. Synonymisation of *H. somsookae* with *H. baujardi*, *H. gerrardi*, *H. pakistanensis* with *H. indica*, and *H. sonorensis* with *H. taysearae*, are confirmed by sequence and phylogenetic analysis. The ITS rRNA and *COI* genes could be considered as informative markers for species identification, barcoding and phylogeographical studies of *Heterorhabditis*.

Keywords – *Bacteriophora* clade, *cmd-1*, *COI*, 28S rRNA, *Indica* clade, ITS rRNA, *Megidis* clade, species delimiting, statistical parsimony, synonymy, *unc*-87.

Species of the monotypic family Heterorhabditidae are frequently used for biological control of insects. Currently, the genus *Heterorhabditis* Poinar, 1976 contains 16 valid species. Evolutionarily, *Heterorhabditis* is a transitional taxon among the Rhabditina. It exhibits ancestral traits shared with its microbivorous ancestors such as *Caenorhabditis elegans*, but has also evolved parasitism and shares the most recent common ancestry with obligate mammalian parasites, such as hookworms and lungworms (Bai *et al*., 2013).

The molecular phylogeny of *Heterorhabditis* has been reconstructed based on the analyses of partial sequences from the 28S rRNA gene (Curran & Driver, 1994; Nguyen *et al*., 2004, 2008; Edgington *et al*., 2011; Li *et al*., 2012; Malan *et al*., 2014), partial 18S rRNA gene (Liu *et al*., 1997), ITS1 of rRNA gene (Adams *et al*., 1998; Phan *et al*., 2003), ITS1-5.8S-ITS2 of rRNA gene (Malan *et al*., 2008, 2014; Nguyen *et al*., 2008; Edgington *et al*., 2011; Li *et al*., 2012; Morales *et al*., 2016), partial *nd*4 gene (Liu *et al*., 1999), partial *COI* mtDNA gene (Kuwata

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et al., 2007) and many protein-coding genes including *cmd-1* and *unc-87* (Regeai *et al*., 2009). Adams *et al*. (1998) conducted a most comprehensive analysis of the genus phylogeny. The phylogenetic estimations based on the *nad*4 gene (Liu *et al*., 1999; Saeb & Grewal, 2014), however, differed in positions of some species and it cannot be excluded that these differences occurred due to some misidentification of nematode samples. Nguyen *et al*. (2008) were the first to propose dividing the genus into three clades (groups): '*Indica*', '*Bacteriophora*' and '*Megidis*', which are presently recognised by the majority of authors. Spiridonov & Subbotin (2016) provided a brief characterisation of these groups with notes on their phylogeography. The biogeographical distribution of *Heterorhabditis* has been also analysed by Hominick *et al*. (1996), Hominick (2002) and Bhat *et al*. (2020).

The objectives of this study were: *i*) to generate new sequences for several species of *Heterorhabditis*; *ii*) to verify the correctness of some sequences from GenBank deposited by a number of authors under various *Heterorhabditis* specific epithets; *iii*) to analyse intra- and inter-specific variations for *Heterorhabditis* using several ribosomal, nuclear and mitochondrial protein coding genes; *iv*) to apply sequence information for species delimiting of *Heterorhabditis* species; and *v*) to reconstruct phylogenetic relationships within species and populations of *Heterorhabditis*.

Materials and methods

Nematode samples

Samples of different species of entomopathogenic nematode were obtained from colleagues from different countries (Table 1). Nematodes were extracted from soil by the *Galleria* baiting method (Bedding & Akhurst, 1975) and then stored to use for molecular study.

DNA extraction, PCR and sequencing

Several nematode specimens were used from each sample. Nematodes were cut with a dental needle in a drop of water and transferred into a 0.2 ml Eppendorf tube. Three μl proteinase K (600 μg ml^{-1}) (Promega) and 2 μl 10$\times$ PCR buffer (*Taq* PCR Core Kit, Qiagen) were added to this tube to a final volume of 25 μl. The tubes were incubated at 65°C for 1 h and then at 95°C for 15 min. After incubation, the tubes were centrifuged and kept at −20°C until use.

Five gene fragments were amplified and sequenced for this study. Primers used in the present study are given in Table 2. For amplification of ribosomal genes, 2 μl of extracted DNA was transferred to 0.2 ml Eppendorf tube containing 2.5 μl 10$\times$ PCR buffer, 5 μl Q solution, 0.5 μl dNTPs mixture (*Taq* PCR Core Kit, Qiagen), 0.15 μl of each primer (1.0 μg μl^{-1}) (synthesised by Integrated DNA Technologies), 0.1 μl *Taq* Polymerase and 12.6 μl distilled water. For amplification of *COI* and protein coding genes, the above described protocol or the protocol with DreamTaq Green PCR Master Mix: 3 μl of extracted DNA was transferred to a 0.2 ml Eppendorf tube containing: 10 μl DreamTaq Green PCR Master Mix (2$\times$) (Thermo Fisher Scientific), 10 μl water and 0.15 μl of each primer (1.0 μg μl^{-1}) were used. PCR was run under one of the following profiles: *i*) for ribosomal genes – 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min 30 s at 55°C, and 2 min at 72°C, followed by a final step of 10 min at 72°C; *ii*) for *COI* and protein-coding genes – 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min 30 s at 72°C, with a final extension at 72°C for 10 min.

PCR products were purified using QIAquick PCR Purification Kit or Gel Extraction Kit (Qiagen) and directly sequenced or cloned. PCR products obtained from some protein-coding genes were cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega). Several clones of each species were isolated using blue/white selection and submitted to PCR and sequencing. Sequencing was made in Quintara Biosciences. New sequences were deposited in GenBank under the accession numbers as indicated in Table 1, phylogenetic trees and networks.

Phylogenetic and sequence analysis

New sequences were aligned with corresponding published gene sequences (Liu *et al*., 1997, 1999; Adams *et al*., 1998; Phan *et al*., 2003; Nguyen *et al*., 2004, 2006; Andaló *et al*., 2007, 2009; López-Núñez *et al*., 2007; Malan *et al*., 2008, 2014; Regeai *et al*., 2009; Stock *et al*., 2009; Edgington *et al*., 2011; Li *et al*., 2012; Zadji *et al*., 2013; Maneesakorn *et al*., 2015; Morales *et al*., 2016; Bruno *et al*., 2020 and others) using ClustalX 1.83 (Chenna *et al*., 2003) with default parameters. Several alignments were created. Sequence alignments were also manually edited using GeneDoc 2.5.0 (Nicholas & Nicholas, 1997). Pairwise divergence between taxa was calculated as the absolute distance value and the percent

Table 1. Species of *Heterorhabditis* used in the present study.

Species	Sample code	Origin	GenBank accession number					Source
			D2-D3 28S rRNA	ITS rRNA	*COI*	*unc-87*	*cmd-1*	
H. amazonensis Andaló, Nguyen & Moino, 2007	CD2510, MC01	Brazil	MT372502	MT372499	MT373738	MT375792	MT406142	V. Andaló
H. bacteriophora Poinar, 1976	CD2504, Hp88	–	MT372509	MT372491	MT373729	MT375796	MT406138	S. Byrd
H. baujardi Phan, Subbotin, Nguyen & Moens, 2003	CD2519, F10	Cameroon	MT372503	MT372500	MT373736	MT375791	MT406139	L. Waeyenberge
H. baujardi	CD2520	Cameroon	–	–	MT373735	–	MT406140	L. Waeyenberge
H. beicherriana Li, Liu, Nermut', Půža & Mráček, 2012	CD2516	China	MT372511	MT372490	MT373730	MT375797	–	Z. Mráček
H. downesi Stock, Griffin & Burnell, 2002	CD2508, 23.9	–	MT372507	MT372494	MT373732	MT375794	MT406135	R.-U. Ehlers
H. floridensis Nguyen, Gozel, Koppenhöfer & Adams, 2006	CD2503, K22	USA	MT372504	MT372501	MT373737	MT375793	MT406141	K.B. Nguyen
H. georgiana Nguyen, Shapiro-Ilan & Mbata, 2008	CD2500	–	MT372510	MT372492	MT373731	MT375798, MT375799	–	S. Byrd
H. indica Poinar, Karunakar & David, 1992	CD2525	–	–	MT372498	–	–	–	R.-U. Ehlers
H. megidis Poinar, Jackson & Klein, 1987	CD2517, Chuvashia	Russia	–	MT372496	–	–	–	S.E. Spiridonov
H. megidis	CD2518, DV	Russia	MT372506	MT372495	MT373733	MT375795	MT406136	S.E. Spiridonov
H. noenieputensis Malan, Knoetze & Tiedt, 2014	CD2506, SF669	South Africa	MT372505	MT372497	MT373728	–	–	A. Malan
H. zealandica Poinar, 1990	CD2507, 23.9	–	MT372508	MT372493	MT373734	–	MT406137	R.-U. Ehlers

Table 2. Primer sets used in the present study.

Amplified gene	Primer code	Sequence (5′ → 3′)	Reference
ITS rRNA	TW81 (f)	GTT TCC GTA GGT GAA CCT GC	Joyce *et al*. (1994)
	AB28 (r)	ATA TGC TTA AGT TCA GCG GGT	Joyce *et al*. (1994)
D2-D3 of 28S rRNA	D2A (f)	ACA AGT ACC GTG AGG GAA AGT TG	Subbotin *et al*. (2006)
	D3B (r)	TCG GAA GGA ACC AGC TAC TA	Subbotin *et al.* (2006)
COI	HCF (f)	TTA CAT GAT ACT TAT TAT G	Kuwata *et al.* (2007)
	HCR (r)	CTG ATA ACT GTG ACC AAA TAC ATA	Kuwata *et al.* (2007)
cmd-1	cmd1F (f)	GGA TAC TGA CAG TGA AGA	Regeai *et al.* (2009)
	cmd1R (r)	CTC TCC CAG ATT CGT CAT TAC	Regeai *et al.* (2009)
unc-87	unc87F (f)	GGA ACT CCC AGG AAC ACC AGC	Regeai *et al.* (2009)
	unc87R (r)	CGT TCC TGA CTG AAG GCG GAC	Regeai *et al.* (2009)

(f), forward primer; (r), reverse primer.

of mean distance, with adjustment for missing data, using PAUP* 4b10 (Swofford, 2003).

The alignments were analysed with maximum likelihood (ML), maximum parsimony (MP) using PAUP* and Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The best fit models of DNA evolution were obtained using the program jModeltest.0.1.1 (Posada, 2008) with the Akaike Information Criterion. Bootstrap support (BS) values for ML and MP trees were calculated by a heuristic search from 100 and 1000 replicates, respectively. The BI analysis for each gene was initiated with a random of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) were given on appropriate clades. The partition homogeneity test for five gene fragments was performed with PAUP*.

The alignments were used to construct phylogenetic network estimations using statistical parsimony (SP) as implemented in POPART software (http://popart.otago.ac.nz) (Bandelt *et al*., 1999). Trees and networks were visualised with the TreeView 1.6.6 program and drawn with Adobe Illustrator v.10.

Results

Phylogenetic and sequence analysis with rRNA and *COI* mtDNA genes

Two approaches were applied to analyse the ITS rRNA, 28S rRNA and *COI* mtDNA gene sequences of *Heterorhabditis* species. The first approach included BI, ML and MP analyses of gene alignments containing only reference sequences of each *Heterorhabditis* species and the second included SP analyses of several alignments of gene sequences for three groups: *Indica*, *Bacteriophora* and *Megidis*.

Phylogenetic and sequence analysis with the ITS rRNA gene

The *Heterorhabditis* phylogeny: The ITS rRNA alignment with reference sequences was 853 bp in length. Maximal sequence diversity within *Heterorhabditis* was 26%. Phylogenetic relationships within 16 valid species as inferred from BI, ML and MP analyses of the ITS rRNA gene sequences are given in Figure 1A. Three major clades corresponding to the three highly supported clades (groups): *Megidis*, *Bacteriophora* and *Indica*, can be seen in the phylogenetic tree.

A total of 911 sequences under the name of *Heterorhabditis* were downloaded from GenBank. However, only 779 sequences were retained in the analysis because they met the quality and length requirements as they were estimated as either not containing, or containing a minimal number of, reading mistakes. Nucleotides suspiciously differing from homologous ones in the conservative 5′ and 3′ ends of sequence were considered as reading mistakes.

The *Indica*-clade: The alignment was 802 bp in length and included 398 sequences. Five new sequences of this group were included in this study. A phylogenetic network for the ITS rRNA sequences, reconstructed using SP with POPART software, is given in Figure 2. This method allowed the discrimination of seven valid species: *H. amazonensis* Andaló, Nguyen & Moino, 2007, *H. baujardi* Phan, Subbotin, Nguyen & Moens, 2003, *H. floridensis* Nguyen, Gozel, Koppenhöfer & Adams, 2006, *H. indica* Poinar, Karunakar & David, 1992, *H. mexicana*

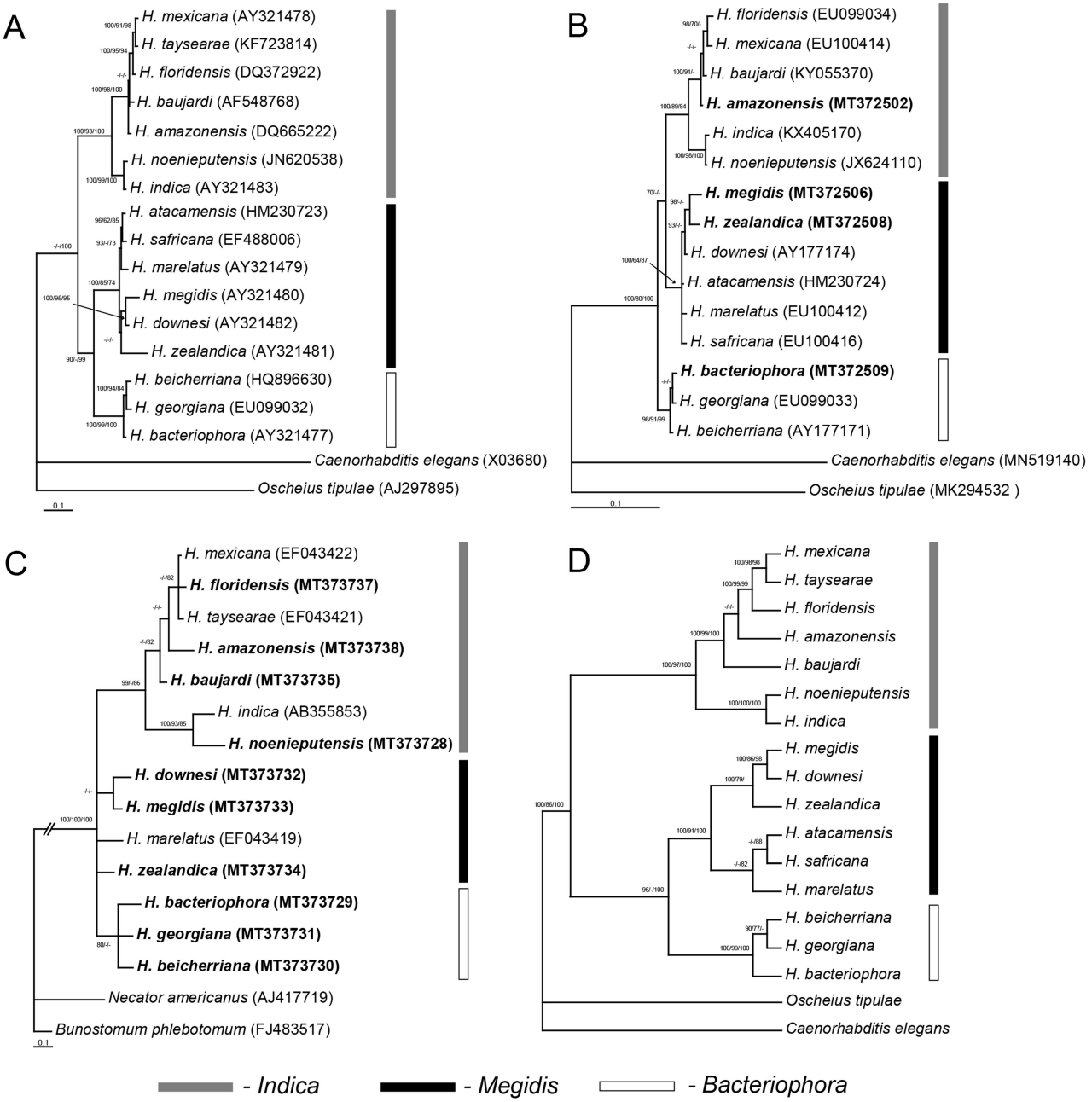

Fig. 1. Phylogenetic relationships within *Heterorhabditis* species as inferred from Bayesian analyses of reference sequences of the ITS rRNA gene (A), the D2-D3 of 28S rRNA gene (B), *COI* mtDNA gene (C) and combined genes (ITS rRNA, D2-D3 of 28S rRNA, *COI*, *cmd*-1 and *unc-87*) (D) using the GTR + I + G model of DNA evolution. Posterior probability and bootstrap support values more than 70% for BI, ML and MP analysis are given for appropriate clades, respectively.

Nguyen, Shapiro-Ilan, Stuart, McCoy, James & Adams, 2004, *H. noenieputensis* Malan, Knoetze & Tiedt, 2014 and *H. taysearae* Shamseldean, Abou El-Sooud, Abd-Elgawad & Saleh, 1996 from one another.

Heterorhabditis indica was represented by three main haplotypes: Hi1 (125 sequences); Hi2 (29 sequences); and Hi3 (11 sequences) and 48 other sequences. Sequences of some samples identified as *H. pakistanensis*

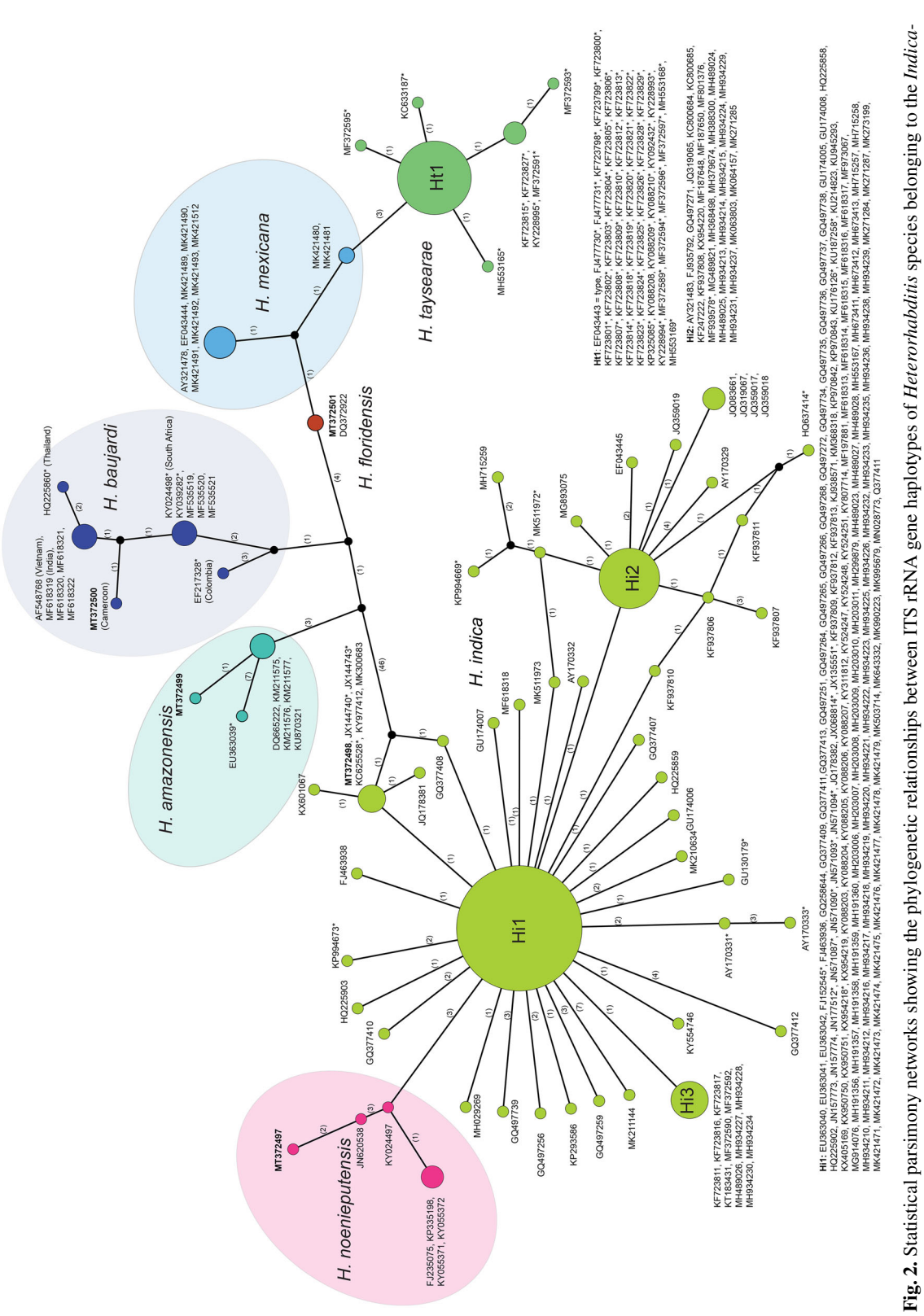

Fig. 2. Statistical parsimony networks showing the phylogenetic relationships between ITS rRNA gene haplotypes of *Heterorhabditis* species belonging to the *Indica*-clade. The sequences of each species are marked by different colours. Pies (circles) represent sequences of each species with the same haplotype and their size is proportional to the number of these sequences in the samples. Numbers of nucleotide differences between the sequences are indicated on lines connecting the pies. Small black circles represent missing haplotypes. *Misidentified sequence. New sequences are indicated by bold font.

Shahina, Tabassum, Salma, Mehreen & Knoetze, 2016 (KX954218), *H. brevicaudis* Liu, 1994 (DQ020278), *H. gerrardi* Plichta, Joyce, Clarke, Waterfield & Stock, 2009 (FJ152545) and *H. hawaiiensis* Gardner, Stock & Kaya, 1994 (AF029707) belong to *H. indica. Heterorhabditis noenieputensis* included seven sequences and differed from *H. indica* by at least four nucleotide changes. *Heterorhabditis taysearae* included 50 sequences. Some sequences of *H. sonorensis* Stock, Rivera-Orduño & Flores-Lara, 2009 were identical to *H. taysearae. Heterorhabditis mexicana* consisted of ten sequences and differed from *H. taysearae* by at least three nucleotide changes. *Heterorhabditis baujardi* included 13 sequences. The sequence of type *H. somsookae* Maneesakorn, An, Grewal & Chandrapatya, 2015 (HQ225860) was identical to that of *H. baujardi*. The sequence of the sample (EF217328) from the central Andean region of Colombia was identified as belonging to *H. baujardi. Heterorhabditis amazonensis* included seven sequences. The sequence of the sample (EU363039) previously identified as *H. baujardi* is considered here as a representative of *H. amazonensis. Heterorhabditis floridensis* contained two sequences and differed from *H. mexicana* by two nucleotide changes. Maximal sequence variation for the *Indica*-clade was 17%.

The *Bacteriophora*-clade: The alignment was 722 bp in length and included 318 sequences. Three new sequences of this group were included in this study. A SP phylogenetic network for the ITS rRNA sequences is given in Figure 3. This method allowed the discrimination of three valid species: *H. bacteriophora* Poinar, 1976, *H. beicherriana* Li, Liu, Nermut', Půža & Mráček, 2012 and *H. georgiana* Nguyen, Shapiro-Ilan & Mbata, 2008 from each other. *Heterorhabditis bacteriophora* contained a total of 284 sequences, 158 of them belonging to the haplotype Hb1. The sequence of *H. argentinensis* Stock, 1993 (AF029706) had one nucleotide change from the haplotype Hb1 and was within the range of intraspecific variation of *H. bacteriophora. Heterorhabditis georgiana*, with 32 sequences, differed by at least 11 nucleotide changes from *H. bacteriophora. Heterorhabditis beicherriana* contained two sequences and differed by at least 14 changes from *H. bacteriophora*. Maximal sequence variation was 5.0%

The *Megidis*-clade: The alignment was 729 bp in length and included 86 sequences. Four new sequences of this group were included in this study. A SP phylogenetic network for the ITS rRNA sequences is given in Figure 4. This method discriminates six valid species: *H. atacamensis* Edgington, Buddie, Moore, France, Merino & Hunt, 2011, *H. downesi* Stock, Griffin & Burnell, 2002, *H. marelatus* Liu & Berry, 1996, *H. megidis* Poinar, Jackson & Klein, 1987, *H. safricana* Malan, Nguyen, De Waal & Tiedt, 2014, and *H. zealandica* Poinar, 1990 from each other. Sequences of *H. atacamensis* differed from those of *H. safricana* by four nucleotide changes, whereas other species differed by ten or more changes. Maximal sequence variation for the *Megidis*-clade was 15.5%.

Phylogenetic and sequence analysis with the D2-D3 of 28S rRNA gene

The *Heterorhabditis* phylogeny: The 28S rRNA gene alignment with reference sequences was 873 bp in length. Maximal sequence diversity within *Heterorhabditi*s was 8.5%. Phylogenetic relationships within 15 valid species as inferred from BI, ML and MP analyses of the D2-D3 of 28S rRNA gene sequences are given in Figure 1B. A total of 105 sequences downloaded from GenBank and ten new sequences were used in the analysis.

The *Indica*-clade: The alignment was 597 bp in length and included 65 sequences. Five new sequences of this group were included in this study. A phylogenetic network for the D2-D3 of 28S rRNA sequences, reconstructed using SP with POPART software, is given in Figure 5A. Maximal sequence variation was 7.3% (30 bp).

The *Bacteriophora*-clade: The alignment was 568 bp in length and included 52 sequences. Two new sequences of this group were included in this study. A SP phylogenetic network for the D2-D3 of 28S rRNA gene sequences is given in Figure 5B. Maximal sequence variation was 1.3% (6 bp).

The *Megidis*-clade: The alignment was 570 bp in length and included 26 sequences. Three new sequences of this group were included in this study. A SP phylogenetic network for the D2-D3 of 28S rRNA sequences is given in Figure 5C. Maximal sequence variation was 2.9% (14 bp).

Phylogenetic and sequence analysis with the COI *gene*

The *Heterorhabditis* phylogeny: The *COI* gene alignment with reference sequences was 377 bp in length. Maximal sequence diversity within *Heterorhabditi*s was 14.8%. Phylogenetic relationships within 14 valid species as inferred from BI, ML and MP analyses of the *COI* gene sequences are given in Figure 1C. A phylogenetic network for the *COI* sequences, reconstructed using SP with POPART software, is given in Figure 6 for three clades.

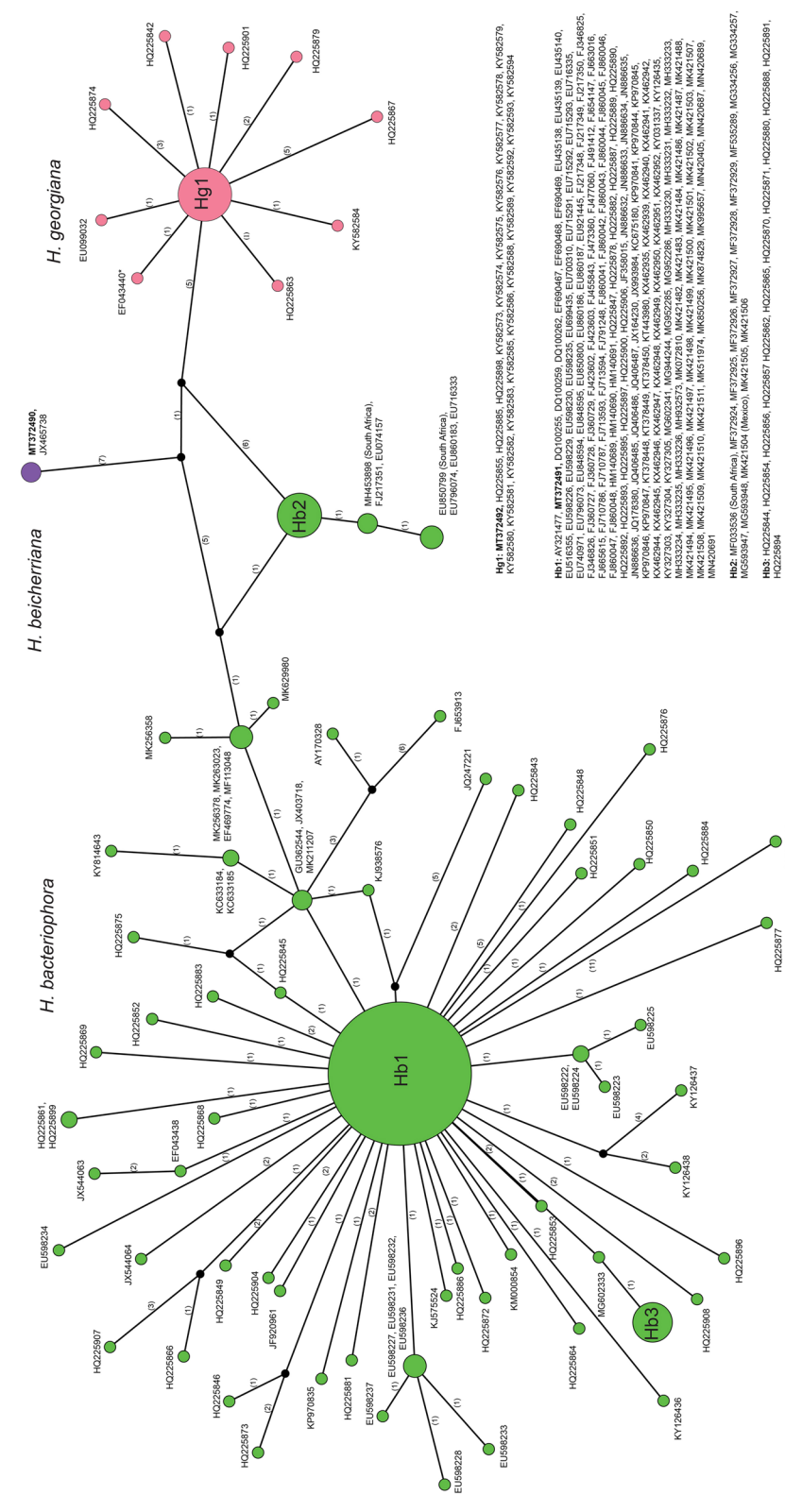

Fig. 3. Statistical parsimony networks showing the phylogenetic relationships between ITS rRNA gene haplotypes of *Heterorhabditis* species belonging to the *Bacteriophora*-clade. The sequences of each species are marked by different colours. Pies (circles) represent sequences of each species with the same haplotype and their size is proportional to the number of these sequences in the samples. Numbers of nucleotide differences between the sequences are indicated on lines connecting the pies. Small black circles represent missing haplotypes. *Misidentified sequence. New sequences are indicated by bold font.

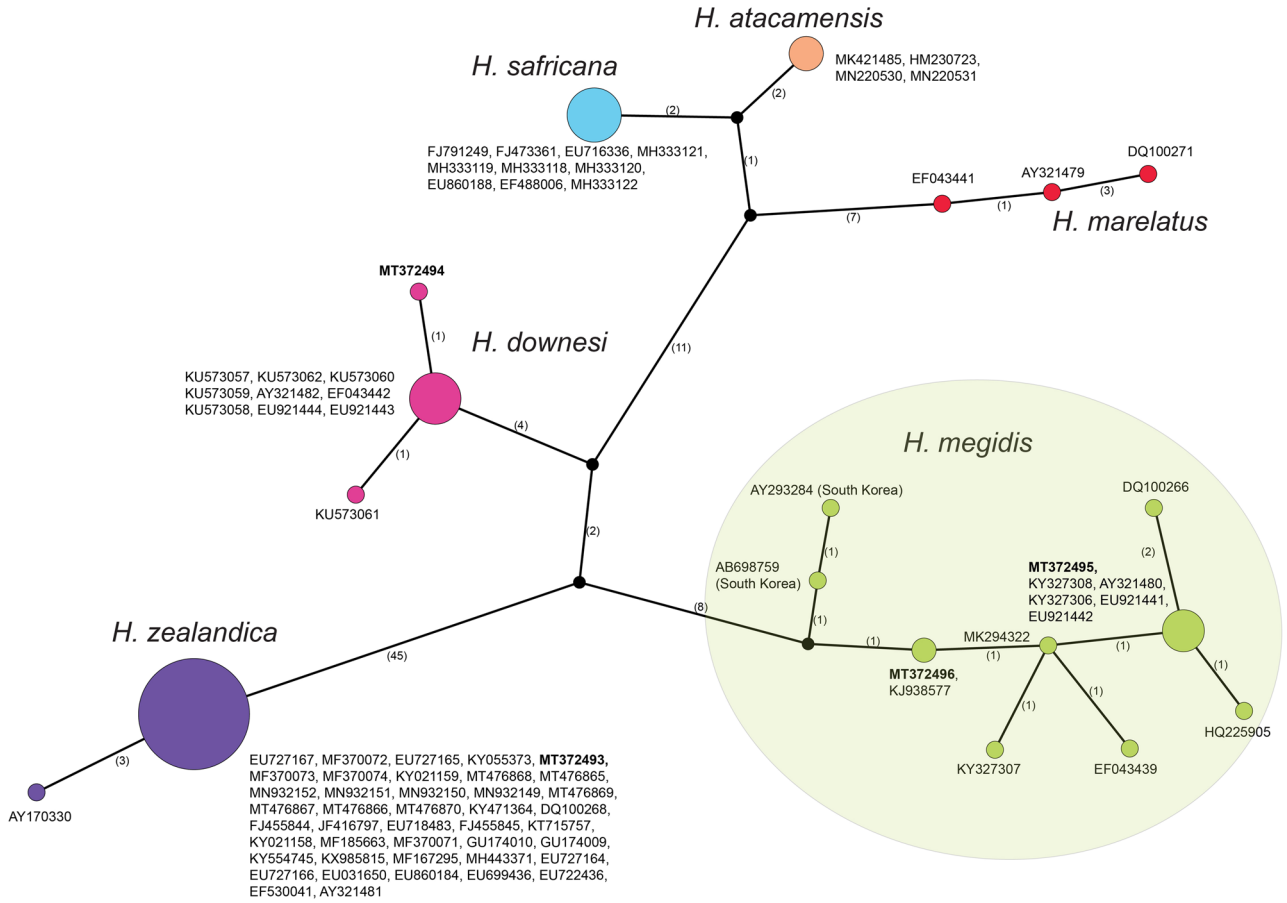

Fig. 4. Statistical parsimony networks showing the phylogenetic relationships between ITS rRNA gene haplotypes of *Heterorhabditis* species belonging to the *Megidis*-clade. The sequences of each species are marked by different colours. Pies (circles) represent sequences of each species with the same haplotype and their size is proportional to the number of these sequences in the samples. Numbers of nucleotide differences between the sequences are indicated on lines connecting the pies. Small black circles represent missing haplotypes. *Misidentified sequence. New sequences are indicated by bold font.

The *Indica*-clade: The alignment was 377 bp in length and included 15 sequences. Five new sequences of this group were included in this study. A phylogenetic network for the *COI* sequences, reconstructed using SP with POPART software, is given in Figure 6A. Maximal sequence diversity was 14.8% (50 bp).

The *Bacteriophora*-clade: The alignment was 377 bp in length and included eight sequences. Three new sequences of this group were included in this study. A phylogenetic network for the *COI* sequences, reconstructed using SP with POPART software, is given in Figure 6B. Maximal sequence diversity was 8.4% (32 bp).

The *Megidis*-clade: The alignment was 377 bp in length and included 11 sequences. Three new sequences of this group were included in this study. A phylogenetic network for the *COI* sequences, reconstructed using SP with POPART software, is given in Figure 6C. Maximal sequence diversity was 10.1% (38 bp).

Phylogenetic and sequence analysis with the *cmd-1* and *unc-87* protein coding genes

The *cmd-1* and *unc-87* gene alignments were 562 bp and 464 bp in length, respectively, and included 13 and 17 sequences, respectively. Eight and nine new sequences were obtained for the *cmd-1* and *unc-87* genes, respectively. Phylogenetic relationships within several valid species as inferred from BI of the *cmd-1* and *unc-87* gene sequences are given in Figure 7.

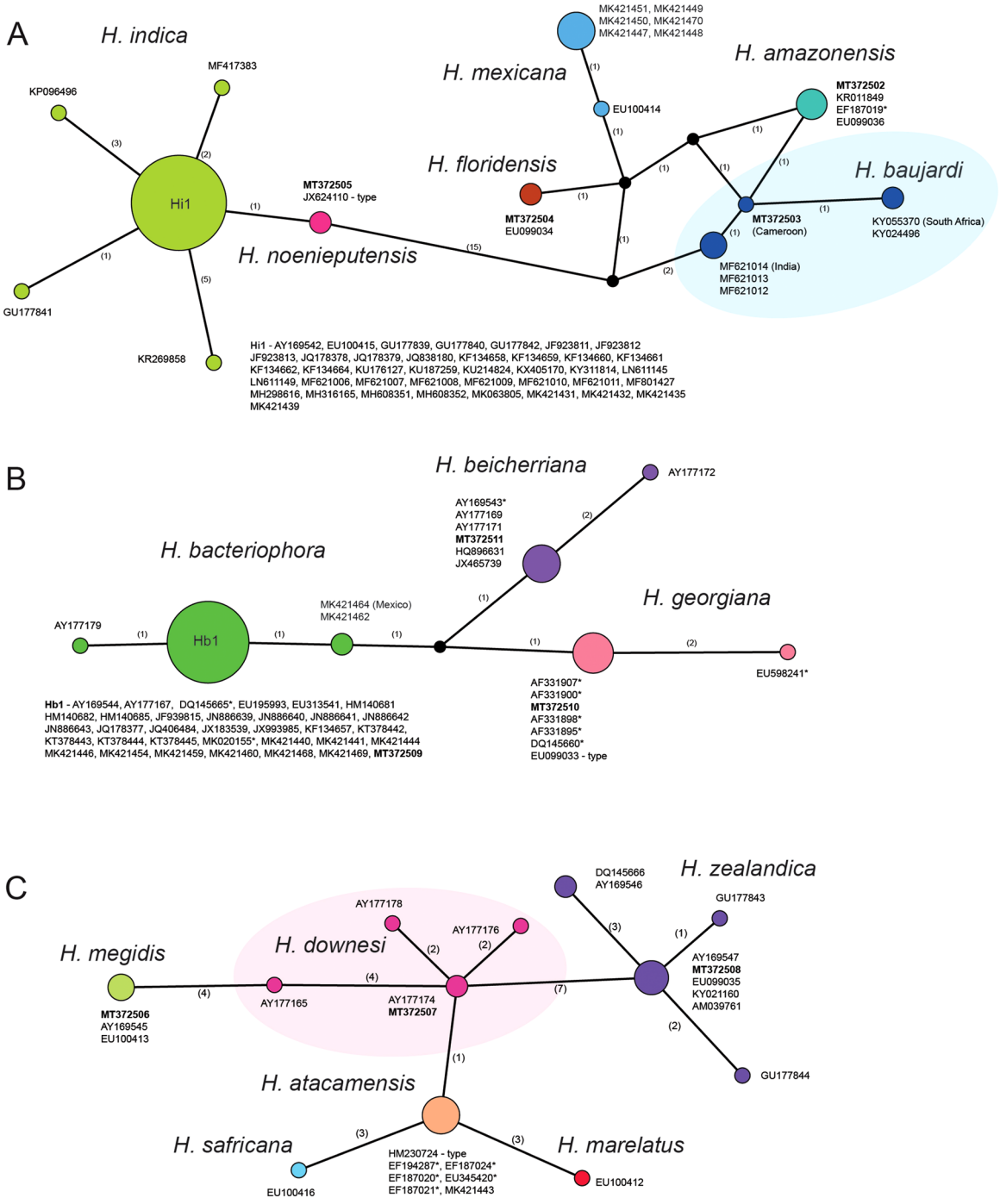

Fig. 5. Statistical parsimony networks showing the phylogenetic relationships between the D2-D3 28S rRNA gene haplotypes of *Heterorhabditis* species. A: The *Indica*-clade; B: The *Bacteriophora*-clade; C: The *Megidis*-clade. The sequences of each species are marked by different colours. Pies (circles) represent sequences of each species with the same haplotype and their size is proportional to the number of these sequences in the samples. Numbers of nucleotide differences between the sequences are indicated on lines connecting the pies. Small black circles represent missing haplotypes. *Misidentified sequence in GenBank. New sequences are indicated by bold font.

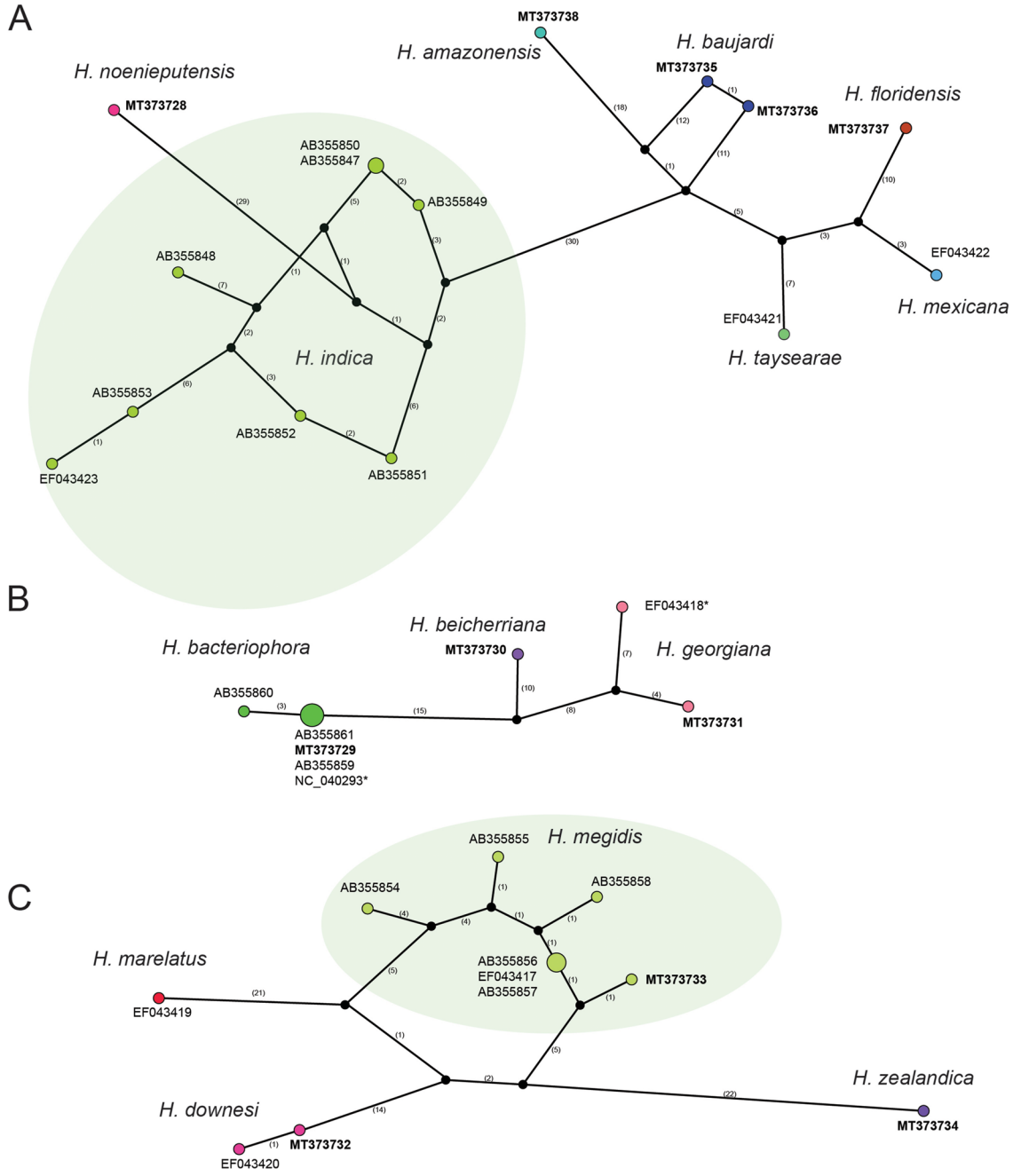

Fig. 6. Statistical parsimony networks showing the phylogenetic relationships between *COI* mtDNA gene haplotypes of *Heterorhabditis* species. A: The *Indica*-clade; B: The *Bacteriophora*-clade; C: The *Megidis*-clade. The sequences of each species are marked by different colours. Pies (circles) represent sequences of each species with the same haplotype and their size is proportional to the number of these sequences in the samples. Numbers of nucleotide differences between the sequences are indicated on lines connecting the pies. Small black circles represent missing haplotypes. *Misidentified sequence in GenBank. New sequences are indicated by bold font.

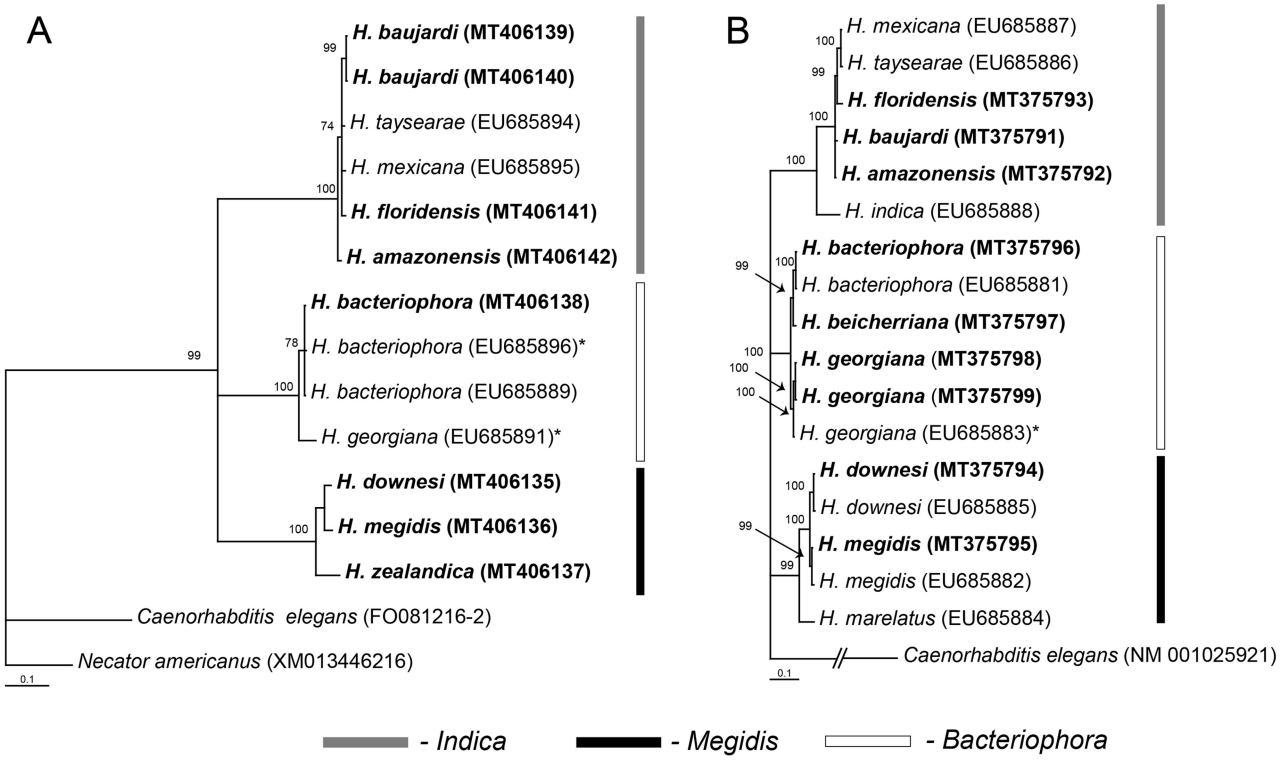

Fig. 7. Phylogenetic relationships within *Heterorhabditis* species and clades as inferred from Bayesian analyses of sequences of protein coding genes: *cmd*-1 (A) and *unc-87* (B) using the GTR + I + G model of DNA evolution. *Misidentified sequence in GenBank. Posterior probabilities equal to or more than 70% are given for appropriate clades. New sequences are indicated by bold font.

Phylogenetic analysis of alignment with combined gene sequences

The ITS rRNA, D2-D3 of 28S rRNA, *COI*, *cmd-1* and *unc-87* gene alignments were combined in one alignment and analysed with BI. The partition homogeneity test showed that the five datasets were not different and could therefore be combined for analysis. Phylogenetic relationships within *Heterorhabditis* species, as inferred from this sequence alignment, are given in Figure 1D.

Discussion

Species synonymisation and identification

The present phylogenetic and sequence analysis revealed many incorrect identifications in the GenBank sequences for several *Heterorhabditis* species and allowed us to correct them. The analysis of the large datasets also allowed us to estimate intraspecific and interspecific sequence variability for several genes of *Heterorhabditis* in more detail, provide important characters for species delimiting, and check the validity of several *Heterorhabditis* species. Among all studied genes, the ITS rRNA and *COI* genes are most informative and offer the best discriminatory power. Our molecular analysis did not reveal nucleotide differences in the ITS rRNA gene sequences of *H. somsookae* with those of *H. baujardi*; *H. sonorensis* with *H. taysearae*; and *H. pakistanensis* with *H. indica*. Thus, this study confirmed the synonymisation of these species as previously proposed by Hunt & Subbotin (2016).

In our study the SP network analysis was used to confirm validity of species. However, it can also provide a rapid and useful tool for detecting putative cryptic species. For example, one possible cryptic species candidate is represented by the presently identified *H. bacteriophora* with the ITS Hb2, and several related haplotypes from Mexico and South Africa. Bruno *et al*. (2020) had already noticed that these Mexican isolates might represent a new species. This hypothesis needs to be tested by morphological, biogeographical, ecological, and phylogenetic analyses with more datasets.

Phylogeny and phylogeography

Phylogenetic analysis of five genes revealed three distinct clades (groups) within the genus: *Megidis*, *Bacteriophora* and *Indica*. This study supports the same evolutionary lines previously found by other authors (Nguyen *et al*., 2008; Spiridonov & Subbotin, 2016). However, well resolved relationships between the clades were revealed only by using ITS rRNA gene sequences.

The reliable phylogeny of *Heterorhabditis* is a good basis for the phylogeographic analysis of the genus. Nevertheless, the efficiency of such analysis can be undermined by factors such as the possible transfer of soil entomopathogenic nematodes by human activity in the course of introducing exotic plants to new regions of the globe. As an important part of the sampling and isolation of new *Heterorhabditis* strains was performed in agricultural and other anthropogenic habitats, the impact of humans on the contemporary geographic distribution of heterorhabditid strains and species cannot be excluded.

All species of the *Indica*-clade are found in the tropics and subtropics or in the warm temperate zone. This clade is divided into two subclades in all trees. The first subclade (*Indica*-subclade) includes *H. noenieputensis* from South Africa and *H. indica*, the only species of the group that is widely distributed in South, southeast, southwest and East Asia, Africa, Australia, North and South America, and the Caribbean region. The second subclade (*Baujardi*-subclade) consists of *H. baujardi* found in Asia: Vietnam (Phan *et al*., 2003), Thailand (Noosidum *et al*., 2010), China (Wang *et al*., 2014), and India (Vanlalhlimpuia *et al*., 2018); Africa: Cameroon (Kanga *et al*., 2012) and South Africa (Abate *et al*., 2018); and South America: Colombia (López-Núñez *et al*., 2007), as identified in the present study. Other species of this subclade include: *H. amazonensis* from South America, *i.e.*, the Amazonian area and other regions in Brazil (Andaló *et al*., 2006, 2009) and Venezuela (Morales *et al*., 2016); *H. taysearae* described in Egypt by Shamseldean *et al*. (1996) and also found in Benin (Zadji *et al*., 2013); and North America: Mexico (Stock *et al*., 2009), *H. floridensis* from southeastern USA (Florida, Georgia), and *H. mexicana* from Mexico (Nguyen *et al*., 2004; Bruno *et al*., 2020). The presence of two lineages and their fragmented distribution indicates a complex colonisation history of the world by the species of this group, which most likely have an Asian origin. It is interesting that in most networks *H. baujardi* relates to *H. amazonensis. Heterorhabditis taysearae*, *H. floridensis* and *H. mexicana* also showed close relationships. Better sampling and more in-depth studies are needed to understand their distribution and the taxonomic status of some populations of these species.

The *Bacteriophora*-clade includes *H. bacteriophora* (= *H. argentinensis*), *H. georgiana* and *H. beicherriana*. *Heterorhabditis bacteriophora* is currently known as the species with the widest cosmopolitan geographical distribution among heterorhabditids and is a highly sequenced species, whereas *H. georgiana* is only known from the USA (Nguyen *et al*., 2008), whilst *Heterorhabditis beicherriana* is known from China and Martinique (Li *et al*., 2012).

The *Megidis*-clade contains *H. zealandica*, *H. downesi*, *H. megidis*, *H. marelatus* (= *H. hepialius*), *H. safricana* and *H. atacamensis*. *Heterorhabditis zealandica* is known from New Zealand, Australia, USA, Syria, South Africa and China. The relationships between some species of this clade are not well resolved with the exception of two pairs of species, *H. downesi* and *H. megidis*, and *H. safricana* and *H. atacamensis*, which come together in most phylogenetic trees with moderate or high statistical support. *Heterorhabditis downesi* is found only in western Europe and *H. megidis* is distributed across the entire Holarctic. *Heterorhabditis safricana* was described from South Africa, whereas *H. atacamensis* was found in the Atacama Desert, Chile, one of the driest deserts in the world, the species being isolated from a sandy bank located more than 2.4 km above sea level (Edgington *et al*., 2011), and also from Mexico (Bruno *et al*., 2020). *Heterorhabditis marelatus*, which often clusters with *H. safricana* and *H. atacamensis*, has a restricted geographic distribution and is found in Oregon and California, USA, although recently reported from Egypt (Saleh *et al*., 2018).

Statistical parsimony yielded network haplotypes delineated by geographical location. In several cases, haplotypes were grouped by nearby geographical locations, whereas haplotypes collected from geographically distinct regions stand apart. For example, ITS rRNA gene haplotypes of *H. baujardi* originating from Vietnam and India, and Thailand, South Africa, Cameroon and Colombia, differed from each other. The ITS rRNA gene haplotypes of *H. megidis* from South Korea are clearly differentiated from European haplotypes of this species.

Some conclusions can be inferred from the known distribution of heterorhabditid species. In each of the three main clades of the genus, at least one widely distributed species is present: *H. indica* in the *Indica*-clade, *H. bacteriophora* in the *Bacteriophora*-clade, and *H. megidis* in the *Megidis*-clade. These three species have been reported from a wide range of habitats, including primary ones pre-

sumably not affected by human activity. *Heterorhabditis megidis* is nearly ubiquitous in the soils of temperate and subarctic belts of the Holarctic, whereas *H. indica* is a circumtropical species, but also present in subtropical habitats. The majority of *H. bacteriophora* records stem from the warmer parts of the temperate zone and the subtropics. It can be presumed that mean annual temperatures can act as a major limiting factor in heterorhabditid distribution. It has been shown that the ability of heterorhabditids to tolerate high or low temperatures, as well as penetrate insect hosts and produce offspring under different temperature conditions, is determined by a whole complex of genes (Mukuka *et al*., 2010). While the highest level of heat tolerance is reported for tropical *H. indica*, a somewhat lower tolerance for the temperate *H. bacteriophora*, and the lowest tolerance for the temperate-subarctic *H. megidis*, intraspecific clades within *H. bacteriophora* and *H. indica* do not show any correlation with the isolation region or average annual temperatures (Mukuka *et al*., 2010). It has previously been suggested that, although heterorhabditid species undoubtedly have well-defined niches of optimal temperatures, soil temperatures below the surface layer are more stable and play a less significant role as limiting factors for the survival of entomopathogenic nematodes (Grewal *et al*., 1994). Different environmental barriers, centres and time of origin, as well as differences in the adaptability of species to climate conditions and their ability to parasitise various insect species, might explain the current known distribution patterns for heterorhabditid species. It is still obscure which biological mechanisms are involved in retaining the genetic entity of such widespread and narrowly distributed species. The nature of the determinants that influence the occurrence of vague but still visible borders between heterorhabditids remains unknown and requires further study.

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