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PERMANENT GENETIC RESOURCES NOTE

Novel primers for the amplification of nuclear DNA introns in the entomopathogenic nematode *Heterorhabditis bacteriophora* and their cross-amplification in seven other *Heterorhabditis* species

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Abstract

We describe 24 novel primers that amplify intron regions in housekeeping and structural genes of *Heterorhabditis bacteriophora*. The cross-amplification potential of these primers in seven other *Heterorhabditis* species was determined. The results obtained showed inter-specific nucleotide, length and splice site variability in the sequenced introns and for one gene, an intron gain was observed. These primers will be useful tools for studying population genetics, genetic diversity and intron DNA evolution within the genus *Heterorhabditis* and other genera of rhabditid nematodes.

Keywords: entomopathogenic, EPIC-PCR, *Heterorhabditis*, intron, molecular markers, nematode

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Heterorhabditid nematodes are obligate and lethal parasites of insects. The infective juvenile (IJ) stages of *Heterorhabditis* are modified third-stage juveniles which occur in soil and are responsible for locating and infecting an insect host. On gaining entry to the insect haemocoel, IJs release cells of a bacterial symbiont of the genus *Photorhabdus* which they harbour in their intestine (Boemare *et al*. 1993). The growth of *Photorhabdus* cells in the infected insect provides a suitable medium for nematode growth and reproduction. Each *Heterorhabditis* IJ matures to become a self-fertile hermaphrodite female, but the progeny of this female contains amphimictic males and females (Dix *et al*. 1992). Approximately 2 weeks post-infection, up to half a million IJs per gram of insect are produced (Akhurst & Bedding 1986). These IJs emerge into the soil where they may survive for several months in the absence of a suitable host. Since a single hermaphrodite IJ can establish a population that undergoes explosive expansion in an insect cadaver, populations of *H. bacteriophora* may be subjected to severe genetic bottleneck effects, but whether these founder effects lead to spatially distinct populations or whether migration between local populations maintains population cohesiveness has not been determined. Entomopathogenic nematodes (EPN) have been studied intensively because of their applications in biological control. Several studies have been published on the molecular phylogeny and molecular diagnostics of these nematodes (reviewed by Adams *et al*. 2006), but studies on the population genetics of EPN are lacking.

We developed novel primer sets for the amplification of introns from 24 structural and housekeeping genes from *H. bacteriophora*, and we tested the ability of these primers to amplify homologous introns in seven additional species of *Heterorhabditis*. Introns harbour high levels of sequence and length polymorphisms (Palumbi & Baker 1994; Graur & Li 2000). Thus, introns are suitable molecular markers for studies of population structure within and among species, and also for reconstructing relationships among closely related species. We constructed a cDNA library from IJs of *H. bacteriophora* (strain HP88) in the vector pBluescript (SK+) (Stratagene) in *Escherichia coli* DH10B cells. Clones

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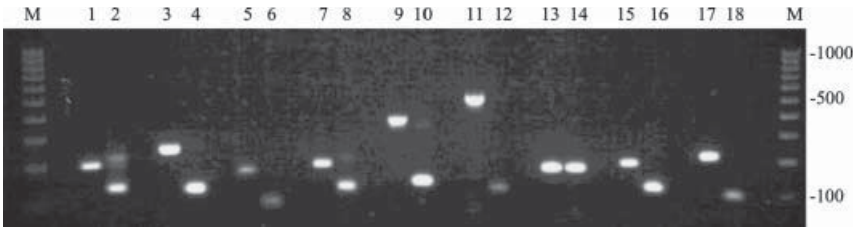

Fig. 1 Agarose gel showing the sizes of gDNA and cDNA amplicons from *Heterorhabditis bacteriophora* (HP88 strain). A larger gDNA amplification product indicates the presence of introns in the gDNA sequence. M (100 bp DNA ladder). Each pair of numbered lanes shows, respectively, gDNA and cDNA amplification products for a single pair of PCR primers. The PCR primers were as follows: 1–2, F16H9.1a (*rgs-2*); 3–4, R11A5.4 (*pepck*); 5–6, M106.5 (*cap-2*); 7–8, T20G5.1 (*chc-1*) (F1/R1); 9–10, C09B8.1 (*ipp-5*); 11–12, F08B6.4b (*unc-87*); 13–14, T20G5.1 (*chc-1*) (F2/R2), bands of equal size indicating absence of an intron; 15–16, F11C3.3 (*unc-54*); 17–18, Y54G11 A5a (*cat*). The primer details are presented in Table 1.

were randomly sequenced from this library, and from these expressed sequence tag (EST) sequences, data for 24 conserved housekeeping and structural genes were used to design exon-primed intron crossing (EPIC) polymerase chain reaction (PCR) primers (Palumbi & Baker 1994).

Putative intron positions in *H. bacteriophora* genes were identified by aligning homologous cDNA and gDNA sequences from *Caenorhabditis elegans* and *C. briggsae* with EST sequences from *H. bacteriophora* and by locating characteristic splice site motifs at the 5′ (e.g. GT) and the 3′ (e.g. AG) ends of these putative intron sites. PCR primers were designed manually to anneal to *H. bacteriophora* sequences in the flanking exons 30–80 base pairs from each putative intron. For each primer pair, the target sequence was amplified in both gDNA and cDNA from *H. bacteriophora* HP88 strain to detect size differences in the PCR product between the two templates (Fig. 1). PCR amplifications were performed with 0.25 μM of each primer, 200 μM of each dNTP, 1 mM $MgCl_2$, 1× reaction buffer, and 1 U of *Taq* DNA polymerase (Promega), in a final volume of 25 μL. Cycling conditions were 94 °C for 1 min, 55–62 °C for 1 min (the annealing temperature ranged from 55 to 62 °C depending on primers used), 72 °C for 1 min, for 30 cycles, and a final extension at 72 °C for 10 min. PCR products were visualized in ethidium bromide-stained 1% agarose gels. When putative introns were detected in the gDNA template, the PCR was repeated using High Fidelity Platinum *Taq* DNA polymerase (Invitrogen). PCR products were purified using a QIAGEN PCR Purification Kit. The purified products were cloned into TOPO TA cloning kit (Invitrogen) and the inserts were sequenced (MWG-BIOTECH).

Twenty-four primer pairs were designed (Table 1) and their cross-amplification in seven other *Heterorhabditis* species was also determined (Table S1, Supporting Information). Two primers (C24F3.2 and C04F6.3) did not amplify in species other than *H. bacteriophora* and another four primers (Y54G11 A5a, M106.5, T20G5.1 and *odr-3*) were tested in *H. bacteriophora* only. A total of 169 introns from eight *Heterorhabditis* species were identified and analysed (Table S2). The *H. bacteriophora* intron borders were determined by aligning the sequences of the *H. bacteriophora* cDNA (EST) and gDNA EPIC-PCR products. The intron borders in the genomic sequences of the seven additional *Heterorhabditis* species were inferred by aligning the gDNA EPIC-PCR products from these species with the homologous *H. bacteriophora* EPIC-PCR sequence in which the intron border had already been determined. As additional checks, the flanking exon sequences were concatenated and translated (using Translate Tool at http://expasy.org/). The resulting amino acid sequences were subjected to BLAST analysis against the GenBank (http://www.ncbi.nlm.nih.gov) and Wormbase (http://www.wormbase.org) databases to confirm gene identify and the correct reading frame. The amino acid translations of the flanking exon sequences of all eight species of *Heterorhabditis* were also aligned with each other and with the homologous sequence from *C. elegans* using Clustal_X (Thompson *et al*. 1997).

The results of EPIC-PCR among *Heterorhabditis* species showed interspecific nucleotide, length and splice site variability in the sequenced introns, and for one gene, an intron gain was observed. These novel intron primers provide molecular markers which will serve as useful tools for studying the population genetics and genetic diversity and intron DNA evolution within the genus *Heterorhabditis*. Because these primers have been identified and designed utilizing their homology to conserved housekeeping and structural gene sequences in *C. elegans*, it is likely that many of these could also be used in studies of other nematode species for which DNA sequence data are currently not available.

Acknowledgements

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Table 1 EPIC-PCR primers used in this study, PCR product sizes and intron lengths in *Heterorhabditis bacteriophora*

Gene name/intron no.	GenBank Accession no.	Primer name	Primer sequence 5′–3′	PCR product size (bp)	Intron size (bp)
ADP-ribosylation factor 1	EE724169	arf1F	CTGATGGTAGGACTAGATGC	483†	76
(*arf*-1) B0336.2, intron 1		arf1R	CCCTAAGTTCATCTTCAGCC		115
Dual specificity phosphatase	EE724178	dspF	GGTGCAAGTTCAGTTATCAGC	798‡	205
C24F3.2, intron 1		dspR	GCCTGATAGTTGAGACTTTCG		118
					63
Catalase (Y54G11A.5a)	EE724180	catF	CATCGTGATGGACCGGCTTGC	220§	100
		catR	CACACCACGGTTCTCCTGGG		
Myosin heavy chain class II	EE724181	unc54F	GCTCAAGCTGGTTCACTAGCAGC	190	76
(*unc*-54) F11C3.3, intron 6		unc54R	ACTGCGCTCTTCGGCAGCC		
Zinc finger protein (K02D7.2), Intron 2	EE724182	zinc FPF	CCAAACGTGTGCGCACTGC	723	625
		zinc FPR	CACACTGCTCACAGATACAAGG		
Inositol polyphosphate 5-phosphatase	EE724183	ipp5F	GAGCAGCTATCCGTCACAGAG	367	240
(*ipp*-5) C09B8.1, intron 5		ipp5R	CGAGAGGATGGGGTGCGG		
Clathrin heavy chain T20G5.1 (*chc*-1)	EE724184	chc1F1	CGATTGGAACGAGCAGAAGCAG	200§	150
		chc1R1	CCATACCTGGACCAGGAGC		
		chc1F2	GGACACCGCAGTATAGTGCCC	180	0
		chc1R2	CCATGCAAGATCGAACCACAAAC		
F-actin capping protein (*cap*-2) M106.5	EE724185	cap2F	CTCCGACGTCTAGAAATCGAAGC	180§	120
		cap2R	GGACGATACTCCACCCTCG		
Vesicle trafficking protein	EE724186	unc18F	GCTAATGTAGCTATGGCTG	429	250
(*unc*-18) F27D9.1a, intron 7		unc18R	GCATCCTCCATGATGTCC		
Phosphoenolpyruvate carboxykinase	EE724187	pepckF	GGCTATGCGTCCATTCATGGG	246	128
(R11A5.4), intron 3		pepckR	GTCCTTGCGGAACCAGTTGAC		
Thin filament (F-actin) associated protein	EE724189	unc87F	GGAACTCCCAGGAACACCAGC	494	386
(*unc-87*) F08B6.4b, intron 2		unc87R	CGTTCCTGACTGAAGGCGGAC		
Calmodulin (*cmd*-1) T21H3.3, Intron 2	EE724190	cmd1F	GGATACTGACAGTGAAGA	579	467
		cmd1R	CTCTCCCAGATTCGTCAT		
Casein kinase 1 (*kin*-19) C03C10.1, intron 1	EE724191	kin19F	CTTCAACTTTGCTCACGCAGG	320	255
		kin19R	CCAATCATCTGATCTGCCAGC		
Chitinase (T05H4.7), intron 2	EE724192	chitF	CACATGCCATTCAAGAAACAGGGG	257	150
		chitR	CCAGTTGAAGAATCCACCCCGG		
Nucleolar protein (*lpd*-7) R13A5.12, intron 1	EE724193	lpd7F	CGGTTAACTATATTTCACGG	226	66
		lpd7R	CGATAGTACCACACACG		
Glycosyl hydrolase	EE724194	cht1F	GGTTATGCTGAGATTATCGC	502	374
(*cht*-1 family 18) C04F6.3, intron 2		cht1R	GAATCGTCTTTCATCTTCCC		
Serine/threonine protein phosphatase	EE724195	gsp2F	CTGACAAGCTCAACTTGG	439†	124
(*gsp*-2) F56C9.1, introns 1 and 2		gsp2R	GAAGGTCATAGTATTGGCC		108
Rab GDP dissociation inhibitor	EE724197	gdi1F	CCGGGAACGAATGATGCG	471†	63
(*gdi*-1) Y57G11C.10, intron 1		gdi1R	CTGTGCCAAGATCAGTCGGCTCG		82
Hypothetical protein T19D7.5, introns 1 and 2	EE24198	hpF	GATCACAGCCGAGGAGGCAGC	381	245
		hpR	CAGTGCGAGGAGCGATGACCG		
Isocitrate lyase (*gei*-7) C05E4.9a, intron 4	EE724201	iclF	CCCGAAGGAACTAGAACAGATGCC	342	169
		iclR	GCATCATGTCTCAGCCACTGCC		
Synaptosome-associated protein	EE724203	snap25F	GAGCTGAAGACTCTCAACTTGC	888‡	318
(*ric*-4) Y22F5A.3, introns 1, 2 and 3		snap25R	GATTAATCGTGTCTAGAGCGCC		126
					258
Regulator of G protein-signalling	EE724204	rgs2F	GCCTGGTCCCAATCCTTTGAC	199	70
(*rgs*-2) F16H9.1a, intron 3		rgs2R	CCGCTTCAACTCTTCACAAGCC		
Elongation factor-1 (*eft*-3) F31E3.5, intron 1	EE724216	eft3F	CATTGGACACGTCGATTCTGG	405	71
		eft3R	GAACTCTCCTGTACCACATGC		
G protein alpha subunit	EU131131	odr3F	GGATCCAGCATCGTCAAGC	617§	470
(*odr*-3) C34D1.3, intron 1		odr3R	GGGAATGTGTAGAATGTTGTCC		

The name of the homologous *Caenorhabditis elegans* sequence is given and, when available; the *C. elegans* gene name is also given in italics. †two introns present; ‡three introns present; §successful PCR but amplicons were not sequenced.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Cross-species comparison of size polymorphisms from 169 introns sequenced from eight species of the EPN genus *Heterorhabditis*. The size in base pairs for each intron is recorded (–, no amplification; **, intron absent) and the intron numbers refer to intron positions in *C. elegans*

Table S2 Gene identities and GenBank Accession numbers of the gDNA EPIC-PCR sequences obtained for *Heterorhabditis bacteriophora* and seven other *Heterorhabditis* species (–, EPIC-PCR product was not obtained)

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