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Diversification of fasting regulated transcription in a cluster of duplicated nuclear hormone receptors in *C. elegans*

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Abstract

The genome of *C. elegans* encodes more than 280 nuclear hormone receptors (NHRs) in contrast to the 48 NHRs in humans and 18 NHRs in *Drosophila*. The majority of the *C. elegans* NHRs are categorized as supplementary nuclear receptors (supnrs) that evolved by successive duplications of a single ancestral gene. The evolutionary pressures that lead to the expansion of NHRs in nematodes, as well as the function of the majority of supnrs, are not known. Here, we have studied the expression of seven genes organized in a cluster on chromosome V: *nhr-206, nhr-208, nhr-207, nhr-209, nhr-154, nhr-153* and *nhr-136*. Reverse transcription – quantitative PCR and analyses using transgenic lines carrying GFP fusion genes with their putative promoters revealed that all seven genes of this cluster are expressed and five have partially overlapping expression patterns including in the pharynx, intestine, certain neurons, the anal sphincter muscle, and male specific cells. Four genes in this cluster are conserved between *C. elegans* and *C. briggsae* whereas three genes are present only in *C. elegans*, the apparent result of a relatively recent expansion. Interestingly, we find that a subset of the conserved and non-conserved genes in this cluster respond transcriptionally to fasting in tissue-specific patterns. Our results reveal the diversification of the temporal, spatial, and metabolic gene expression patterns coupled with evolutionary drift within supnr family members.

Keywords

Caenorhabditis elegans; *Caenorhabditis briggsae*; nuclear hormone receptor; gene expression; fasting

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1. Results and Discussion

1.1 Rhabditidae genomes encode far more NHRs than insects or mammals

While the *Drosophila* genome contains only 18 *nhr* genes and the human genome contains 48 such genes, sequence homology identifies more that 284 *nhrs* in the *C. elegans* genome (Antebi, 2006; Haerty et al., 2008; King-Jones and Thummel, 2005; Sluder et al., 1999), 232 in *C briggsae* (reported by (Haerty et al., 2008)) and 256 in *C. remanei* (Haerty et al., 2008). Of the 284 *nhr* genes in *C. elegans*, only 13 (Robinson-Rechavi et al., 2003; Robinson-Rechavi et al., 2005) can be regarded as highly conserved nuclear receptors. The remaining *C. elegans nhr* genes encode receptors that are categorized as supplementary nuclear receptors (supnrs), the majority of which appear to have been derived from successive duplications of a single ancestral gene related to vertebrate HNF-4 (Robinson-Rechavi et al., 2005). A few of these supnrs have been ascribed functions. For example, the HNF-4-related NHR encoded by *nhr-49* is a key regulator of metabolic genes in response to fasting and feeding (Van Gilst et al., 2005a) while supnr *nhr-60* plays a role in epidermal cell development (Simeckova et al., 2007). Characterizing the pattern of expression and functions of additional supnrs is an important step in understanding the evolutionary pressures that have led to the explosion of these receptors in Rhabditidae genomes. In this study, we have chosen to focus on a cluster of seven supplementary *nhr* genes for an analysis of transcription, fasting response, and function.

1.2. The genomic organization of a *nhr* gene cluster localized on chromosome V

Seven *nhr* genes are organized as a direct tandem repeat within a 17 kb region of Chromosome V represented on the cosmids R07B7.13 and C13C4.3 (V: 12,092,022 – 12,109,114; WS207). The genes localized in this region are in close proximity to each other and include the following *nhrs* starting from the conventional chromosomal left end: *nhr-206*, *nhr-208*, *nhr-207*, *nhr-209*, *nhr-154*, *nhr-153* and *nhr-136* (Fig. 1A).

In order to characterize the mRNAs transcribed from this cluster, we prepared cDNAs from mixed cultures of *C. elegans* wild type (N2) worms and performed polymerase chain reactions (PCR) with primers derived from each gene as identified by the GeneFinder program. To capture the 5' mRNA ends, we included the SL1 and SL2 splice leader primers or used rapid amplification of cDNA ends (RACE). PCR or RACE products were cloned into pCR4 and/or PCRII vectors and sequenced. This strategy confirmed the expression of mRNAs for all seven genes in the cluster with exon-intron boundaries as depicted in WormBase (WS207).

An alignment of amino acid sequences of all NHRs from this cluster was generated by the Clustal program (Larkin et al., 2007) (Fig. 1B). The analysis shows that the strongest homology between receptors is within the DNA binding domains (DBD) and at the C-terminal regions of the receptors spanning the putative ligand binding domain (LBD). Outside of the DBD and LBD, these NHRs show striking sequence diversity, although Blast searches identified genes of this cluster as the closest homologues in the *C. elegans* genome. The genomic organization and sequence similarities strongly suggest that these genes arose by successive duplications with subsequent sequence divergence. The phylogenic and cladistic analyses indicated that the genes in the cluster can be divided to two subgroups, the first containing the genes most related to *nhr-209* and the second related to a common ancestor of *nhr-153* and *nhr-136* (Fig. 2A). The analysis also indicated that the later cluster is more ancient and that genes *nhr-206*, *nhr-208* and *nhr-207* were formed by more recent duplications. All seven NHRs within this cluster belong to Class I *C. elegans* nuclear hormone receptors, based on their P-box sequences (Van Gilst et al., 2002). P-box sequence relatedness is in agreement with the overall homology estimated by Blast: NHR-206,

NHR-208 and NHR-207, have the P box sequence CNGCKA and form a small set of receptors in subgroup 12 whereas NHR-209, NHR-154, NHR-153 and NHR-136 have the P box sequence CNGCKT and form subgroup 8 (Van Gilst et al., 2002).

We compared the sequences of the clustered *C. elegans* receptors with their closest homologues in *C. briggsae* and found that the receptors with P box sequence CNGCKT are evolutionarily conserved, including identical P-box sequences. Comparison of the *C. elegans* and *C. briggsae* genomic sequences indicates the following relationships: the ortholog of NHR-209 is CBP 20617 (CBG 23379), the ortholog of NHR-154 is CBP 24460 (CBG23380), the ortholog of NHR-153 is CBP24461 (CBG23381) and the ortholog of NHR-136 is CBP 24462 (CBG23383). The clear orthology defines these genes as *Cbr-nhr-209*, *Cbr- nhr-154*, *Cbr-nhr-153* and *Cbr-nhr-136*. The amino aid sequences of this set of conserved NHRs in *C. elegans* are 66% to 76% identical to the orthologous receptors in *C. briggsae* (NHR-209, 66%; NHR-154, 76%; NHR-153, 69 %, NHR-136, 75%). We also found exon size conservation among orthologs in these two species (Fig. 2B). The sequences and the sizes of the introns are not conserved, nor are the sequences of the intergenic regions. In addition, the first three receptors in the cluster (NHR-206, -208 and -207) do not have orthologs in *C. briggsae*.

1.3 *nhr-207* and *nhr- 209* are organized in an operon

The intergenic regions between genes of this *nhr* gene cluster vary from 270 to 1277 bp in length. Short distances between genes transcribed in the same direction in *C. elegans* are suggestive of an operon in which co-transcribed mRNAs are processed into individual coding messages via trans-splicing (Blumenthal, 2005; Blumenthal et al., 2002). Preceding *nhr-206*, Wormbase identifies a gene *R07B7.12a* that is transcribed from the same strand, raising the possibility that is also part of an operon including the *nhrs*; *R07B7.12a* encodes a large, predicted extracellular protein unrelated to NHRs. In order to distinguish between individually transcribed genes and genes expressed from operons, we prepared cDNA from mixed stages of N2 animals and performed multiple PCRs with primers specific for Splice Leader 1 (SL1) and Splice Leader 2 (SL2) in combination with gene-specific, reverse strand primers. Approximately 75% of genes in *C. elegans* are trans-spliced to SL1 and when present, this leader sequence is indicative of transcription as a single gene or the first gene in an operon. SL2 trans-splicing predominantly occurs on the second, and subsequent, genes in an operon. We identified trans-splicing with SL1 for *nhr-154* resulting in a 13 bp long 5'UTR (tatagtggcagcc), consistent with WormBase information. Trans-splicing with SL2 was detected for *nhr-209* resulting in a 257 bp long 5'UTR. Trans-splicing of *nhr-209* has not previously been reported and demonstrates it is co-transcribed with one or more flanking genes oriented on the same coding strand. Surprisingly, trans-splicing to SL1 or SL2 was not detected for any of the remaining genes of this *nhr* cluster, although the expression of the predicted mRNAs was readily amplified using gene specific primers derived from the predicted sequences. Our results demonstrate that *nhr-207* and *nhr-209* are part of a novel two-gene operon while all other *nhrs* within this cluster are transcribed individually.

1.4. *nhr* cluster gene expression during *C. elegans* and *C. briggsae* development

We prepared cDNA from individual stages of *C. elegans* and *C. briggsae* animals as follows: embryos, larval stages L1, L2, L3, L4 and young adults. Quantitative Reverse Transcriptase PCR (RT-qPCR) was done using SYBR Green technology. The *ama-1* gene in *C. elegans*, encoding the large subunit of RNA Polymerase II and the ortholog of *ama-1* in *C. briggsae* (CBG05355) were used for normalization of the data.

All seven *C. elegans* genes of the studied cluster, and all four *C. briggsae* homologs, were expressed during all developmental stages. *C. elegans* genes *nhr-206*, *nhr-208* and *nhr-207*

were detected in hundreds of units (Fig. 3A), while the evolutionarily conserved genes (*nhr-209*, *nhr-154*, *nhr-153* and *nhr-136*) were detected at much higher relative levels (10,000 to 20,000 units) (Fig. 3B). All *C. elegans* genes, with the exception of *nhr-154*, and all *C. briggsae* genes showed relatively stable expression during development under normal growth conditions. In contrast, *nhr-154* was expressed at high levels in embryos, L1s, and young adults, but decreased during mid-larval stages (Fig. 3B). It is possible that the rise in expression in the adult stage sample is due to embryos within gravid adults, rather than adult expression per se (see below). The expression values detected for *C. brigssae* orthologs *Cbr-nhr-209*, *Cbr-nhr-154*, *Cbr-nhr-153* and *Cbr-nhr-136* were similar to that observed for their *C. elegans* counterparts (Fig. 3C) with a slight dip in mid-larval stages. These results demonstrate that the seven *nhrs* of the cluster are expressed throughout development and that the recent expansion in *C. elegans* of *nhr-209*-related genes was accompanied by a change in expression levels. Our results are largely consistent with whole genome expression array data from early embryogenesis (Yanai and Hunter, 2009), demonstrating detectable expression of most genes in the cluster in both species. An exception was *nhr-209* for which we found qPCR evidence for embryonic expression while Yanai and Hunter detected essentially no early embryonic expression; late embryonic onset of expression would be consistent with both results

1.5. The expression of *gfp*-fusion reporter genes shows partially overlapping patterns for the clustered *nhrs*

In order to determine in which tissues and cells the individual receptors are expressed, we prepared transgenic lines carrying putative promoters of each gene fused with the gene encoding green fluorescence protein (GFP) (Fig. 4). At least three independent lines for each of two different promoter constructs were generated for each *nhr* gene in the cluster. A "short" promoter construct used a 500 – 600 bp segment immediately upstream of the coding region of each gene. A second "long" promoter construct used a 1,000–2,000 bp segment upstream of the coding region that often included part of the upstream, flanking gene. One exception to this strategy was *nhr-153*, for which only a 968 bp segment upstream of the coding region was used with the reporter gene. Five of the seven *nhr* genes tested showed *gfp* expression: *nhr-206*, *nhr-208*, *nhr-207*, *nhr-154*, and *nhr-153*. Transgenes for *nhr-209* and *nhr-136* failed to yield reporter gene expression. As detailed above, *nhr-209* is the second gene of an operon. Consequently, we tried several different promoter regions for this reporter transgene, including one with the putative promoter and entire coding region of the upstream flanking gene (*nhr-207*), all without success. The lack of expression of the *nhr-136* reporter transgenes in 12 independent lines was unexpected because of its relatively high expression when assayed by RT-qPCR. It is possible that this gene is regulated by a distant enhancer located far upstream, or downstream, of the coding region.

A. Expression of the evolutionarily conserved subgroup of clustered *nhrs* at standard laboratory conditions—The results of all GFP reporter transgenes are summarized in Table 1. As noted above, only two of the evolutionarily conserved *nhrs* within this cluster yielded reporter gene expression.

The expression of ***nhr-154*** GFP reporter transgenes was first detected in the 2-fold stage embryo within the developing pharynx and in precursors of several unidentified head neurons. By the three-fold stage of embryogenesis, expression was seen in the pharynx and throughout the intestine, a pattern reminiscent of the developmental transcription factor *pha-4* (Smith and Mango, 2007; Updike and Mango, 2006) (Fig. 5A). In L1 and L2 stages, the reporter gene expression was strong in the pharyngeal muscles (anterior and posterior bulbs, predominantly), in unidentified head neurons, the intestine, and in the intestinal-rectal valve or sphincter cell (Fig. 5B). Consistent with our RT-qPCR analysis, GFP reporter gene

expression decreased in subsequent larval stages (L3, L4) so that by the adult stage only pharyngeal expression persistence is reproducibly observed. This suggested that the peak of expression in adults detected by RT-qPCR might be due to the embryos inside gravid adults in these samples (Fig. 5C). No obvious differences in pattern were seen between the two promoter lengths tested. The expression of *nhr-154* GFP reporter was not observed in male specific structures.

The expression of ***nhr-153*** GFP reporter transgenes was first detected in the 2-fold stage embryo in pharyngeal and intestinal cells (Fig. 5D). In all larval stages, the reporter gene expression was very strong in the posterior bulb of pharynx and in all intestinal cells as well as the intestinal-rectal valve or rectal gland cells. Expression was also seen in several unidentified neurons near the posterior pharyngeal bulb and in the tail (Fig. 5E). In males, very strong reporter expression was observed in some of the ray-associated neurons (Fig. 5F).

B. Expression of recently duplicated clustered *nhrs* at standard laboratory conditions—The expression of ***nhr-206*** GFP reporter transgenes starts during the comma stage of embryogenesis, and is initially seen in four unidentified cells localized in the head region. By the 2-fold stage, embryonic expression is observed in the pharynx with weaker expression in intestine (Fig. 5G). This expression pattern continues throughout all larval stages and in adults with pronounced anterior pharyngeal expression. The reporter genes were also strongly expressed in rectal gland cells, the anal sphincter, and in epidermal cells in the tail (Fig. 5H). Weaker expression was also observed in the vulva and spermatheca. In males, expression was visible in male specific neurons of the tail and rays (Fig. 5I). The pattern of expression is very similar to that described by Reece-Hoyes and coworkers in their high throughput screen (Reece-Hoyes et al., 2007).

The expression of ***nhr-208*** GFP reporters started in embryos at the 1.5-fold stage within the pharynx, intestinal sphincter, and epidermal cells in the tail. By the 3-fold stage of embryogenesis, additional expression was observed in rectal gland and surrounding cells (Fig. 5J). During all larval stages, strong expression of the transgenes was visible in pharyngeal and unidentified head neurons, the pharyngeal-intestinal valve cell, the posterior part of the intestine, the intestinal sphincter, two rectal gland cells, the intestinal-rectal valve cell, and the epidermal hyp10 cell (Fig. 5K). In males, the expression was seen in several rays (6–8) and other male specific neurons (Fig. 5L). This pattern was similar to that described by Reece-Hoyes and coworkers (using a 653 bp long promoter), although our reporters did not result in expression in the excretory cell and vulva.

The expression of ***nhr-207*** GFP reporters began in 1.5-fold embryos in pharyngeal and epidermal cells (Fig. 5M). In 3-fold stage embryos, expression was observed in pharyngeal neurons, intestinal cells, the intestinal-rectal valve, and the sphincter. This pattern of expression was present during all larval stages and in adults (Fig. 5N). In larvae and in adults, additional expression was observed in the pharyngeal-intestinal valve (Fig. 5O) and spermathecae. In males, the expression was seen in male specific neurons, including rays (Fig. 5P). Our results overlap those reported by Reece-Hoyes and coworkers (using a 340 bp long promoter).

To summarize, the functional *nhr* GFP fusion reporter genes had many common features. Onset of expression was similar in all cases (comma to 2-fold embryos) with common expression in the intestine, the intestinal-rectal valve, the sphincter, and unidentified head neurons. All but one (*nhr-154:gfp*) also showed male tail specific neuron expression, including within a common subset of the rays. The recently duplicated *nhrs* (*nhr-206, -208, -207*) showed a strong bias of expression in the intestine at the posterior end and two had

additional expression in the vulva and spermatheca. We concluded that this gene cluster shares many common sites of expression, although there are clear differences in the patterns between the evolutionarily conserved versus the recently expanded *nhrs* within this cluster.

1.6. The expression of clustered nuclear hormone receptors responds to fasting

The function of several *C. elegans* NHRs have been linked to metabolism (Magner and Antebi, 2008; Pohludka et al., 2008; Van Gilst et al., 2005a; Van Gilst et al., 2005b). For example, our previous work on NHR-40 demonstrated developmental phenotypes that were dependent on their nutritional status and environmental conditions such as temperature (Brozova et al., 2006; Pohludka et al., 2008). Since the clustered *nhr*s we focused on in the current study belong to the same subgroup of NHRs as NHR-40, we tested if their expression was similarly affected by alterations in feeding status. To eliminate developmental effects, we used L1s synchronized by hatching in the absence of food; such animals arrest development until fed.

We assayed the expression of the clustered *nhrs* by RT-qPCR in L1 populations of *C. elegans* that had been hatched and starved for either six or 54 hours using the expression of *ama-1* to normalize the data. The expression of *C. elegans nhr-206*, *nhr-208*, *nhr-207* showed a dramatic up regulation (5 to10-fold) under both fasting conditions. There also was a slight increase (2-fold) in expression of *nhr-153* and *nhr-136* during fasting (Fig. 6). As controls, we assayed the expression of genes that are known to be increased (*acs-2*), decreased (*fat-7*) or unaffected (*nhr-49*) by fasting (Van Gilst et al., 2005b). As expected, *acs-2* was up-regulated approximately 2-fold by fasting, *fat-7* decreased approximately 5 to 10-fold and the expression of *nhr-49* did not change in our experimental protocols (Fig. 6). Analysis of the expression of *C. briggsae* orthologs after six hours of starvation showed that only *Cbr-nhr-209* increased (2-fold) in fasting larvae (Fig. 6).

The substantial up-regulation of several of the clustered *nhrs* in response to starvation, as detected by RT-qPCR, suggested corresponding GFP reporter genes might behave similarly. Indeed, the elevated expression of GFP fusion transgenes was clearly visible and was tissue restricted, as seen for the intestinal cell expression for *nhr-206*, *nhr-208* and *nhr-207* (Fig. 7A, B). We quantitated changes in expression using densitometric analyses of images captured at constant settings in conditions of feeding versus fasting for the two major sites of expression for the transgenes; the pharynx and the intestine. The analyses confirmed the elevated expression in *gfp* fusion genes in intestinal cells of transgenic animals expressing *nhr-206*, *nhr-208* and *nhr-207* and in pharynx of animals expressing *gfp* fusion genes of *nhr-153* and *nhr-154* (Fig. 7). These results confirmed the RT-qPCR results and demonstrated that the recent expansion of *nhr* genes within this cluster in *C. elegans* was coupled to altered transcriptional responses, showing dramatic up-regulation in response to fasting that is restricted to intestinal cells. The evolutionarily conserved *nhrs* of the cluster showed more modest increases that were predominantly seen in the pharynx.

1.7. Inhibition of clustered *nhrs*, either alone or in combination, does not affect viability

We studied the effect of gene inhibition for all seven *C. elegans* clustered *nhrs* by RNA-mediated interference (RNAi) induced by soaking of larvae in solutions of dsRNA as well as by microinjection into the gonads of parental hermaphrodites. All genes were inhibited individually as well as simultaneously in combinations of two, three or four genes or all seven genes together. We did not observed any morphological phenotypes as a result of RNAi, but there was a slight increase (up to ~5%) of embryonic lethality when all evolutionarily conserved genes were inhibited simultaneously (see Supplementary Table 1). We concluded that none of the genes on their own was essential for viability, consistent with

redundancy of function among many *nhr* family members and their common roles in metabolic regulation in response to changing environmental conditions.

In conclusion, in an effort to further understand the evolutionary pressures leading to the explosive expansion of NHRs in nematodes, we studied set of tightly clustered *nhrs* in *C. elegans*. Our results demonstrate that four of the clustered *nhrs* are evolutionarily conserved between *C. elegans* and *C. briggsae*, sharing identical DNA binding P-box domains between orthologs as well as exon-intron structures. Recent duplications of one of these genes has led to the expansion of this cluster in *C. elegans* to include three additional *nhrs* that are not present in *C. briggsae*, providing an example of the continued evolutionary flux of this gene family. We found that all genes of these clusters in both species are expressed and GFP reporters demonstrate conserved temporal and spatial patterns for some aspects of expression. The conserved expression patterns, coupled with high sequence conservation among orthologs, strongly argue that these *nhrs* are functional in the organism. However, RNAi knockdown of these seven *nhr* genes, either alone or in combinations, failed to reveal any essential function, suggesting these genes act redundantly and/or that they are involved in processes that are not vital for development and viability. Interestingly, the most recently duplicated genes within the cluster that are present in *C. elegans* are distinct from their evolutionarily conserved paralogs in their expression. Each gene in this triplet is dramatically responsive to fasting, whereas the evolutionarily conserved quartet of genes is not or only slightly so. In addition, the triplet and quartet of *nhrs* show different tissue responses to fasting. This data demonstrates that cluster expansion in *C. elegans* was accompanied by an alteration in gene regulation at the level of metabolic response and cell type specificity. Our results support the concept that *nhrs* are a dynamically fluxing set of transcription factors that display plasticity in regulation, allowing them to rapidly adapt to novel metabolic and developmental roles.

2. Experimental Procedures

2.1 Worm cultures and lines

C. elegans and *C. briggsae* worms were obtained from *C. elegans* Stock Center and cultures were maintained as described (Brenner, 1974). Stock cultures were frozen in liquid nitrogen and fresh cultures were started from frozen stocks when necessary. Larvae were grown on nematode growth medium (NGM) plates or on 2% agarose-capped plates and fed the OP50 bacterial strain of *E. coli* (Brenner, 1974).

Transgenic lines were prepared as described (Mello, 1995) from N2 wild type worms. In general, the cloning strategy was designed to keep the first exon, the first intron and a part of the second exon in the transgene. Two different lengths of putative promoters were chosen for promoter analysis using green fluorescent protein (GFP)-fusion transgenes. Short constructs used approximately 500 to 600 bp of putative promoter sequences where long construct used from 1,000 to 2,000 bp. In general, the short promoter regions included usually only intergenic sequences, but long promoter sequences partially overlapped with the coding region of the preceding gene. Each promoter was analyzed with minimum of three independent lines prepared from short and long promoter regions. The exception was *nhr-153*, for which only one size of promoter sequence was used, 968 bp and six transgenic lines were prepared.

All constructs were prepared by PCR amplification of genomic DNA using primers with appropriate restriction sites (see Supplementary Table 2). The PCR products were digested and cloned in frame with GFP into the corresponding restriction sites in the vector pPD95.67 which includes a nuclear localization site (NLS).

All transgenic lines were prepared by co-injection of plasmid DNA containing pPD95.67 vector with cloned inserts (at the concentration 50 ng/μl) and a marker, pRF4 plasmid (50ng/μl) carrying the mutated *rol-6* gene (Su1006). The mixture was injected into gonads of young adult hermaphrodites and the progeny of injected mothers were screened for a rolling (Rol) phenotype.

2. 2 Cloning of complete cDNAs and selected cDNA regions for RNAi

Total RNA isolation: *C. elegans* and *C. briggsae* animals from all stages of development (mixed wild type worms) were grown on 2% agarose plates. Worms were washed with M9 medium and frozen at −80°C. Total RNA was prepared following the protocol described by Johnstone and Barry (Johnstone and Barry, 1996) with modifications (Kostrouchova et al., 2001). Briefly, the frozen pellets were re-suspended in 0.5 ml of re-suspension buffer (0.5% SDS; 5% 2-mercaptoethanol; 10 mM EDTA; 10 mM Tris/HCl (pH 7.5) with 12.5 μl of proteinase K (20 mg/ml), vortexed for 1 min. and incubated for 1 hour at 55°C. The samples were treated by phenol-chloroform extraction and ethanol precipitation and dried pellets were dissolved in water. Contaminating genomic DNA was eliminated by DNase treatment (Promega, Madison,WI), using 1 μl of solution containing 1 unit/μl per 1 μg total RNA and samples were incubated for 30 min. at 37°C. An additional phenol-choroform extraction and ethanol precipitation step was included. Total RNA was dissolved in DEPC water and frozen at −80°C before further use.

C. elegans and *C. briggsae* cDNA was prepared from total RNA by reverse transcription reaction using the Superscript II kit (Invitrogen, Carlsbad, CA). For reverse transcription, approximately equal amounts of RNA were used as assayed by a Nanodrop 2000c (Thermo Scientific, Wilmington, DE). One microgram of RNA was mixed with 100ng of random hexamers, heated for 5 min. at 65°C and 4 μl of 1st strand buffer, 1 μl of 100mM DTT, 1μl of RNasin, 1 μl of dNTPs and 1 μl of Superscript II were added. The mixture was incubated 10 min. at 25°C, 50 min. at 42°C and 15 min. at 70°C. PCR reactions were done with gene specific primers derived from the predicted mRNA sequence from WormBase (see Supplementary Table 2). Attempts to amplify all cDNAs of clustered *nhrs* with either SL1 (ggtttaattacccaagtttgag) or SL2 (ggttttaacccagttactcaag) splice leader sequence and primers derived from predicted gene sequences.

nhr-209 5’ end RACE: In order to confirm the trans-splicing of the gene *nhr-209* by an unbiased method, we used 5' RACE Kit, 2nd Generation (Hoffman-La Roche, Basel, Switzerland). Amplicons were cloned in pCR4 and or pCRII vectors (Invitrogen, Carlsbad, CA), individual plasmid DNA mini preparations were assayed by restriction analysis and by agarose gel electrophoresis and selected clones were sequenced.

2.3 RNA interference

For RNA interference, selected regions of cDNA clones (excluding DNA binding domains) were used as templates. Primers used for amplification of selected regions are listed in Supplementary Table 2. PCR products were cloned to pCR4 and or pCRII vectors (Invitrogen, Carlsbad, CA) and sequenced. dsRNA was prepared by in vitro transcription using bacterial polymerases T7 or T3 or SP6 from linearized plasmids (Promega, Madison, WI). The resulting ssRNAs were checked by agarose electrophoresis for quality, annealed with an equal molar amount of the corresponding opposite ssRNA, and purified by phenol/ chloroform extraction and ethanol precipitation. Purified dsRNA was used for RNAi by microinjections to the gonad of young adult N2 worms at a concentration 2 mg/ml using a Narishige (Olympus, Tokyo, Japan) system coupled with the Olympus IX 70 (Olympus, Tokyo, Japan) inverted microscope.

Double stranded RNA was also used for induction of RNA interference using the soaking method at concentration 2 mg/ml. Hermaphrodites were soaked in dsRNA and transferred each 12 hours to new plates for total of 4 days period. The embryonic lethality and changes of larval phenotype were recorded at least twice a day on individual plates kept at 22°C. To check a possible non-specific effect of prepared dsRNA a control dsRNA, which was prepared from the (non-coding) promoter region of *nhr-60*, was used (Simeckova et al., 2007).

2.4 Analysis of the expression of clustered *nhr* genes in *C. elegans* and *C. briggsae* during development by RT-qPCR

RNA from individual developmental stages (embryos, L1, L2, L3, L4, and young adult) of *C. elegans* and *C. briggsae* was isolated and used in reverse transcription reactions as described above. The experiments were performed on ChromoIV instrument from Bio-Rad (Bio-Rad Laboratories, Hercules, Ca) and data were calculated by the Opticon monitor TM version 3 software. A single pair of primers for qPCR analysis of the developmental expression pattern were prepared from regions specific for individual genes as described in Supplementary Table 2. We used the DyNAmo™ SYBR® Green qPCR kit (New England Biolabs, Ipswich, Ma). Because this is an intercalating technique, which labels all amplicons present in a solution, we included Tm calling analyses to assure that we detected only one amplification product and standard curves were determined using a purified standard (prepared by PCR from a full length cDNA cloned in plasmid) to ensure linearity of amplification over the range of detected values. The following protocol was used: Pre-denaturation 95°C for 10 min, and 40 cycles consisting of denaturation at 94°C for 10 s, annealing at 58°C for 20 s and elongation step at 72°C for 20 s. Fluorescence signal was recorded at the end of a each 72°C step. Melting curves were determined with the following parameters: 95°C for 60 s, 40°C for 2 min. and continuous heating from 40°C to 95°C. The data and Cp values were exported and the copy number was calculated manually as described (Pfaffl, 2001).

2. 5 Analysis of the expression of clustered *nhrs* genes in *C. elegans* and *C. briggsae* during fasting and feeding by RT-qPCR

The fasting-feeding dependence of expression of studied genes was analyzed by two different primer sets for each studied gene in two different methods. For the 6 hr fasting protocol, all genes were analyzed using SYBR green technology and analyzed with a PTC 0200 DNA Engine R Thermal Cycler equipped with ALS 0296 96-well sample block (MJ Research, Watertown, MA, USA) and normalized against *ama-1*. Samples were also analyzed by the Universal Probe Library (Hoffman-La Roche, Basel, Switzerland) technique normalized against *ama-1* or *Cbr-ama-1*. Each method employed a different amplicon for each of the *nhr* genes assayed and primer sequences for each are listed in Supplementary Table 2. Both methods yielded nearly identical results demonstrating that the results were consistent for both amplicons tested for the *nhr* genes. Normalization against *ama-1* expression was chosen based on a preceding analysis in which we found in three independent experiments, analyzed by multiple measurements, that the expression of *ama-1* is not altered by fasting. The 54 hrs fasting was assayed by the Universal Probe Library (Hoffman-La Roche, Basel, Switzerland) technique only. All experiments were done in triplicate at a minimum.

The combination of software Probe Finder and 165 pre-validated probes enabled us to design intron-spanning amplicons for the assays. We used the protocols with the LightCycler TaqMan Master (Hoffman-La Roche, Basel, Switzerland) with internal reference gene amplifications. Specific primers for *ama-1* in *C. elegans* and *Cbr-ama-1* CBG05355 in *C. briggsae* served as a normalization control and fluorescent probes were

added following first strand cDNA synthesis. The following protocol was used: Pre-denaturation 95°C for 10 minutes and 45 cycles consisting of a denaturation step at 95°C for 15 s, annealing step at 60°C for 30 s and elongation step at 72°C for 1 s. The fluorescence was measured at the end of each 60°C step. Finally, the reactions were cooled for 15 s at 37°C. All UPL probes are detected using the 480–530 nm channel while reference genes were recorded using the 640 nm channel. All UPL experiments were performed on a LightCycler 1.2 instrument with the software LightCycler 4.1 (Hoffman-La Roche, Basel, Switzerland). As additional controls, probes and corresponding specific primers for *fat-7*, *nhr-49*, and *acs-2* were selected and expression of these genes determined at standard laboratory conditions and in fasting using the UPL strategy.

Laboratory conditions for starvation/fasting experiments were done as follows: Sychronized L1 larvae were prepared and kept in deionized water overnight (approximately 20 hr). Two different conditions of fasting were used. For short fasting, the larvae were divided to equal portions and seeded on agarose plates with or without bacteria. The larvae were kept on plates for 6 hr at 20°C. Larvae were harvested using repeated washes with deionized water and put to container were they were incubated for another 20 minutes on rocking plate at 20°C. Following this step, larvae were collected by centrifugation for 5 minutes at (200 ×g) at room temperature. Pelleted larvae were frozen at −80°C before further use. For long fasting, the synchronized larvae were kept after hatching in deionized water for 48 hr at 20°C and then treated as described for short fasting, resulting in a total time without food of 54 hours.

2.6 Microscopy

Fluorescence microscopy and Nomarski optics were done using an Olympus BX60 microscope equipped with DP30BW CD camera (Olympus, Tokyo, Japan). For densitometric analysis of expression of *gfp* fusion genes, the worms were started as small synchronized cultures and all worms that expressed any GFP in the particular culture were photographed at constant settings (objective 40×, exposure 100 ms or 50 in case *nhr-206*). The exposure time for each paired experiment was determined. For analysis of transgene expression of all analyzed lines, a minimum set of 20 photographs with a range of exposure times was prepared. The exposure was set to the highest possible exposure that resulted in images that showed less than 5% of area at saturation. The pictures were analyzed using the Image J program (Abramoff et al., 2004). Total areas of body containing the gut without the pharynx or pharyngeal areas were analyzed by densitometry after subtraction of the background that was calculated as a mean of three measurements taken outside the body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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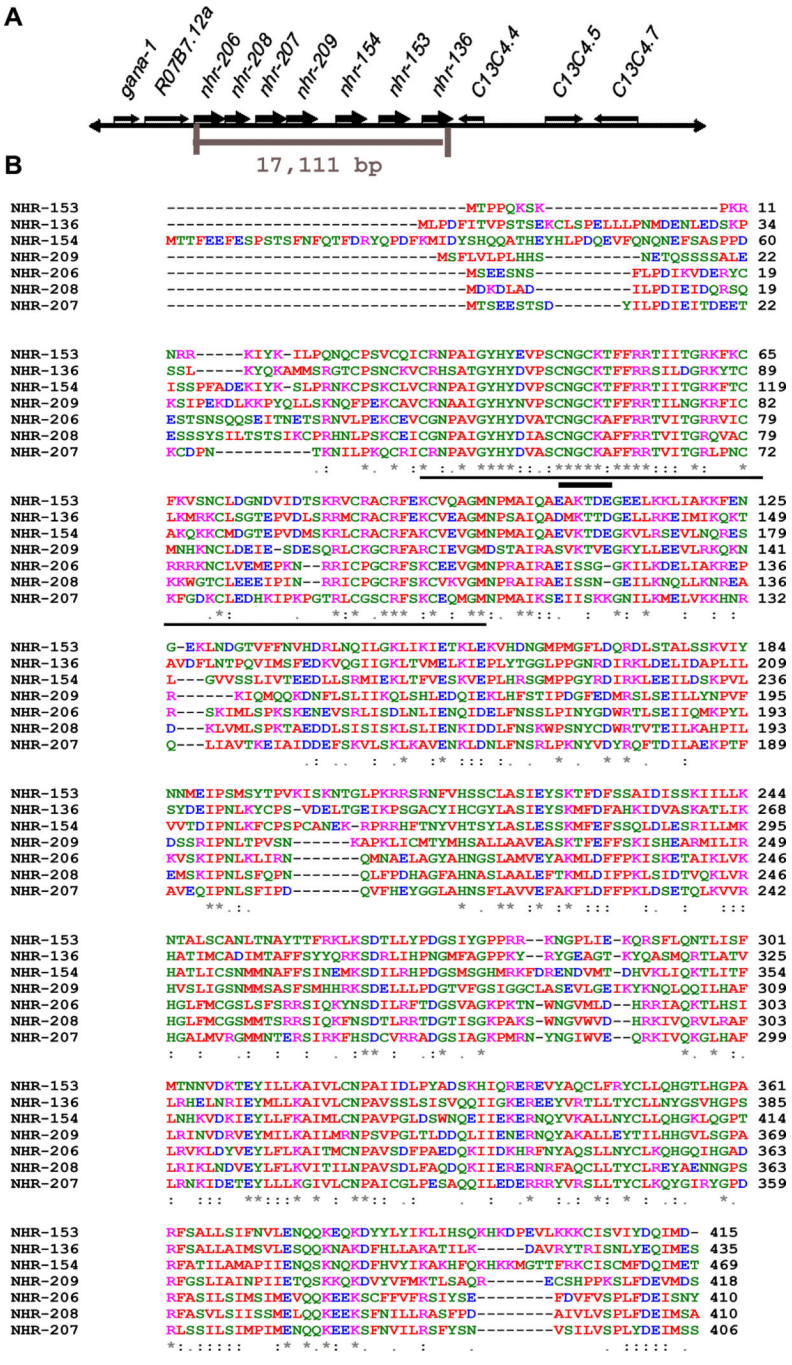

Figure 1. Genomic organization and sequence similarity of seven clustered NHRs

A) A schematic diagram of genes localized in a region of Chromosome V. A cluster of seven *nhr* genes (thick back arrows) spanning 17 kb are bracketed by the unrelated genes *R07B7.12a* at the 5' end *C13C4.4* at the 3' end.

B) The Clustal analysis of the amino acid sequences corresponding to the seven clustered nuclear hormone receptors: NHR-206, NHR-208, NHR-207, NHR-209, NHR-154, NHR-153 and NHR-136. The alignment shows regions of high sequence conservation and regions that have substantially diversified. The highly conserved DNA binding domain (DBD, thin underline) and the sub domain involved in the contact of nuclear hormone receptors with DNA response elements (P box, thick underline). The ligand binding domains

constituting the majority of approximate C- terminal halves of receptors show substantial diversification (visualized using standard color parameters, representing small and hydrophobic amino acids in red, acidic amino acids in blue, basic in magenta and hydroxyl- or amine- group containing amino acids in green color and the remaining amino acids in grey color).

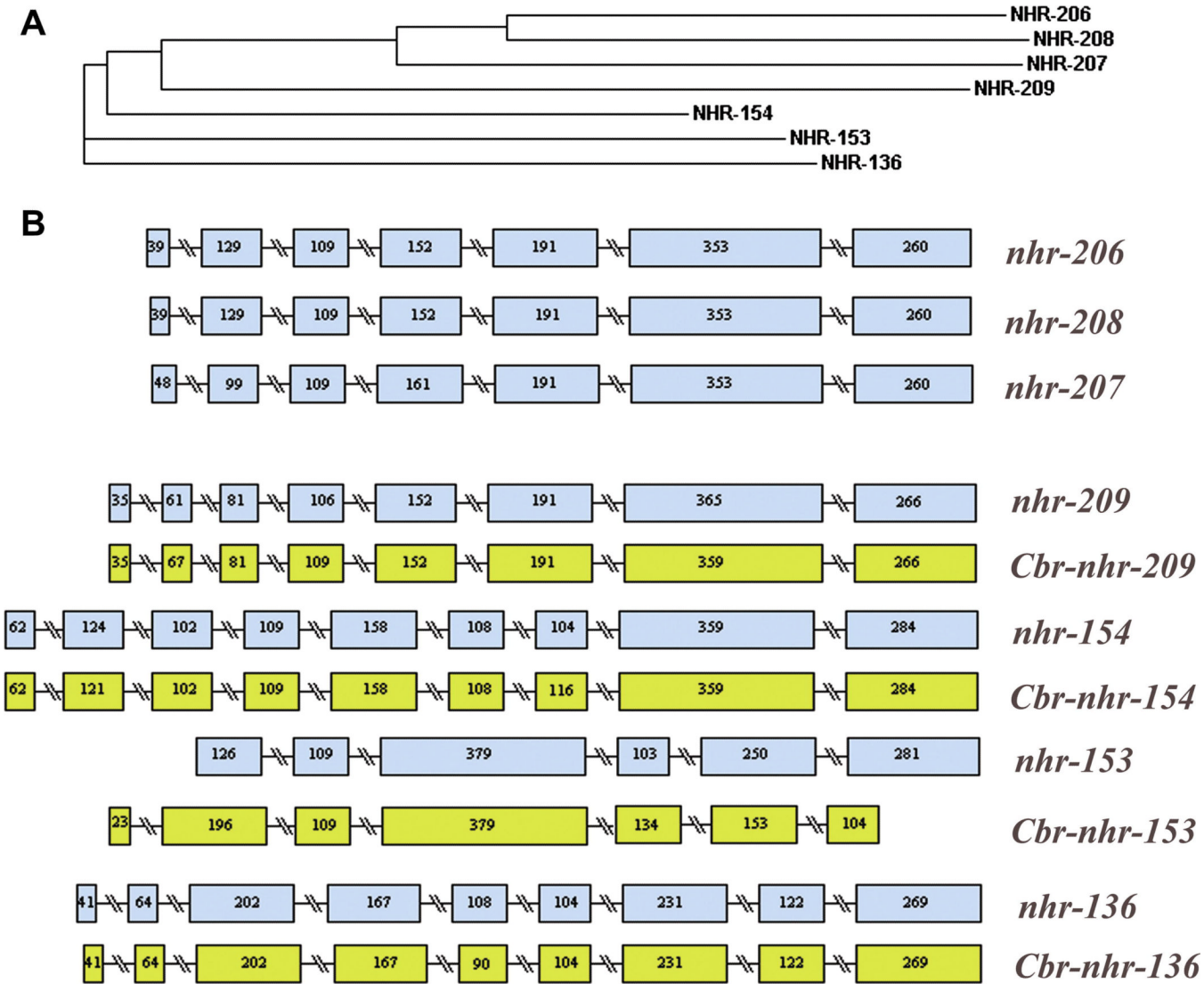

Figure 2. Evolutionary relatedness and intron-exon similarities among the seven clustered *nhrs*

A) The phylogram of the seven clustered NHRs, as calculated from protein sequences using the Clustal program, indicated that NHR-209, NHR-154, NHR-153 and NHR-136 group together while NHR-206, NHR-208 and NHR-207 represent more recent duplications and are most closely related to NHR-209.

B) The coding region organization of the seven clustered *C. elegans nhr* genes and their closest homologs in *C. briggsae*. The diagram shows differences between recently duplicated genes present only in *C. elegans* and genes conserved between *C. elegans* and *C. briggsae*. While the recently duplicated genes have almost identical exon sizes, the conserved genes show wider diversity of exon sizes between paralogs compared to interspecies orthologs.

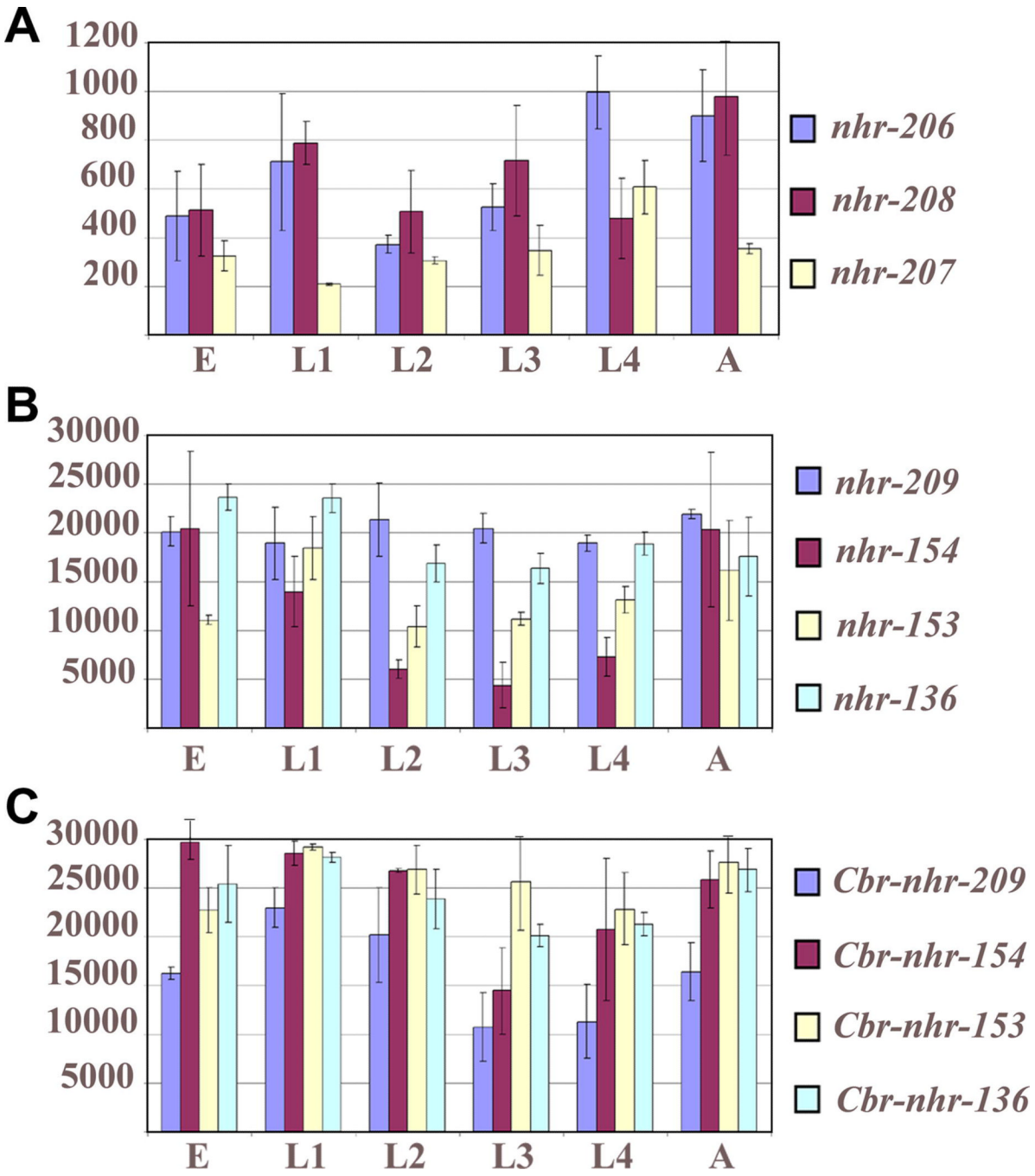

Figure 3. The developmental profile of clustered *nhr* gene expression

A & B) The expression of the seven clustered *C. elegans nhr* genes during development was analyzed by RT-qPCR revealing all are expressed under standard laboratory growth conditions. The recently duplicated genes within the cluster (*nhr-206*, *nhr-208* and *nhr-207*) are expressed at substantially lower levels than the genes that are conserved between *C. elegans* and *C. briggsae*. All genes, with the exception of *nhr-154*, are expressed at relatively constant levels throughout development in *C. elegans*. The expression of *nhr-154* decreases in mid-larval stages with the lowest levels detected at the L3 stage. C) Analysis of the expression of *C. briggsae nhr* cluster orthologs by RT-qPCR. The expression of *Cbr-*

nhr-209, Cbr-nhr-154, Cbr-nhr-153, Cbr-nhr-136 is relatively stable during development and shows levels comparable to their *C. elegans* orthologs.

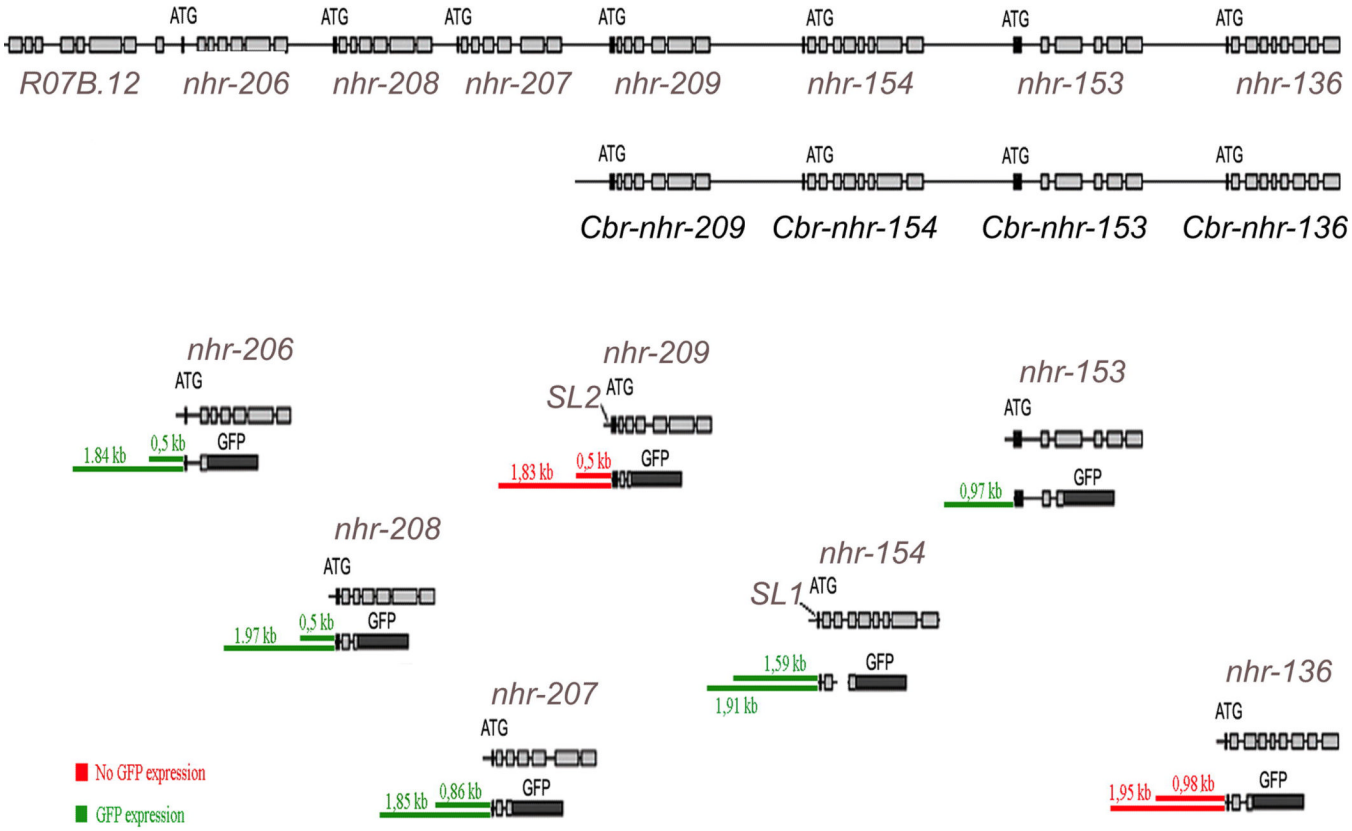

Figure 4. Promoter segments used for GFP reporter gene analysis

The upper part of the figure shows a schematic representation of the seven clustered nuclear hormone receptors in *C. elegans* (top) in comparison with the corresponding *C. briggsae* ortholog (bottom). The lower part of the figure shows a schematic representation of the genomic regions used as potential promoters in transgenic lines of *C. elegans* carrying *5* fusion reporter genes. The SL1 and SL2 splice leader sequences that were identified are indicated. Putative promoter segments that resulted in GFP expression are indicated by green lines while those yielding no reporter gene expression are indicated by red lines.

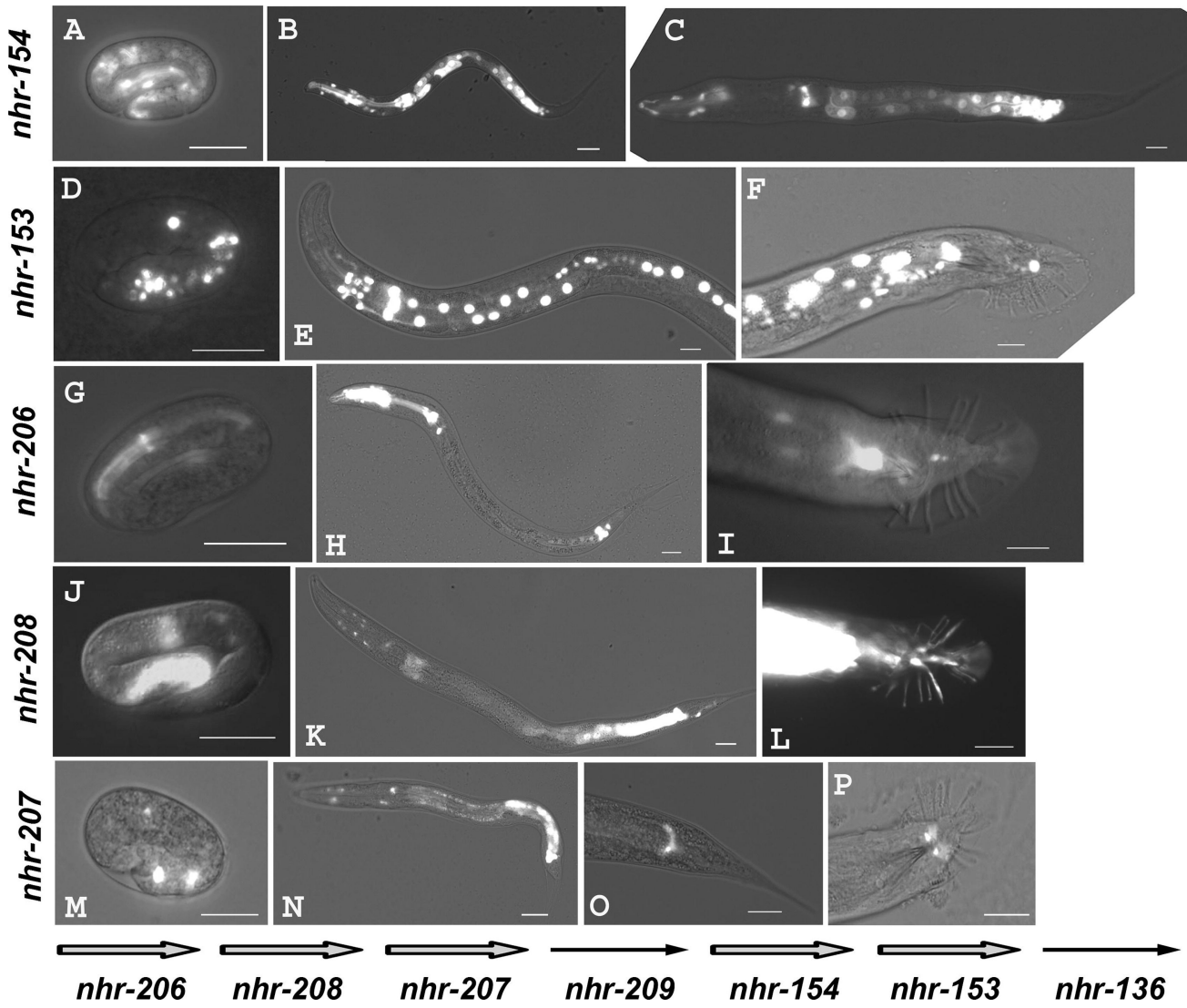

Figure 5. Examples of expression patterns for five of the seven clustered *nhrs* in *C. elegans* for which GFP reporter genes resulted in a signal in transgenic lines

A–C) *nhr-154* reporter expression in a 3-fold embryo, L1, and L2 larvae, respectively. Strong expression was observed in pharyngeal and intestinal cells.

D–F) *nhr-153* reporter expression in a 2-fold embryo, L4, and adult male tail, respectively. Pharyngeal and intestinal cell expression was strong throughout development with additional expression observed in the vulval muscles in the L4 hermaphrodite, male tail specific neurons and rays.

G–I) *nhr-206* reporter expression in a 3-fold embryo, L1, and adult male tail (ventral view), respectively. Strong expression in the pharyngeal cells with weak intestinal cell expression is seen throughout development. By the L1 stage, expression is also detected in head neurons, the sphincter, and rectal gland cells. Reporter gene expression was also detected in male specific neurons and rays.

J–L) *nhr-208* reporter expression in a 3-fold embryo, L2, and adult male tail (ventral view), respectively. Strong embryonic expression was seen in the pharyngeal cells with subsequent intestinal expression in larvae that was particularly pronounced in the posterior. Reporter gene expression was also observed in male specific neurons and ray (6–8).

M–P) *nhr-208* reporter expression in a 1.5-fold embryo, L1, L2, and adult male tail (ventral view), respectively. Expression was seen in the intestinal sphincter and posterior epidermal cells during embryogenesis. By L1, unidentified head neurons and strong posterior intestinal expression were observed. Expression was also seen in the intestinal-rectal valve cell (O) and in male specific neurons and rays. Scale: 20 μm.

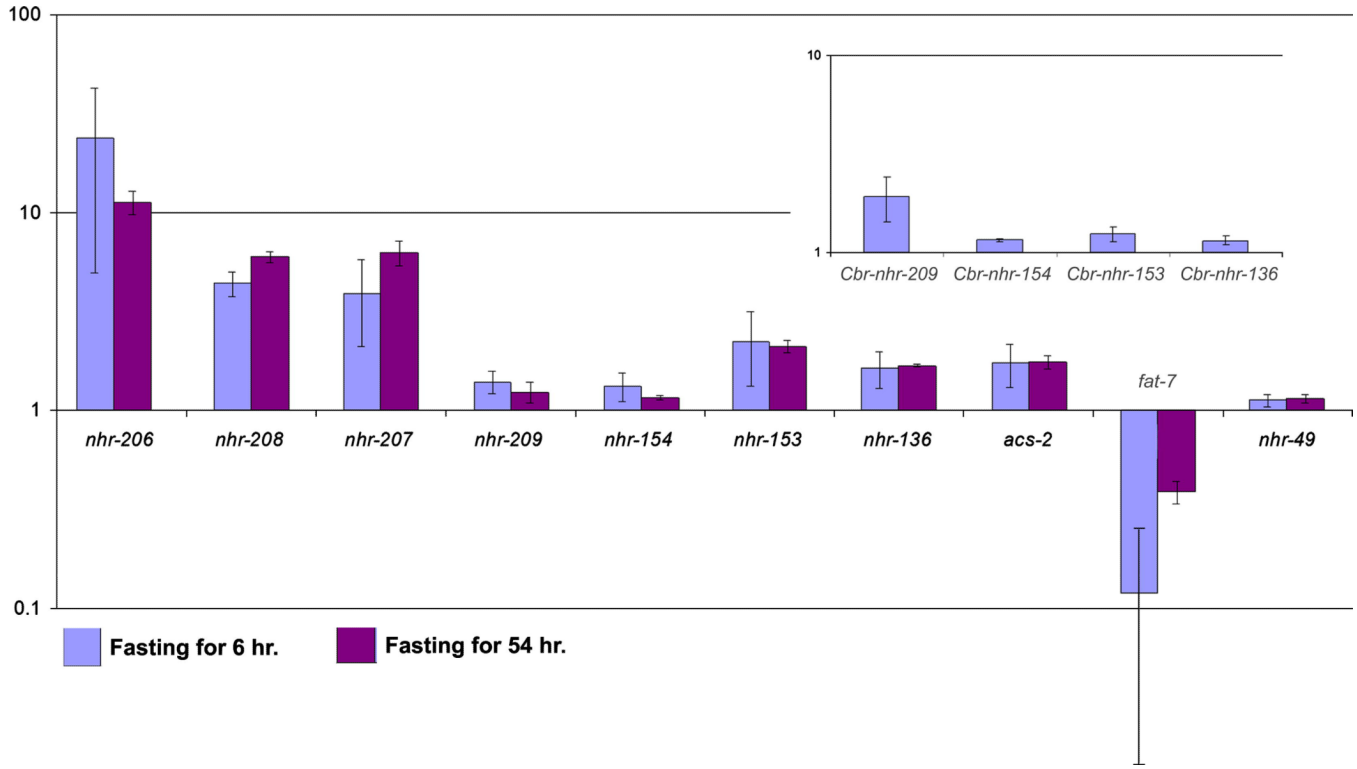

Figure 6. The expression of seven clustered *C. elegans nhrs* during fasting was analyzed by RT-qPCR

Expression of the recently duplicated *C. elegans* genes *nhr-206, nhr-208 and nhr-207* is strongly induced in animals after either 6 hours (grey columns) or 54 hours (dark columns) of fasting. The results are shown in logarithmic scale where a value of one is equal to unchanged expression compared to fed control animals. Values greater than one indicate up-regulation and levels less than one represent down-regulation during fasting conditions. The expression of genes reported previously to be affected by fasting (Van Gilst et al., 2005b), *acs-2* (increased), *fat-7* (decreased) and *nhr-49* (unchanged) were included as additional controls for these experiments. Inset: The expression of *C. briggsae* homologues *Cbr-nhr-209*, *Cbr-nhr-154*, *Cbr-nhr-153* and *Cbr-nhr-136* was analyzed by RT-qPCR in animals after 6 hours of fasting. Only *Cbr-nhr-209* showed an increase of expression (2-fold) during fasting.

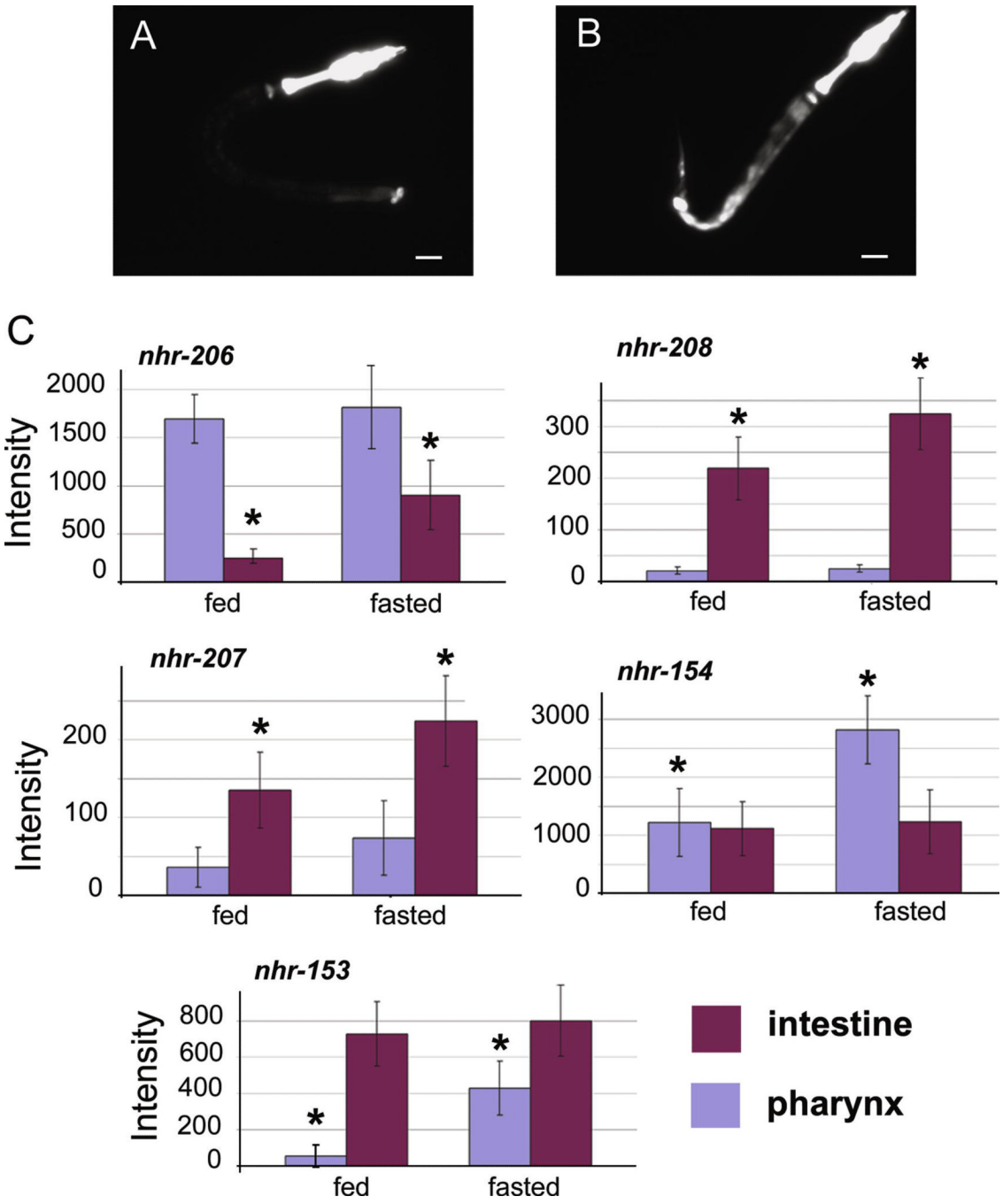

Figure 7. Responses of GFP reporter genes to fasting and feeding conditions

Changes in reporter gene expression in response to feeding (A) or fasting (B) could be easily visualized, as seen in this representative image for *nhr-206::gfp*. The change in GFP expression in either the pharynx or intestine was quantified by densitometric analysis during fed or fasting conditions for the reporters *nhr-206::gfp*, *nhr-208::gfp*, *nhr-207::gfp*, *nhr-154::gfp*, *nhr-153::gfp*. Calculations were based on capturing the anterior area of animals coving the pharynx (gray columns) or the whole body and intestine, excluding the pharynx (dark columns); note that the intensity scale was adjusted for each reporter gene. The data revealed that *nhr-206*, *nhr-208*, and *nhr-207* reporters each showed significant (2- to 4-fold), intestinal-specific up-regulation of expression in response to fasting. In contrast,

nhr-154 and *nhr-153* reporters showed significant (2- to 8-fold), pharyngeal-specific increases in expression in response to fasting. Standard deviations are indicated in each column. An asterisk (*) indicates the probability of the result in the Student T test at $P<0.05$ compared with null hypothesis. Scale: 20 μm.

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Table 1

Summarized results of reporter gene expression for *C. elegans* clustered *nhrs*. The intensity of expression was scored from low (+) to high (+++) in each cell or tissue type. Absence of expression is indicated (−). If specific developmental stages are not reported, expression was observed in all larval stages and in adults.

Reporter Gene	***nhr-206***	***nhr-208***	***nhr-207***	***nhr-209***	***nhr-154***	***nhr-153***	***nhr-136***
Start of emb. expression	Comma	1.5-fold	1.5-fold	−	2-fold	2-fold	−
Phar. Muscles	+++	−	+	−	+++	−	−
Head Neurons	+++	+++	++	−	++	+++	−
Phar. – Intest. Valve	−	−	+++	−	−	−	−
Intestine	+	+	+	−	+++ L1, L2, L3	+++	−
Int.- Rectal Valve	−	+	++	−	+	+	−
Rectal Glands	+++	+++	+	−	+	+	−
Int. Sphincter	+	+	+	−	+	−	−
Tail Neurons	−	+	−	−	−	+	−
Epidermal cells in head	++	+	−	−	+	−	−
Epidermal cells in tail	++	++	−	−	−	+	−
Spermatheca	+++	−	+++	−	−	−	−
Vulva	++	−	++	−	−	−	−
Rays	+ R6	+ R6, 7, 8	+ R6	−	−	−	−
Ray Neurons	+	+	+	−	−	+	−
Male Specific Neurons	++	−	++	−	−	−	−