Multiple losses of ecdysone receptor genes in nematodes: an alternative evolutionary scenario of molting regulation

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Abstract

Molting is a hallmark feature of ecdysozoans, including arthropods, tardigrades, and nematodes. Ecdysone hormones play a crucial role in regulating the molting process of different ecdysozoan taxa. Interestingly, despite this highly conserved function of ecdysone, the model nematode *Caenorhabditis elegans* has lost the ecdysone receptor (ECR) genes and their molting appears to be ecdysone-independent. The loss of *ecr* has only been reported in *Caenorhabditis* within ecdysozoans, and the evolutionary background behind this loss has remained enigmatic. Here, we show that loss of *ecr* is not exceptional in *Caenorhabditis*, but has occurred at least three times in Rhabditina and Tylenchina nematodes. Our genome-wide analysis of 160 nematode species revealed the multiple losses of *ecr* and its typical heterodimer partner *usp* during nematode evolution. Furthermore, using genomic and transcriptomic analyses, we identified two factors that likely underlie and buffer the loss of ECR gene/function: molting regulation by an alternative nuclear receptor HR3 (NHR-23) and a lineage-specific expansion of nuclear receptors in the *ecr*-deficient taxa. Taken together, it is suggested that ecdysone/ECR function in nematodes is only minor or redundant. We propose a novel scenario for the evolution of molting regulation, highlighting the importance of HR3 as the potential core regulator.

Introduction

Ecdysone hormone is a key regulator governing various developmental processes, such as molting and reproduction, in Ecdysozoa including arthropods, tardigrades, and nematodes[1-5]. Interestingly, however, the model nematode *Caenorhabditis elegans* is known to lack any genes coding the ecdysone hormone receptor (ECR)[6,7], which is so far the only reported case among ecdysozoans (see also Fig. S1). Molting of *C. elegans* must be therefore independent of ecdysone along with loss of *ecr*[8], and this contrasts with the involvement of ecdysone/ECR in the molting regulation of some parasitic nematodes[3-5]. The loss of *ecr in C. elegans* also seems to contradict the conserved stereotypic molting in nematodes, in which all species consistently molt four times regardless of the divergence in the life cycles[9]. Thus, it is generally considered that the loss of *ecr* in *C. elegans* is an extremely specific and rare event[7], and elucidating the evolutionary background of *ecr* loss has been a long-standing challenge.

Result and Discussion

Motivated by the insufficient exploration of the specificity of *ecr* loss within a phylogenetic framework, we first searched for *ecr* genes in 160 nematode species including major nematode taxa (Fig. 1A, 159 genomic and 1 transcriptomic data, see also the methods). Our search identified *ecr* genes from all major nematode taxa, including the sister group of all remining nematodes, Enoplida (Fig. 1A). Nevertheless, not only in *C. elegans*, but in all *Caenorhabditis* species examined, *ecr* was not found (Fig. 1A). Considering that *ecr* is present in all other Rhabditina species (Fig. 1A), the loss of *ecr* is specific to the *Caenorhabditis* lineage. Unexpectedly, *ecr* is also absent in 26 of 41 Tylenchina species examined (Fig. 1A). Based on the currently supported phylogeny[10,11], the loss of *ecr* is indicated as occurring twice in Tylenchina (Fig. 1A). Together, the loss of *ecr* occured at least three times during nematode evolution.

C. elegans is also known to lack the gene encoding USP[7], a typical heterodimer partner of ECR. Our gene mining revealed that *usp* is also absent in several lineages of Rhabditina and Tylenchina (Fig. 1A). Especially, the loss of *usp* is detected not only in the species lacking *ecr* but more broadly observed as no *usp* was identified in some Rhabditina species having *ecr* (ex., Strongylida: Fig. 1A). The loss of *usp* also precedes the loss of *ecr* in Tylenchina as only Steinernematidae have both *ecr* and *usp* (Fig. 1A). The correlation of the loss of *ecr* and *usp* suggests that the ECR-USP functionality has been lost several times in nematodes. It should

also be noted that a correlation of *ecr* and *usp* losses with specific lifestyle/habitat is not detected (Fig. 1A).

Given that the ecdysone hormone is reportedly involved in the regulation of molting in some nematodes such as Spirurina parasitic nematodes[3-5], it is likely that the function of ecdysone as a molting trigger is specifically lost in Rhabditina and Tylenchina. To test this hypothesis, we examined the temporal expression of *ecr* during the molting cycles of four representative nematode species (Fig. 1C, D, F, and G, Rhabditina: *Pristionchus pacificus*, *Ancylostoma ceylanicum*, Spirurina: *Brugia malayi*, and Trichinellida: *Trichuris suis*), for which transcriptome data during the molting cycle are publicly available (see methods). In *T. suis*, *ecr* expression showed a sharp peak pattern before L3-L4 and L4-adult molting (Fig. 1G). Similar (but more moderate) peaked expression was observed in L3-L4 molting of *B. malayi* (Fig. 1F), and these expression patterns are consistent with the molting trigger function of ecdysone/ECR. On the other hand, although a pulsed expression of *ecr* was detected in L2-L3 and L3-L4 molting of *P. pacificus*, the expression peaks did not precede a start of molting but showed at the same time (or later) as ecdysis, a final step of molting (Fig. 1D, see also the method and Fig. S3). Furthermore, the expression of *ecr* in *A. ceylanisum* which lacks *usp* was comparatively minorly increased during L3-L4 molting (Fig. 1C). In summary, it is suggested that the molting trigger function of ECR is lost in a stepwise manner in parallel with the loss of *ecr* and *usp*.

Regardless of the divergent molting function of ECR, nematodes consistently molt four times in all nematodes[9], suggesting the presence of other key regulators. In this context, the nuclear receptor HR3 (NHR-23 or ROR) is interesting because it plays a critical role in the molting regulation in *C. elegans*[8]. Indeed, our survey revealed that HR3 is highly conserved across nematode lineages (Fig. 1A). We then examined the expression pattern of *hr3* during the molting cycle in six species (Fig. 1B–G, the above four species, *ecr*-lacking Rhabditina *C. elegans*, and *ecr*-lacking Tylenchina *Globodera pallida*). Notably, pulsed expression of *hr3* prior to molting was observed in all six species (Fig. 1B–G). For example, *hr3* expression shows a peaked pattern prior to molting in *A. ceylanisum* which does not show peaked *ecr* expression, and *G. pallida* which lacks *ecr* (Fig. 1C, E, respectively). We also systematically screened and compared the transcriptome data of the six species using fuzzy c-mean clustering and OrthoFinder (see Methods, Fig. S4); conservation of such peaked expression among six species was not found in any other NRs. This supports that HR3 has an evolutionarily

conserved function in the regulation of molting in nematodes, which likely allows to buffer multiple losses of the *ecr* gene and its function.

Since pleiotropic functions of the ecdysone hormone are suggested in nematodes (i.e., reproduction)[3], different genes may also take over the ECR function (which may include molting trigger) in *ecr*-lacking species. In this context, it is interesting that Rhabditina and Tylenchina species extensively expanded nuclear receptor (NR) genes (HNF4-subtype)[12]. Plotting the number of NRs in the phylogenetic tree shows an evolutionary tendency to increase NR number (Fig. 1A). We then extracted orthogroups (OGs) of NRs from represented 11 species (1–3 species from each Rhabditina, Tylenchina, Spirurina, Trichinellida, and Enoplida: Fig. 1A, H). This analysis showed that the common ancestor of nematodes has 12 OGs, and NRs increased to 34 OGs in the ancestor of Rhabditida (Figs 1H, S5: Spirurina/Rhabditina/Tylenchina). Finally, the ancestor of Rhabditina/Tylenchina acquired additional 22 OGs (total 56 OGs), and Rhabditina and Tylenchina ancestrally had 136 and 75 OGs, respectively (Figs 1H, S5). Some nematodes such as *Plectus sambesii* also independently increased NRs (Fig. 1A), while *ecr* is not missing. Thus, although expansions of NRs did not strictly correlate with the loss of *ecr* in some lineages, it is plausible that Rhabditina and Tylenchina are unique to have abundant "resource" to take over ECR function (this may also underlie the loss of the USP gene/function).

We propose that the loss of ECR genes/functions multiply occurred in Rhabditina and Tylenchina buffered by conserved HR3 molting regulatory function and lineage-specific expanded NRs (Fig. 1I). This opens the hypothesis of a dominant role for HR3 as an ancestral and conserved molting regulator in nematodes and possibly even ecdysozoans (note that HR3 is also a core molting regulator in arthropods[2] and putatively in tardigrades[1]). Considering that HR3 orthologues ROR and NHR-23 are known to be involved in regulation of circadian clocks among bilaterians[13,14], the ancestry of HR3 shows a correlation with the origin of molting regulation, which control biological and developmental time. Finally, our results provide the possible use of HR3 regulation of molting for medical applications and pest control, in plant-parasitic and some human-parasitic nematodes, which have lost ECR (Fig. 1A: Tylenchomorpha and Strongylida, respectively).

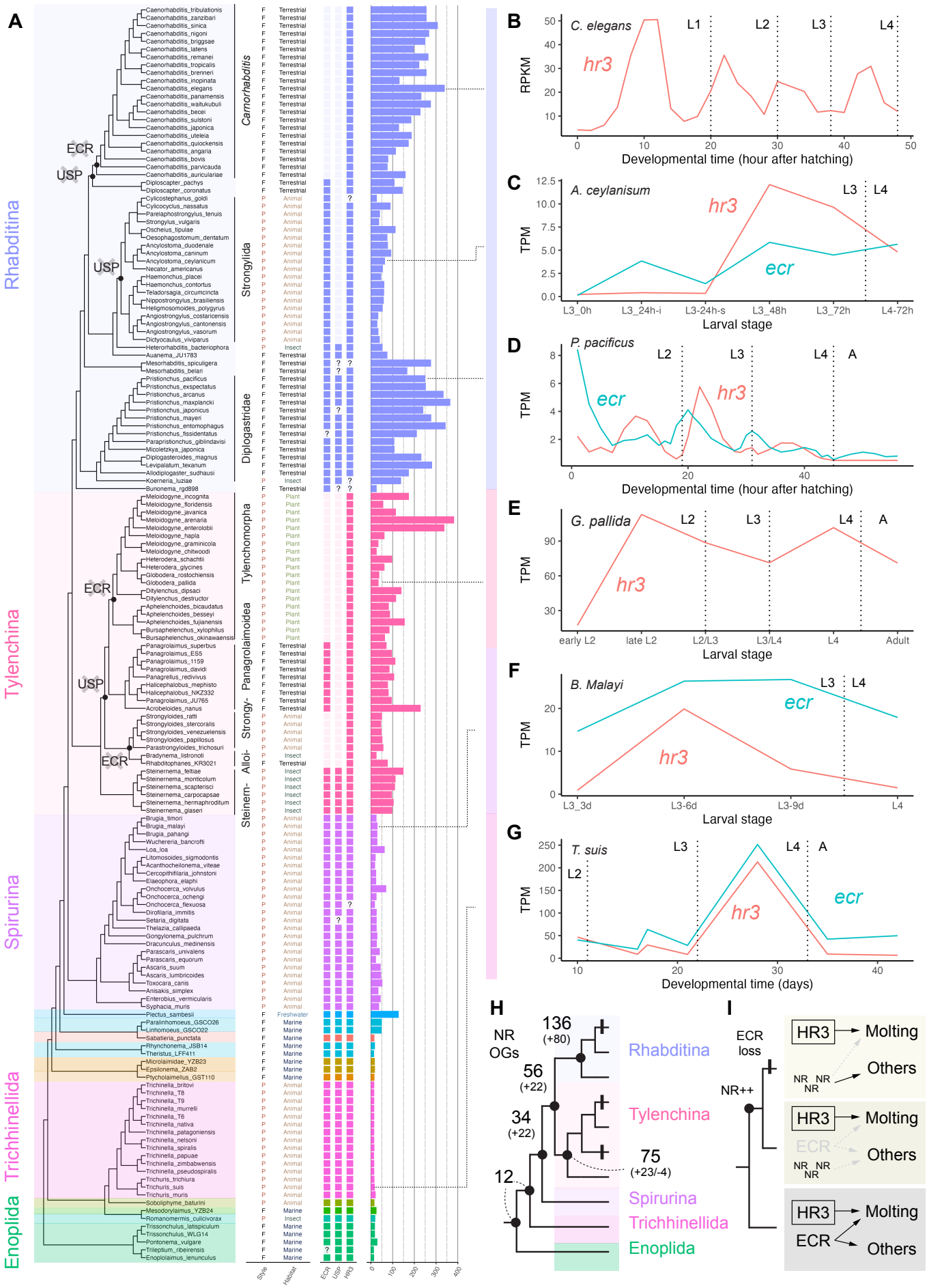
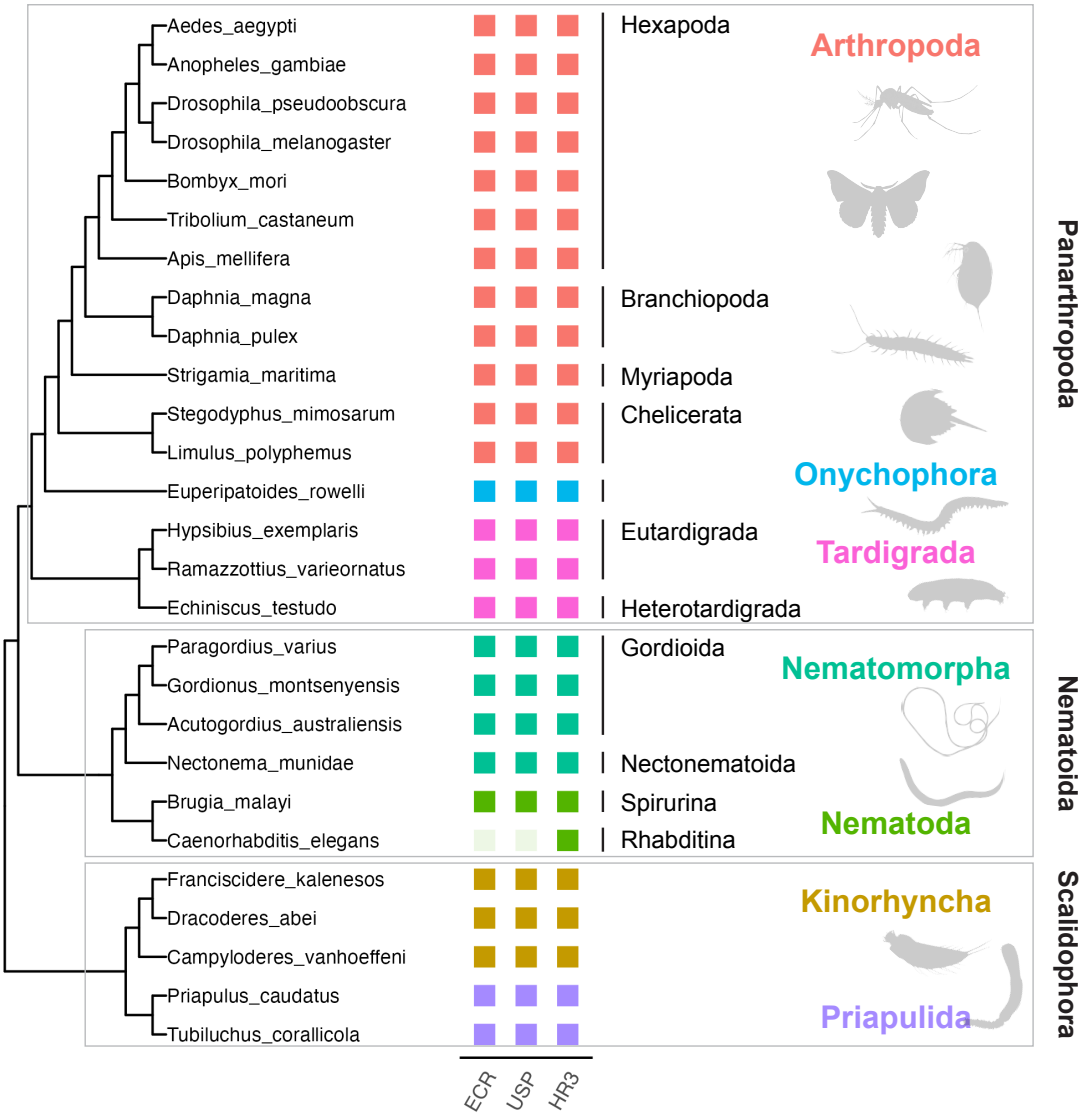

A
Rhabditina
Tylenchina
Spirurina
Trichinellida
Enoplida
ECR
USP
Carnorhabditis
Strongylida
Diplogastridae
Tylenchomorpha
Panagrolaimoidea
Strongy-
Alloi-
Steinern-
Style
Habitat
ECR
USP
HR3
B
C. elegans
hr3
L1
L2
L3
L4
RPKM
Developmental time (hour after hatching)
C
A. ceylanisum
hr3
ecr
L3
L4
TPM
L3_0h
L3_24h-i
L3-24h-s
L3_48h
L3_72h
L4-72h
Larval stage
D
P. pacificus
ecr
hr3
L2
L3
L4
A
TPM
Developmental time (hour after hatching)
E
G. pallida
hr3
L2
L3
L4
A
TPM
early L2
late L2
L2/L3
L3/L4
L4
Adult
Larval stage
F
B. Malayi
ecr
hr3
L3
L4
TPM
L3_3d
L3-6d
L3-9d
L4
Larval stage
G
T. suis
L2
L3
L4
A
ecr
hr3
TPM
Developmental time (days)
H
NR OGs
136 (+80)
56 (+22)
34 (+22)
12
75 (+23/-4)
Rhabditina
Tylenchina
Spirurina
Trichhinellida
Enoplida
I
ECR loss
NR++
HR3
Molting
Others
NR NR NR
ECR

Fig. 1: Summary of gene identifications, expression analysis, and putative evolutionary scenario

A: Lifestyle (P: parasite and F: free-living), habitat, gene repertories (*ecr*, *usp*, and *hr3*), and number of nuclear receptors (NRs) are indicated with the phylogeny of the target nematode species (color represents the taxa [Fig. S2], question marks represent missing information, see the Methods). Abbreviation: Storngy-/Strongyloididae, Alloi-/Alloionematidae, and Stein-: Steinernematidae. The nodes of ECR and USP loss events are highlighted in the tree. **B**–**G**: Temporal expression pattern of *ecr* (blue) and *hr3* (red) in the six nematode species. The timing of molting (or ecdysis) is indicated by the dotted lines in each panel (L1–L4: first to fourth larval stages and A: juveniles/adults). **H**: Estimated number of orthogroups (OGs) of NRs at each node of the represented nematode taxa. The numbers in the brackets indicate the number of NRs gained or lost at the node. **I**: Hypothetical evolution of molting regulation (top: ECR-lacking group [ex. *Caenorhabditis*], middle: the group showing no ECR function in molting trigger [ex. *Ancylostoma*], bottom: the group having ECR and USP [ex. *Trichuris*]). HR3 may function as a "core" regulator of molting (squared) in all groups, and ECR/HR3 may ancestrally co-function to regulate molting and other functions. After expansion of NRs (NR++), ECR function may be taken over by other NRs in a stepwise manner (gray arrows), resulting in loss of ECR.

141 **Supplementary figures**



142 **Fig. S1. Presence of *ecr*, *usp*, and *hr3* in ecdysozoans**

143 Phylogeny of Panarthropoda, Nematoida, and Scalidophora is based on Howard et al., 2022[15].
144 The presence of *ecr*, *usp*, and *hr3* was indicated in the boxes next to each species name. Gene
145 identification was referred to the previous annotation and this study (see Methods)[6,16].

Fig. S2. Representative taxonomic color in Fig. 1

Each color represents the nematode taxa which were used in Fig. 1A.

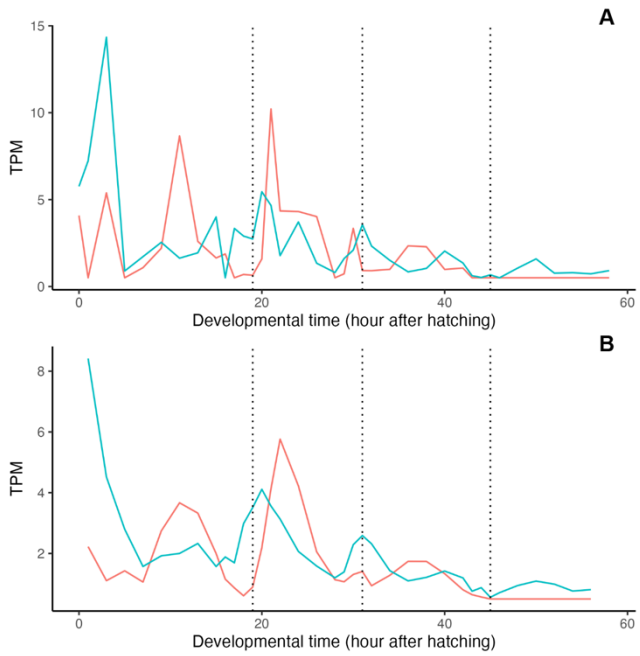


Fig. S3. Comparison of original and corrected expression pattern of *ecr* and *hr3* in *P. pacificus*

A: Original expression profiles of *ecr* (blue) and *hr3* (red) from Sun et al., 2021[17]. B: corrected expression profiles of *ecr* and *hr3* by calculating the average expression levels (TPM) between the three time point windows from Sun *et al.*, 2021 (see Methods)[17].

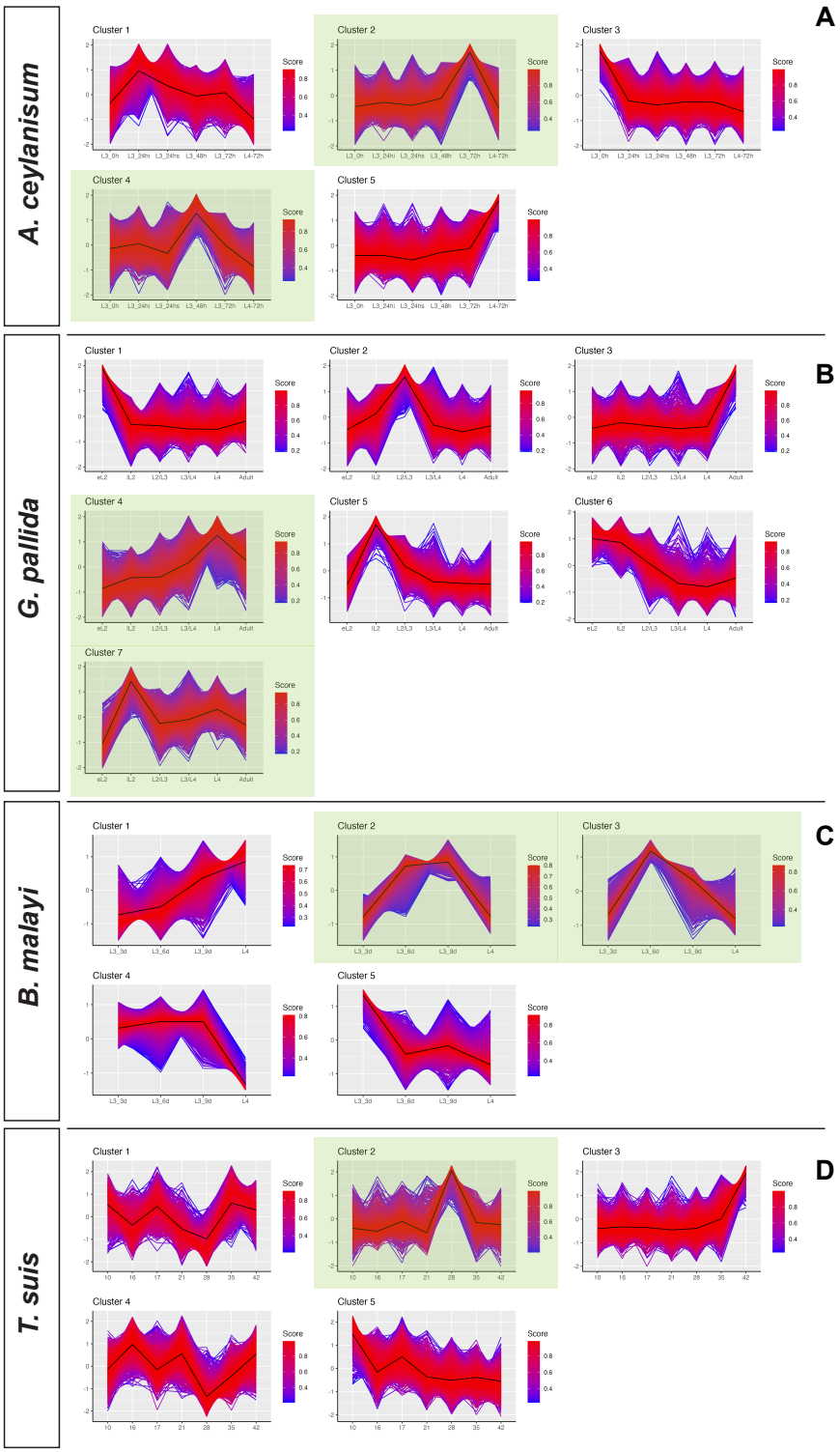


Fig. S4. RNA-seq clustering of *A. ceylanisum*, *G. pallida*, *B. Malayi*, and *T. suis*

A-D: Fuzzy c-mean clustering of each transcriptome data set (*A. ceylanisum*, *G. pallida*, *B. Malayi*, and *T. suis*, respectively). Green shades indicate the clusters showing correlated expression with molting cycle.

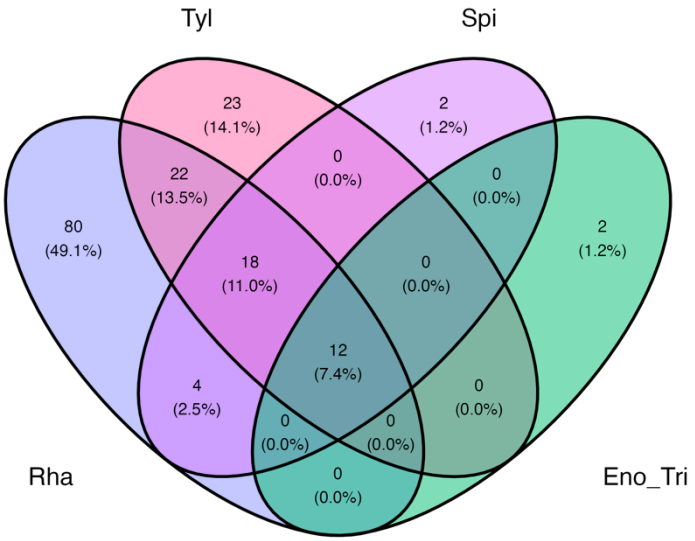


Fig. 5. Venn diagram of orthogroups of nuclear receptors in the representative nematode groups

Rha: Rhabditina, Tyl: Tylenchina, Spi: Spirurina, Eno_Tri: Enoplida and Trichinellida.

173 **Supplementary dataset**

174 Maximum-likelihood phylogenic trees for the identification of ECR, USP, and HR3

175 Sequences of the genes identified

176 Sequences of the genes which were used for the phylogenic analysis

177 Sequences of NRs

178 The list of the genes and their accession numbers

179 OrthoFinder output file/folder (NRs / clustering)

180 Results of clustering

181 Scripts for the analysis and visualization

Methods

Data collection and target species

We obtained protein sequences of 159 nematode species from WormBase Parasite (version: WBPS19, WS291)[18]. These sequences/protein models were reconstructed from the genomic data. All accession numbers of the original dataset are available in the supplementary dataset. In this study, the gene ID was referenced to the original dataset, while also incorporating some modifications. For example, species identifiers were added to the beginning of the gene ID (i.e. *GeneID* → *NEMA_CaenoElega_GeneID*). Some characters, such as curly brackets, were also removed for later analysis. In addition, the transcriptome data of the Enoplida nematode *Pontonema vulgare* was obtained from NCBI (accession number: PRJNA504396). Quality of raw reads were examined using FastQC (version: v0.11.9; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The subsequent filtering was performed using trimmomatic (version: 0.39)[19], and *de novo* assembly was conducted using Trinity (version: v2.13.2)[20]. Transdecoder (version: 5.5.0; https://github.com/TransDecoder/TransDecoder) was used to obtain the peptide sequences of *P. vulgare* transcript models. The assembled sequences are available in the supplementary dataset. Phylogenic relationship of the target species was referred from previous works[10,11].

Gene identification

Screening

Gene mining of *ecr*, *usp,* and *hr3* was conducted based on the sequence similarity by combination of BLAST search and phylogenetic analysis with the following screening. All sequences and phylogenetic trees are available in the supplementary datasets.

We first performed a BLAST search using the query sequences of ECR, USP, and HR3 from *Drosophila melanogaster* against the database of each nematode species. The query sequences are from UniProt (ECR: P34021, USP: P20153, and HR3: P31396). The peptides showing the hit with less than 1E-20 e-value were extracted and used as query sequences for the second BLAST against the *D. melanogaster* database. This database was build using the CDS sequences from FlyBase (dmel-all-CDS-r6.45.fasta)[21]. Finally, the peptides showing the reciprocal top hit with less than 1E-10 e-value were extracted. Each candidate gene set of ECR, USP, and HR3 (181, 256, and 342 peptides, respectively) was aligned with other known nuclear

213 receptors (NRs) from different animals (the sequences were previously used to identify NR
214 genes[1,22], see also the supplementary dataset) using MAFFT (version: v7.526, --auto option)[23],
215 and the alignments were filtered using trimAl (version: 1.4.1, threshold: 0.8)[24]. Phylogenetic
216 analysis of each data set was performed using RAxML (version: 8.2.12, bootstrap: 1,000
217 generations, raxmlHPC-PTHREADS-SSE3 -f a -x 12345 -p 12345 -# 1000 -m
218 PROTGAMMAAUTO —-auto-prot=aic -s *input_file* -n *output_file*)[25]. Gene identity was
219 determined by examining, whether the candidate genes were included in the clade of target
220 genes with high bootstrap values. By removing the genes that did not belong to the target clade,
221 we finally identified 128, 74, and 276 peptide models as ECR, USP, and HR3, respectively (see
222 the trees in the supplementary dataset). Note that some species have multiple copies.

223 Next, the above identified nematode ECR, USP, and HR3 were used as query sequences,
224 and a BLAST search was performed against the database of each nematode species in which
225 the target genes were not found in the first screening. Top-hit sequences with less than 1E-20
226 e-values were collected, and each sequence was used as a query for the second BLAST search
227 against the database of the original species from which the first query originated. Then, genes
228 showing the reciprocal one-to-one top-hit were finally accumulated as candidates (ECR: 197,
229 USP: 277, and HR3: 28 peptides). After selecting the peptides having DNA binding domain of
230 NRs (HMMER domain search [version 3.4][26], see the below for the details) and manually
231 checking the accuracy of the gene domain (e.g., NCBI conserved domain search:
232 https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), we finally investigated the identity of
233 the total 277 peptides by phylogenetic analysis as mentioned above. In this analysis, 5, 21, and
234 1 peptide(s) were additionally identified as ECR, USP, and HR3, respectively. Finally, a total
235 of 27 peptides obtained in the 2nd screening were used for the BLAST query sequences, and
236 we repeated the BLAST analysis as performed in the 2nd screening. However, no genes were
237 newly identified as ECR, USP, and HR3.

238 Target genes were also searched by BLAST, Hmmer domain search (screening of the
239 genes having DNA or ligand binding domain of NRs, see also the below), and phylogenetic
240 analysis. For example, the BLAST search was performed with a higher e-value threshold (ex.
241 1E-05). Different database was also explored for the species missing the target genes, and ECR
242 of *Steinema feltiae* was identified using the other dataset in WormBase Parasite (version:
243 WBPS19, WS291) from the one which we originally used (accession number: PRJNA204661).
244 Finally, eight additional genes (nine peptides) were identified to compensate for the missing
245 target genes, and the results are summarized in Fig. 1.

Criteria for the presence and absence of target genes

The summary of the screening and gene identification of 160 species is shown in Fig. 1A. In some species, the target genes were missing even though the genes were found in species of the same genus (or closely related species). Since this study avoided to distinguish between species-specific loss and technical errors such as low data quality, gene missing in these species were considered as missing information in Fig. 1A (10 species in total, question marks in Fig. 1A). We considered the loss to be reliable if the loss of the genes was observed in all the same genus or closely related species. According to these criteria, we detected loss of *ecr* only in *Caenorhabditis*, Tylenchomorpha, Strongyloididae and Alloionematidae (Fig. 1A). Loss of *usp* was also reliable in Strongylida, Anarhabditis, non-Steinernematidae Tylenchina (Fig. 1A).

Gene identification in other ecdysozoans

Previous works identified *ecr*, *usp* and *hr3* in insects (*Drosophila melanogaster*, *Drosophila pseudoobscura*, *Anopheles gambiae*, *Aedes aegypti*, *Bombyx mori*, *Tribolium castaneum*, and *Apis mellifera*), other arthropods (Myriapoda: *Strigamia maritima*; Chelicerata: *Limulus polyphemus* and *Stegodyphus mimosarum*; and Branchiopoda: *Daphnia pulex* and *Daphnia magna*), tardigrades (*Ramazzottius varieornatus* and *Hypsibius exemplaris*), onychophorans (*Euperipatoides rowelli*), nematodes (*Brugia malayi*, *Caenorhabditis elegans* [only *hr3*]), and priapulids (*Priaplus caudutus*)[6,16]. In addition, we newly identified *ecr*, *usp*, and *hr3* in tardigrades (*Echiniscus testudo*), nematomorphs (*Acutogordius australiensis*, *Gordionus montsenyensis*, *Nectonema munidae*, *Paragordius varius*), kinorhynchs (*Campyloderes vanhoeffeni*, *Franciscidere kalenesos*, *Dracoderes abei*) and priapulids (*Tubiluchus corallicola*). For gene identification, we collected genomic or transcriptomic data from publications or public databases (*E. testudo*: Murai et al., 2021[27], *A. australiensis* and *N. munidae*: Cunha et al., 2023[28] [FigShare: doi.org/10.6084/m9.figshare.23419922], *G. montsenyensis*: Eleftheriadi et al., 2024[29], *P. varius*: ERR1817116, ERR1817117, ERR1817118, and ERR1817119 [NCBI SRA]; kinorhynchs: Herranz et al., 2022[30] [*C. vanhoeffeni*: SRR14509480, *F. kalenesos*: SRR14509483, and *D. abei*: SRR14509486]). Raw read data from *P. varius* and kinorhynch species were *de novo* assembled as described above. Gene identity was confirmed by BLAST and phylogenetic analysis using the same methods as above.

277 The results are summarized in Fig. S1, and the gene tree and sequences are available in the
278 supplemental dataset.

279

280 **Extraction of NRs based on the domain conservation**

281 The HMM profile of NRs (Hormone_recep: PF00104) was obtained from the Pfam database.
282 Using this profile as a query, hmmscan was performed against all peptide data of the target
283 species (threshold 1E-05)[26], and the identified genes were considered as NRs. The number of
284 genes was counted considering the isoforms and shown in the linear bar graph of Fig. 1A.

285

286 **OrthoFinder analysis**

287 OrthoFinder (version: 2.5.4, with default parameters)[31] was used to examine the orthogroups
288 (OGs) of NRs in the represented nematode species of five major groups (Fig. 1A): Rhabditina
289 (*Ancylostoma ceylanicum*, *Caenorhabditis elegans*, *Heligmosomoides polygyrus*, and
290 *Pristionchus pacificus*), Tylenchina (*Globodera pallida*, *Meloidogyne arenaria*, and
291 *Steinernema monticolum*), Spirurina (*Ascaris suum* and *Brugia malayi*), Trichinellida
292 (*Trichuris muris*), and Enoplida (*Pontonema vulgare*). Peptide sequences of NRs from each
293 species were obtained as described above. After identifying 163 OGs in this comparison, we
294 investigated the taxonomic origin of the genes in each OG (Rhabditina, Tylenchina, Spirurina,
295 Trichinellida, and Enoplida). If the OG contained one or more genes from all taxa, the OG was
296 considered to be the one that the common ancestor of the taxa had. Using these criteria, the
297 evolutionary gain/loss events of NR OGs were resolved, as shown in Fig. 1H. Output file of
298 the analysis is available in the supplementary dataset.

299

300 **Transcriptome analysis**

301 *Data collection*

302 We collected transcriptome data, including molting stages, for a total of six nematode species
303 from public databases. The expression matrix (RPKM) for *Caenorhabditis elegans* was
304 obtained from Kim et al., 2013 (Gene Expression Omnibus, accession number: GSE49043)[32].
305 WormBase ParaSite (version: WBPS19, WS291)[18] was also the resource of this study to obtain
306 the calculated expression matrix (TPM) for *Trichuris suis* (NCBI Sequence Read Archive

307 [SRA] accession number: SRP039506) and *Globodera pallida* (NCBI SRA accession number:
308 ERP001236). The expression matrix (TPM) for *Pristionchus pacificus* was obtained from the
309 supplementary file of Sun et al. 2021[17]. In addition, raw read data of *Brugia malayi* and
310 *Ancylostoma ceylanicum* were obtained from SRA (NCBI SRA, accession number:
311 SRR4308250-51 and SRR9858310-17 [*B. malayi*][33] and SRR2125572, SRR2125581-86,
312 SRR2125588-90, SRR2125594, SRR2125597, SRR2125600, SRR2125602, SRR2125606-7,
313 SRR2125610-SRR2125611, SRR2125614, and SRR2125627 [*A. ceylanicum*][34]), and the
314 expression levels were calculated by us. As mentioned above, quality control and filtering of
315 raw reads were performed by FastQC and trimmomatic, respectively. We also used samtools
316 (version: 1.15.1) to remove the ribosomal RNA-derived sequences in the *B. malayi*[35]. The
317 filtered reads were mapped to the reference transcript models (*B. malayi*: PRJNA1072 and *A.*
318 *ceylanicum*: PRJNA231479, WormBase Parasite[18]) using RSEM/bowtie2 (version: v1.3.1,
319 rsem-calculate-expression --paired-end --bowtie2) to calculate expression levels (TPM)[36].
320 Molting stages were referred from original/previous papers[17,32-34,37-39]. Expression levels of the
321 transcripts corresponding to the *ecr* and *hr3,* which were identified in the above screenings,
322 were obtained from each transcriptome dataset (the average of TPM was calculated in the case
323 of having replicates at the same stage). In the transcriptome data of *G. pallida*, the gene model
324 of *hr3* was inappropriately reconstructed into two models (id: GPLIN_000052400 and
325 GPLIN_000052600). Thus, the expression levels were calculated by summing the expression
326 levels of the two models at each time point.

327

328 *Data curation*

329 Sun et al., 2021 originally provided the 1–2 h interval transcriptome data from 0 to 58 h after
330 hatching (i.e., 0, 1, 3, 5, ... 58 h)[17]. In our preliminary analysis, we found that the data contained
331 an overall fluctuating expression pattern throughout the examined stages (Fig. S3A). Therefore,
332 we divided the time points into overlapping windows of three time points each, and the mean
333 was calculated for each window. For example, the first time-window was grouped by the data
334 at 0, 1, and 3 h, and the mean value represents the data at 1 h. The second window included the
335 data at 1, 3, and 5 h, and the mean value represents the data at 3 h. Using this method, we
336 obtained the corrected expression profiles, which tends to remove the effects of fluctuating
337 expression due to biological or technical differences on individual development (Fig. S3B).

338

Unbiased screening of NRs for identifying molting regulators

With reference to our previous work[1], the expression profiles (TPM matrices) of four nematode transcriptome data were classified into 5-7 clusters using fuzzy c-mean clustering (Fig. S3: *A. ceylanicum*, *G. pallida*, *B. malayi*, and *T. suis*). Analysis was carried out using R and its package e1071 (version: 1.7.11: https://cran.r-project.org/web/packages/e1071/index.html). From such clustering, the genes in the cluster(s) that showed a positively correlated expression pattern with molting cycles were extracted (Fig. S3, green shades, *A. ceylanicum*: 8110, *G. pallida*: 3383, *B. malayi*: 7114, and *T. suis*: 2896 genes). In addition, Sun et al. 2021 and Kim et al. 2013 extracted the genes whose expression showed an oscillatory pattern along the molting cycle in *P. pacificus* (2,964 genes) and *C. elegans* (1,592 genes: assigned to clusters 1–6 in Kim et al., 2013), respectively[17,32]. As described above, OrthoFinder[31] was used to extract OGs among the screened genes, and this result identified 5,180 OGs. Among 5,180 OGs, 55 OGs contain one or more genes from all species. Finally, we searched for the presence of NR domains in the represented genes of each OG using Hmmer search[26] (see above).

Data visualization

We used R (version: 4.3.3)[40] and its packages such as Tydyverse (version: 2.0.0)[41], ggtree (version: 3.10.1)[42], ggstance (version: 0.3.7, https://github.com/lionel-/ggstance), ggvenn (version: 0.1.10, https://github.com/yanlinlin82/ggvenn), and ape (version: 5.8, https://cran.r-project.org/web/packages/ape/index.html) to analyze and visualize the results. Images for Fig. S1 are from PHYLOPIC (https://www.phylopic.org/).

Data availability

Scripts and datasets (e.g. assembly sequences, phylogenetic trees, data accession numbers) are available in the supplementary dataset.

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Competing interests

The authors declare no competing interests

Author contributions

Conceptualization, S.Y..; methodology, S.Y. and L.-M.B.; software, S.Y. and L.-M.B.; formal analysis, S.Y.; investigation, S.Y and L.-M.B..; writing—original draft, S.Y.; writing—review & editing, S.Y., L.-M.B., and A.H.; visualization, S.Y.; funding acquisition, S.Y. and A.H.

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