Supplemental Methods

Crossing strategy and phenotyping

To minimize heterozygosity in *C. nigoni* wild isolates, each strain was inbred for at least 10 generations via single male-female crossings, with triplet sets used in every round. Plates showing significantly reduced progeny numbers or severe phenotypic defects were excluded. The resulting inbred strains exhibited low heterozygosity, ranging from 0.117% to 0.514%, comparable to that of the *C. nigoni* reference strain JU1421 (Supplemental Table 1). For crosses involving *Cni-shls-1(-/-)* mutant males and either AF16 or ZF1220 females, five ZZY0997 males (*Cni-shls-1* mutant in the JU1421 background) (Xie et al. 2024) were placed on a crossing plate (with an OP50 bacterial lawn at the center) together with at least ten L4 females (either AF16 *she-1(VC43)* mutant or ZF1220) overnight. Fertilized females were then transferred to new plates to lay eggs for 4-6 hours before their removal. The total number of eggs laid was recorded initially and re-counted after at least 12 hours to determine the hatching rate. Each cross was performed in at least three biological replicates. In microinjection experiments, *Cni-neib-1* genomic DNA (20 ng/µl in sterilized Milli-Q water) was injected into a minimum of 10 young adult ZF1220 females. After approximately three hours of recovery, injected worms were transferred to crossing plates and allowed to lay eggs for 4–5 hours, after which progeny hatching rates were similarly calculated, following an incubation period of at least 12 hours. The *Cni-neib-1* genomic sequence was PCR amplified using previously described primers (Xie et al. 2024). Milli-Q water without DNA served as the injection control.

De novo genome assembly of reference strains

High-quality, chromosome-level genome assemblies for the reference strains *C. briggsae* (AF16) and *C. nigoni* (JU1421), referred to as CB5 and CN3, respectively, were previously generated using ONT long-read DNA sequencing in combination with chromatin conformation capture (Hi-C) data (Xie et al. 2023). Briefly, long-read sequencing data at approximately 80× coverage were initially assembled into contigs using Flye (v2.9.3) (Kolmogorov et al. 2019). Raw Hi-C reads were subsequently aligned to

these contigs using Juicer (v1.6) (Durand et al. 2016) to generate contact matrices in HIC format. The contigs were arranged into chromosome-level scaffolds by analyzing contact frequencies from the matrices, with correct order and orientation determined using the 3D-DNA pipeline (v180419) and subsequently curated manually with JuiceBox (v1.11.08). Finally, bacterial contamination was removed and genome polishing was performed using short-read NGS data, following the same procedures described for the **Genome assembly of *C. nigoni* and *C. briggsae* wild isolates**.

Genome assembly of *C. nigoni* and *C. briggsae* wild isolates

The raw ONT long reads for each *C. nigoni* wild isolate were first subjected to read correction using Canu (v2.2) (Koren et al. 2017) with the parameters "-correct genomeSize=130m". The corrected reads were then assembled using Flye (2.9.3) (Kolmogorov et al. 2019) with the "-nano-raw" option optimized for nanopore sequencing. For *C. briggsae* wild isolates (QR24, ED3036, JU439 and HK104), Flye-assembled contigs generated from ONT long-read sequencing were retrieved from *Widen et al.* (Widen et al. 2023). Subsequently, both the *C. nigoni* and *C. briggsae* contigs were subjected to reference-based correction and scaffolding to produce chromosome-level assemblies using RagTag (v2.1.0) (Alonge et al. 2022) with default settings. Briefly, the "correct" module in RagTag was used to fix misassemblies in the contigs via whole-genome alignment, using the ragtag.py correct command with MUMmer (v4.0.0) (Marcais et al. 2018) as the aligner. The alignment was carried out using the NUCmer tool from the MUMmer toolkit with the parameters "--mum --mincluster 100 --maxgap 300." Next, the "scaffold" module in RagTag was employed to anchor the corrected contigs to the reference genomes (*C. nigoni* JU1421 or *C. briggsae* AF16) using the same alignment parameters. Scaffolds identified as bacterial contaminations through BLAST (v2.11.0) searches against the NCBI nucleotide (nt) database (using an E-value threshold of >1e-09) were removed. The filtered scaffolds were then polished using Pilon (v1.24) (Walker et al. 2014) over five rounds to improve sequence accuracy. Polishing was performed by mapping Illumina short reads to the assemblies using BWA (v2.2.1) (Li and Durbin 2009) with default parameters. Two *C. briggsae* wild isolates, VX34 and QX1410, with pre-existing chromosome-level assemblies (Stevens et al. 2022), were utilized directly. Notably, these genomes do

not include unassigned contigs, whereas the assemblies of all the other strains include unassigned ones. Heterozygosity levels for AF16, JU1421, as well as *C. nigoni* wild isolates were estimated using Merqury (v1.4.1) (Rhie et al. 2020), which applies *k*-mer-based methods (Supplemental Table 1).

Gene prediction and annotation

Repetitive sequences in the assembled genomes were processed similarly to previous *C. nigoni* genome studies (Yin et al. 2018). Specifically, *de novo* repeat libraries were generated for all 16 genomes of *C. nigoni* and *C. briggsae* using the BuildDatabase command from RepeatModeler (v2.0.3) (Flynn et al. 2020). Potential protein-coding sequences (peptides containing 30 or more residues) in the repeat library were initially predicted with the getorf command from EMBOSS (v6.6.0.0) (Rice et al. 2000). True protein-coding sequences were identified using two complementary approaches: (1) Each predicted peptide sequence was aligned to the *C. elegans* proteome (WormBase WS280) (Sternberg et al. 2024) via BLASTP (v2.11.0) with an E-value threshold of 1e-09. (2) Protein domains within each predicted peptide were identified using InterProScan from InterPro (Blum et al. 2024). Any peptide that aligned to the *C. elegans* proteome or was annotated with a protein domain was classified as true protein-coding, and repeats containing these peptides were filtered out. The remaining repetitive DNA sequences were masked with RepeatMasker (v4.1.2) using the parameters "-xsmall -gff -e ncbi -gccalc". *Ab initio* protein-coding gene prediction was then performed with AUGUSTUS (v3.5.0) using the following parameters: "--extrinsicCfgFile=$AUGUSTUS_PATH/config/extrinsic/extrinsic.M.RM.E.W.cfg --softmasking=True --hintsfile=combined.hints.gff --uniqueGeneId=true --protein=on --introns=on --start=on --stop=on --cds=on --codingseq=on --gff3=on --progress=true --species=caenorhabditis." For the *C. nigoni* strains and *C. briggsae* AF16, hints files were derived from RNA-seq data of mixed-stage worms. In contrast, gene prediction for the *C. briggsae* wild isolates was performed without hints files. Specifically, raw RNA-seq reads were trimmed using Trim Galore! (v0.6.10) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and aligned to the corresponding genome using STAR (v2.7.8a) (Dobin et al. 2013) with default parameters. Exon hints were obtained by converting mapped reads into wig files with the AUGUSTUS bam2wig tool and processing them with wig2hints.pl using the parameters "--width=10 --margin=10 --minthresh=2 --minscore=4 --src=W

--type=ep --radius=4.5". Intron hints were generated using AUGUSTUS bam2hints tool with default parameters. The concatenated exon and intron hints were provided as a combined hints file to AUGUSTUS for gene prediction, and the predicted protein sequences were subsequently extracted using the getAnnoFasta.pl script from AUGUSTUS.

Sequence rearrangement detection by SyRI

Aligned regions were classified as either syntenic or rearranged, with rearranged regions including inversions, translocations, duplications, and inverted translocations/duplications. Within both syntenic and rearranged blocks, sequence variations such as single nucleotide polymorphisms (SNPs), copy number gains and losses, tandem repeats, insertions, and deletions were also identified. The indel identification using SyRI distinguishes two classes: small and large indels. The small indels are detected by local whole-genome alignment tools. Specifically, by using the '--maxgap 300' parameter during pairwise genome alignment with NUCmer, we ensure that these indels are typically less than 300 bp. In contrast, larger indels are identified by analyzing the gaps and overlaps between consecutive alignments within the same structural variation annotation block detected by SyRI. The results were visualized using Circos (v0.69-9) (Krzywinski et al. 2009), generating Circos plots that comprehensively illustrate synteny and rearrangement patterns.

Multiple-genome alignment and phyloP calculation

To assess evolutionary conservation at single-nucleotide resolution, reference-free whole-genome alignments of all *C. nigoni* or *C. briggsae* strains were performed using Progressive Cactus (v2.6.9-20.04) (Armstrong et al. 2020).Each chromosome was split and aligned separately to create single-sequence alignments, a prerequisite for subsequent phyloP analysis. The resulting HAL file was converted to MAF format via the cactus-hal2maf tool. The multiple-genome MAF alignment, along with the phylogenetic tree models for intraspecific strains (see **Phylogeny Analysis**), was then analyzed using phyloFit from the PHAST (Phylogenetic Analysis with Space/Time models) software package (v1.6) (http://compgen.cshl.edu/phast/). PhyloFit was executed with the following parameters: "--target-coverage 0.25 --expected-length 12 --rho 0.4", fitting the alignment via maximum likelihood.

Subsequent conservation or acceleration *p*-values (phyloP) were then computed using the phyloP program within PHAST based on the alignments and the phylogenetic model. For visualization, phyloP values were averaged and binned into 10 bp intervals for plotting 2 kb upstream/downstream sequences of genes in different gene family groups, and into 100 kb intervals for chromosome-wide plots.

Graph plotting and statistical analyses

All graphs were generated using RStudio (R version 4.3.2) (https://www.R-project.org/), unless otherwise specified. Preliminary figures were subsequently refined, adjusting sizes and fonts, to produce publication-ready panels using Adobe Illustrator. Statistical analyses were performed as follows. The Wilcoxon rank-sum test was used to compare average pi nucleotide, Tajima's D values, and average gene expression levels between adults and embryos of *C. nigoni* (JU1421). Confidence intervals for worm hatching rates were calculated using the Agresti-Coull method, and differences in hatching rates were assessed for significance using Fisher's exact test, with a significance threshold of $P < 0.05$.

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