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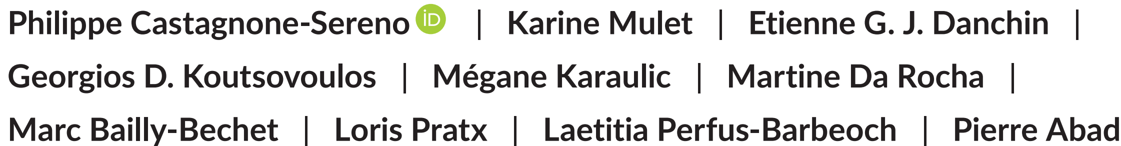
Gene copy number variations as signatures of adaptive evolution in the parthenogenetic, plant-parasitic nematode *Meloidogyne incognita*



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Abstract

Adaptation to changing environmental conditions represents a challenge to parthenogenetic organisms, and until now, how phenotypic variants are generated in clones in response to the selection pressure of their environment remains poorly known. The obligatory parthenogenetic root-knot nematode species *Meloidogyne incognita* has a worldwide distribution and is the most devastating plant-parasitic nematode. Despite its asexual reproduction, this species exhibits an unexpected capacity of adaptation to environmental constraints, for example, resistant hosts. Here, we used a genomewide comparative hybridization strategy to evaluate variations in gene copy numbers between genotypes of *M. incognita* resulting from two parallel experimental evolution assays on a susceptible vs. resistant host plant. We detected gene copy number variations (CNVs) associated with the ability of the nematodes to overcome resistance of the host plant, and this genetic variation may reflect an adaptive response to host resistance in this parthenogenetic species. The CNV distribution throughout the nematode genome is not random and suggests the occurrence of genomic regions more prone to undergo duplications and losses in response to the selection pressure of the host resistance. Furthermore, our analysis revealed an outstanding level of gene loss events in nematode genotypes that have overcome the resistance. Overall, our results support the view that gene loss could be a common class of adaptive genetic mechanism in response to a challenging new biotic environment in clonal animals.

KEYWORDS
adaptive evolution, array comparative genomic hybridization, experimental evolution, gene copy number variations, parthenogenesis, root-knot nematodes

1 | INTRODUCTION

For over a century, the paradigm has been that the lack of recombination associated with asexual reproduction should reduce additive genetic variance and produce clonal progenies, thus resulting in poor capacities to generate new combination of genotypes (Crow, 1992; Edhan, Hellman, & Sherill-Rofe, 2017; Song, Scheu, & Drossel, 2012). In this context, adaptation to changing environmental conditions

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represents a challenge to parthenogenetic organisms. However, evidence collected over the last decade indicates a considerable intraclonal variation in many asexually reproducing eukaryotic animals (e.g., Badaeva, Malysheva, Korchagin, & Ryskov, 2008; Fontaneto, Kaya, Herniou, & Barraclough, 2009; Monti, Mandrioli, Rivi, & Manicardi, 2012) and raises question about the very notion of clone (see Loxdale, 2009 for review). Moreover, artificial selection studies under laboratory conditions showed that populations reproducing by obligate parthenogenesis are able to rapidly respond to strong selective constraints such as abiotic stress or thermal adaptation (Doroszuk, Wojewodzic, & Kammenga, 2006; Lombardo & Elkinton, 2017; Robin, Andanson, Saint-Jean, Fabreguettes, & Dutech, 2017).

In the case of plant–parasite interactions, another corpus of studies illustrated the adaptation of various asexual species to their hosts, for example, in aphids (Agarwala & Choudhuri, 2014; Loxdale, 2008) or in fungi (de Jonge et al., 2013; Seidl & Thomma, 2014). However, it remains enigmatic how phenotypic variants are generated in these a priori clonal populations that are known to respond at least partially to selection, including changes in the host plant when plant parasites/pathogens are considered. The root-knot nematode (RKN) *Meloidogyne incognita* is a plant parasite of worldwide agricultural importance. It reproduces in an asexual way by obligate parthenogenesis without meiosis (i.e., apomixis), and several hundreds of offspring are produced by a single female that form virtually clonal populations (Castagnone-Sereno, Danchin, Perfus-Barbeoch, & Abad, 2013). However, although these clones share a priori the same genetic heritage, they can exhibit phenotypic variations when exposed to unfavourable environments, for example, when they are in interaction with host plants harbouring resistance genes. Indeed, virulent populations (i.e., able to reproduce on resistant plants) have been reported in the field (reviewed in Barbary, Djian-Caporalino, Palloix, & Castagnone-Sereno, 2015). To avoid confusion in terminology, the term "virulence" as used for the purpose of this study will designate the ability of the nematode to overcome host resistance and successfully establish infection. Experimental studies have clearly demonstrated the emergence of virulent specimens in the progeny of *M. incognita* avirulent females and that inheritance of virulence is not Mendelian (Bost & Triantaphyllou, 1982; Castagnone-Sereno, Wajnberg, Bongiovanni, Leroy, & Dalmasso, 1994; Jarquin-Barberena, Dalmasso, De Guiran, & Cardin, 1991). Indeed, at the phenotypic level, the proportion of virulent specimens in the originally avirulent population increases over generations, but never reaches 100% (as should be expected in case of strict asexual reproduction and with the exception of the rare escape cases that may occur). Some nucleotidic variations have been found between avirulent and virulent nematodes resulting from experimental evolution (Neveu, Jaubert, Abad, & Castagnone-Sereno, 2003b; Semblat, Rosso, Hussey, Abad, & Castagnone-Sereno, 2001). However, they probably do not represent the only factor that could be responsible for the switch from avirulence to virulence, since their distribution proved to be random in natural virulent populations (P. Castagnone-Sereno, unpublished data). From this perspective, *M. incognita* thus provides an important model for the comprehensive study of the various molecular mechanisms that might promote adaptation of parthenogenetic animals to environmental changes. In addition, the *M. incognita* genome sequence and annotation have been deciphered (Abad et al., 2008), which reinforces the asset of this experimental system.

In recent years, whole-genome sequencing has shown that besides point mutations, copy number variations (CNVs) constitute another important mechanism of genomic variation submitted to selection. This feature has been identified in most model organisms across the plant or animal kingdom (Brown et al., 2012; Locke et al., 2015; Yu et al., 2013; Zmienko, Samelak-Czajka, Kozlowski, & Figlerowicz, 2016) and consists of DNA segments typically exceeding 1 kb that are variable in copy number in comparison with a reference genome, share a sequence identity higher than 95% and are dispersed throughout the genome (Alkan, Coe, & Eichler, 2011). CNVs can either be inherited from the previous generation or appear de novo through duplication/deletion events, and their fixation by drift or selection may contribute to the creation of genetic novelty resulting in species adaptation to stressful or novel environments (Katju & Bergthorsson, 2013; Kondrashov, 2012). For example, it has been shown that CNVs may lead to adaptive phenotypes such as copper resistance in yeast (Hull, Cruz, Jack, & Houseley, 2017) or insecticide resistance in the dengue mosquito *Aedes aegypti* (Faucon et al., 2017).

In nematodes, CNVs have been essentially documented at the genome scale in the model species *Caenorhabditis elegans*. In this model nematode, the rate of CNV per gene and per generation is two orders of magnitude higher than the spontaneous rate of point mutation per coding nucleotide (Lipinski et al., 2011). In addition, CNV has been extensively detected in 12 natural populations of *C. elegans*, affecting over 5% of the genes in the genome, thus allowing even very closely related strains to be distinguished (Maydan, Lorch, Edgley, Flibotte, & Moerman, 2010). Deletions relative to the canonical N2 strain appeared more common in these genetically different populations than duplications, affecting gene families involved in environmental responses and innate immunity (Maydan et al., 2010). A further investigation on experimental *C. elegans* lineages indicated that multiple duplications and deletions can reach intermediate to high frequencies in independent genotypes, and several lines of evidence suggest that some of these changes were adaptive to laboratory conditions (Farslow et al., 2015). Thus, CNV likely represents an important, yet incompletely characterized, source of de novo genetic variation and adaptive potential in many eukaryotes, including parasitic species.

Here, we tested the hypothesis that CNV may contribute to the adaptive capacities of the parthenogenetic RKN *Meloidogyne incognita*, that is, its ability to overcome plant resistance genes. For that purpose, we performed an array comparative genomic hybridization (aCGH) analysis of two independent pairs of avirulent vs. virulent genotypes (i.e., nonadapted vs. adapted to the host resistance, respectively), taking advantage of the parthenogenetic reproduction of the nematode to set up near-isogenic lines (NILs) through experimental evolution on susceptible or resistant host plants, respectively. This strategy made it possible to draw up a comprehensive genomewide landscape of gene CNVs associated

with the virulence of the nematode and resulted in a set of 184 genes differentially affected by CNV between avirulent and virulent NILs. We further filtered this list of candidate CNVs and identified 33 genes that systematically showed the same highly supported changes in copy number across replicates between the two pairs of virulent vs. virulent NILs. The fold change values indicated gene copy losses in virulent nematodes, which were then tested by qPCR. This validation step confirmed a subset of 18 genes exhibiting CNVs conserved between two independent sets of avirulent and virulent lines. To our knowledge, this study represents the first genomewide analysis of the distribution of CNVs in the genome of a nonmodel nematode and provides new insights into the genetic mechanisms that may promote adaptation of a parthenogenetic animal to changing environmental conditions.

2 | MATERIALS AND METHODS

2.1 | Nematode avirulent and virulent near-isogenic lines

Experimental evolution was conducted in the laboratory on two *M. incognita* isolates from the living RKN collection of the Institut Sophia Agrobiotech, both originally sampled in the field and avirulent against the tomato *Mi-1.2* resistance gene (Figure 1a). In order to ensure that the observed phenotypes were the result of our experimental evolution protocol (independently of the genetic background of the nematodes), we selected isolates with very different geographic origins (Kursk, Russia and Morelos, Mexico, respectively). In addition, to eliminate any potential within-population

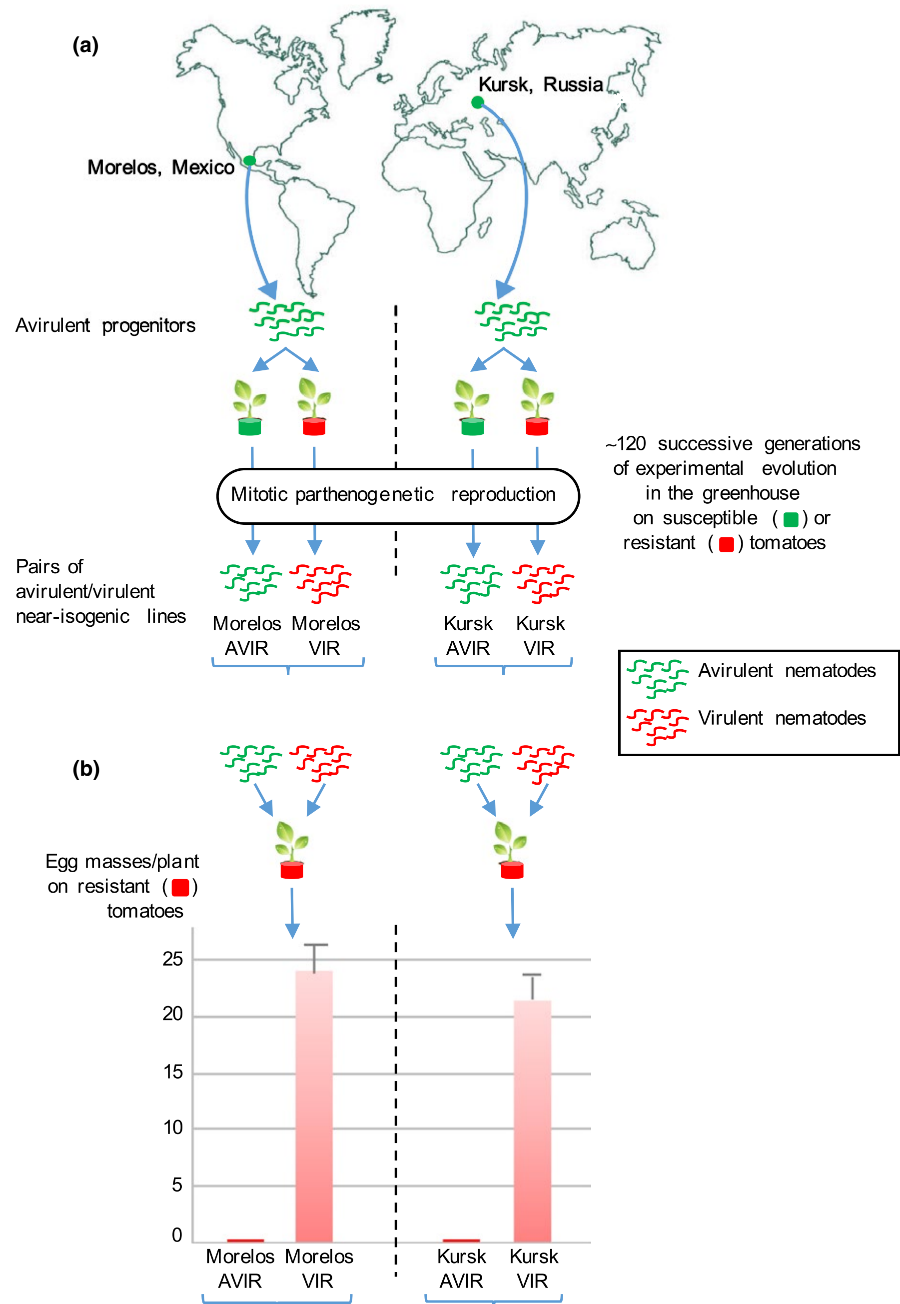

FIGURE 1 Nematode avirulent and virulent lines used in this study. (a) The experimental evolution procedure designed to generate independent pairs of *Meloidogyne incognita* near-isogenic lines avirulent or virulent against the tomato *Mi-1.2* resistance gene. (b) Reproduction of *Meloidogyne incognita* avirulent and virulent near-isogenic lines on the resistant tomato cv. Piersol

heterogeneity, a line was raised from each field population, starting from the progeny of a single female carefully dissected from the root tissues along with its egg mass, which was then used to reinoculate a tomato plant. Because of the obligatory mitotic, parthenogenetic mode of reproduction of *M. incognita*, the second-stage juveniles (J2s) that hatched from each egg mass were considered as a clonal line (Castagnone-Sereno et al., 2013). From this starting material, sets of replicated lines were repeatedly inoculated under controlled conditions on susceptible (cv. Saint Pierre) or *Mi-1.2*-resistant (cv. Piersol) tomatoes, according to described experimental evolution procedures (Castagnone-Sereno, Bongiovanni, & Wajnberg, 2007; Jarquin-Barberena et al., 1991). This gave rise to two pairs of originally avirulent vs. derived virulent near-isogenic lines (NILs). Due to their apomictic reproduction, these pairs of NILs were considered to vary only in their (a) virulence against the *Mi-1.2* resistance gene.

2.2 | DNA sequencing, SNP calling and phylogenetic analysis

For each of the two pairs of avirulent vs. virulent NILs from Kursk and Morelos, DNA was extracted from eggs as described in Perfus-Barbeoch et al. (2014). In summary, eggs were grinded for 5 min in a Dounce homogenizer to access nuclei. Nuclei were purified by 20-min centrifugation at 7800 *g* and incubated at 37°C for 5 min with 1 µl MNase (15 U). Digested DNA was extracted with phenol/chloroform, precipitated with isoamyl alcohol, washed with 70% ethanol and suspended in 40 µl qPCR grade water. To generate the 4 libraries to be sequenced, MicroPlex Library Preparation™ kit (Diagenode) was used. Each barcoded library was then quantified by qPCR (KAPA Library Quantification Kit, KAPA Biosystems) and standardized to 4 nM before being pooled altogether. Illumina sequencing was performed at UCAGenomiX-IPMC platform (Sophia Antipolis, France) on NextSeq 500 to generate 75-bp single reads.

After the filtering of low-quality bases, each library was aligned to the *M. incognita* reference genome with BWA (Li, 2013) and alignments with mapping quality score <20 were removed with SAMtools (Li et al., 2009). We used the FreeBayes variant detection tool (Garrison & Marth, 2012) to call SNPs and small-scale insertions/deletions, incorporating all the library alignment files simultaneously, and produced a variant call file (VCF). We filtered the resulting VCF file with the vcffilter function of vcflib (Garrison, 2018), retaining the positions that had more than 20 Phred-scaled probability and a coverage depth > 10. Finally, we used SnpEff (Cingolani et al., 2012) to annotate variants at coding regions. SNPs were concatenated into a supermatrix that was used as input to IQ-TREE (Nguyen, Schmidt, von Haeseler, & Minh, 2015) to estimate the phylogeny under maximum-likelihood criterion using the GTR+ASC+G model. Bootstrap was used to assess branch support. The resulting tree was visualized in FigTree (Rambaut, 2018).

2.3 | Evaluation of the ability to reproduce on resistant tomato

At the end of the selection procedure, the two pairs of avirulent vs. virulent NILs from Kursk and Morelos were compared for their ability to reproduce on resistant tomatoes. Experiments were conducted in a climatic chamber maintained at 22°C (±2°C) with a 14-hr light cycle. *Mi-1.2*-resistant Piersol tomato seedlings were grown individually in 50-ml plastic tubes containing a steam-sterilized sandy soil, and 4-6 true leaves plants were inoculated with a calibrated water suspension of 25 J2s, in order to avoid any influence of density-dependent effects on nematode reproduction parameters. Seven weeks after inoculation, the root systems of 10 to 13 plants for each NIL were gently washed free from soil in tap water, immersed in cold eosin yellow (0.1 g/L water) and stirred for 30 min to stain nematode egg masses. Numbers of egg masses per root system were then counted under a magnifying glass. Preplanned comparisons between means were done with Student's *t* least significant difference test at $p = 0.01$. All computations were done using analysis of variance models implemented in the PROC GLM procedures of the sas/stat package (SAS Institute Inc., 1990).

2.4 | Oligonucleotide array CGH design

A 4 × 180 K custom CGH array (Agilent Technologies) was generated based on the 2,995 super-contigs of the *M. incognita* Mi1V1 reference genome sequence obtained from an avirulent isolate (Abad et al., 2008). A total of 173,539 probes of 60-bp length, representing a global coverage of ~10.4 Mb, were designed from the 19,212 protein-coding genes according to Agilent probe design procedure at an average rate of ~9 probes/gene (https://earray.chem.agilent.com/suredesign/). In addition, 380 probes randomly selected from the global set, 10× replicated, together with 3,440 Agilent Technologies control probes were included for hybridization quality control.

2.5 | DNA isolation, DNA labelling and array hybridization

For each sample, genomic DNA was purified from ~200 µl of nematode eggs according to a standard phenol/chloroform protocol (Sambrook, Fitsch, & Maniatis, 1989) and stored at −80°C until use. Quality of DNA was checked by PCR using the *Meloidogyne*-specific primers MelF and MelR (Tigano, Carneiro, Jeyaprakash, Dickson, & Adams, 2005). Sample identity to the species level was further confirmed using a species-specific SCAR marker as described previously (Randig, Bongiovanni, Carneiro, & Castagnone-Sereno, 2002). For each of the four genotypes tested (i.e., two geographic origins × two (a)virulence phenotypes), three biological replicates were processed.

The sample preparation and hybridization were performed as specified by Agilent Technologies on the HELIXIO platform (Helixio, Saint-Beauzire, France). Briefly, pairs of test (virulent NILs) and reference (avirulent NILs) DNAs (500 ng) were digested with *Alu*I

and *Rsa*I and labelled with fluorescent dyes Cy5 and Cy3, respectively. Absorbance was measured at 260 nm (DNA), 550 nm (Cy3) or 650 nm (Cy5) to calculate the specific activity. Array cohybridization was then performed at 65°C for 24 hr. After washing, arrays were scanned at a 3 µm resolution on the G2505C Microarray Scanner and images were processed using the Feature Extraction software v. 11.5.1.1 (Agilent Technologies). Quality control reports with a set of evaluation metrics were generated from each of the images to assess the quality of the various laboratory steps (label, hybridization, wash, scan steps), and only images that showed metrics that are within the suggested threshold ranges were considered for further analysis.

2.6 | Data normalization and statistical analysis

Before CNV prediction, intra-array normalization of the data was performed using specific algorithms of the Feature Extraction software, and the average $\log_2$ ratio of each probe (i.e., $\log_2$ [cy5 processed signal/cy3 processed signal]) was computed. CNVs were called as segments with a mean |ratio| > 1.50 (i.e., fold change |FC| > 1.50). In order to reduce the number of false positives, probes were filtered; that is, only those showing the same flag of intensity signal for all the three biological replicates of a given nematode genotype were considered for further statistical analysis. Comparisons between reference (avirulent) and test (virulent) samples were carried out using *t* tests. To take into account the occurrence of false positives, *p*-values < 0.05 after FDR correction (false discovery rate; Benjamini & Hochberg, 1995) were considered statistically significant.

2.7 | Functional characterization of genes affected by CNVs

Genes corresponding to probes that were affected by CNVs were retrieved from the *M. incognita* Mi1V1 genome sequence (http://meloidogyne.inra.fr). To determine which functional categories were found in these genes, we performed a gene ontology (GO) annotation inferred from the InterPro protein domains annotation using AmiGO, the GO Consortium's annotation and ontology toolkit (Carbon et al., 2009). The GO-slim annotations were split into three ontologies (biochemical function, cellular component and molecular function). In addition, we used a hypergeometric test as implemented in FUNC v0.4.7 (Prüfer et al., 2007) to detect enriched GO terms in the genes showing significant variation in copy numbers between avirulent and virulent genotypes. We considered the GO terms that returned a FDR threshold < 0.05 as significantly enriched. To further characterize these genes, we compared them to a list of > 100 *M. incognita* putative effectors (i.e., genes encoding secreted proteins that mediate interactions with the host), as recently reviewed in Nguyen et al. (2018).

2.8 | Validation of CNVs by qPCR

In order to select a short list of genes systematically affected by CNVs, an additional custom CNV-calling pipeline with higher stringency was designed with the following parameters: (a) probes exhibiting the same fold change direction (positive or negative) simultaneously in the two AVIR_vs_VIR comparisons and with fold change > 2 and (b) genes tagged by at least two probes exhibiting the features listed in 1). We then validated by qPCR the CNVs resulting from the filtering pipeline. Amplification and detection were performed on an Agilent AriaMX qPCR system. Primers used for qPCR analyses were designed using Primer3 online (Untergasser et al., 2012) and are listed in Table S1. Reactions were in a final volume of 15 µl containing 7.5 µl of qPCR MasterMix Plus For SYBRGreen I No Rox (Eurogentec), 0.45 µl 10 µM of each primer and 15 ng of DNA template. PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s. At the end of the program, a melting curve (from 60 to 95°C, read every 0.5°C) was determined to ensure that only single products were formed. For each nematode NIL, two technical replicates and three biological replicates were assayed. The copy number of each CNV was further normalized against the GAPDH housekeeping gene, a control gene that did not vary in copy number in the *M. incognita* genome (Kozera & Rapacz, 2013). Finally, the relative copy number fold changes between avirulent and virulent NILs were compared according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), based on Ct values for the target genes and GAPDH for both the avirulent and virulent samples. Values of $p < 0.05$ after FDR correction were considered as statistically significant.

3 | RESULTS

3.1 | Phenotypic and genetic characterization of nematode NILs after experimental evolution

The experimental evolution system was originally set up in the laboratory in 1995 and constantly maintained under the same controlled conditions since that time. Considering that about 45 days are required at 20°C for *M. incognita* to complete its life cycle (Ploeg & Maris, 1999), the two pairs of avirulent vs. virulent NILs from Kursk and Morelos used in this study have independently evolved for 160 successive generations on the susceptible and resistant tomato cv. Saint Pierre and Piersol, respectively. At the end of the process, the phenotype of the four NILs was characterized on the resistant tomato. As expected, both avirulent NILs were controlled by the *Mi-1.2* resistant cv. Piersol; that is, no egg masses were observed on the root systems of inoculated plants. Conversely, the virulent NILs exhibited a high reproduction rate on the resistant tomatoes (average egg mass number per root system = 20.85 ± 2.33 and 23.08 ± 2.47 for the virulent NILs from Kursk and Morelos, respectively; Figure 1b). This result indicates that the evolution towards nematode virulence was indeed successful, virulence being defined here as the ability of the parasite to reproduce (i.e., for a *M. incognita* given female to produce one egg mass) on a resistant cultivar.

In order to estimate whether the two avirulent lines used as the ancestors of the two virulent lines are independent or share a recent evolutionary history, we performed a phylogenetic analysis

of the two avirulent/virulent pairs of *M. incognita* lines from Kursk and Morelos. For that purpose, we sequenced the four genomes in question, which led to 24 to 49 millions 75-bp single reads per library, respectively. After alignment against the *M. incognita* reference genome, the SNPs in coding regions were identified, concatenated and used as markers to estimate the phylogeny. The topology of the obtained tree clearly shows that the two original lines from Kursk and Morelos are independent (Figure S1), which in turn supports the independent origin of the virulent (adapted) strains, and thus adaptive parallel evolution of the virulence phenotype. Overall, the observed, unambiguous phenotypic and genotypic differentiation between avirulent and virulent NILs confirmed that these lineages are thus a biological material of choice for the aCGH strategy performed here.

3.2 | Detection of gene CNVs associated with nematode (a)virulence

Following normalization of the hybridization signals, the average $\log_2$ ratio of each of the 60-bp 173,539 probes was computed. All together, these probes covered 11.6% and 45.8% of the *M. incognita* V1 genome (i.e., 86 Mb; Abad et al., 2008) and predicted protein-coding regions, respectively. About 75% of the sequences from the avirulent and virulent NILs from Kursk and Morelos corresponding to these probes exhibited no variation when aligned against the *M. incognita* reference genome (Table S2). We identified a total of 2,400 and 1,463 probes exhibiting CNVs between avirulent and virulent NILs from Kursk and Morelos, respectively (Tables S3 and S4). Probes exhibiting a positive FC ratio have higher copy number in the avirulent strain vs. the virulent strain and represented 74.3% and 80.8% of the total number of probes exhibiting CNVs for the NILs from Kursk and Morelos, respectively. Overall, 776 probes with CNV between avirulent and virulent nematodes were shared by NILs from Morelos and Kursk, 92.8% of which exhibited a positive FC ratio (Figure 2; Table S5). Notably, alignment of the Illumina reads of the virulent/avirulent Morelos and Kursk isolates on the *M. incognita* reference genome revealed no SNP at all in 91.4% of these 776 probes (Table S5). This confirmed that the probes designed on the Morelos reference genome should be able to detect CNVs on Kursk with a relatively similar sensitivity. The 776 probes correspond to 184 genes in the *M. incognita* genome. Only these common, differential probes/genes were taken into account for further analysis.

When scanned for InterPro domains, 103 out of the 184 (~56%) genes varying in copy numbers in avirulent vs. virulent NILs were found to harbour at least one known domain and were further assigned corresponding Gene Ontology (GO) terms. Overall, Cellular Component, Molecular Function and Biological Process GO terms could be assigned to 26, 70 and 99 genes, respectively (Figure S2). We identified 18 and 4 significantly enriched GO terms, in the Molecular Function and Biological Process ontologies, respectively, in the 184 genes showing variations in copy numbers. No Cellular Component GO term was significantly enriched (Table S6). Interestingly, 6 of the 18 enriched Molecular Function terms were related to peptidase

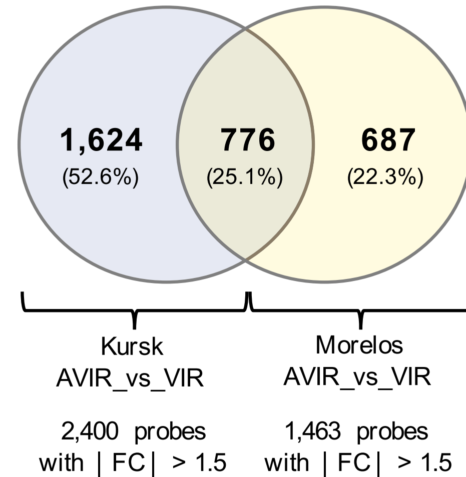

FIGURE 2 Venn diagram illustrating the overlap between probes with |fold change (FC)| > 1.5 in the CGH comparison between *Meloidogyne incognita* avirulent (AVIR) and virulent (VIR) near-isogenic lines from Kursk and Morelos

activity and this is further highlighted by the “proteolysis” term also significantly enriched in the Biological Process Ontology.

In addition, the comparison of the 184 genes exhibiting CNVs to > 100 characterized *M. incognita* putative effectors (Nguyen et al., 2018) revealed three positive matches: a metallopeptidase (Minc00108), a gene of unknown function expressed in the subventral glands (Minc13292) and a gene of unknown function expressed in the amphids (Minc00158).

3.3 | Distribution of genes affected by CNVs in the nematode genome

Further analysis of the 184 genes varying in terms of copy number in both couples of avirulent vs. virulent NILs revealed that they are distributed on 33 super-contigs, ranging from one to 23 genes per super-contig (Figure 3a). The rate of distribution appeared highly variable, with more than half of the genes (51.1%) present on five super-contigs only, while 15 super-contigs harbour one single gene affected by CNVs. In addition, no significant correlation was observed between the occurrence of genes affected by CNVs and the super-contig length (R^2 = 0.38686; Figure 3b). Moreover, 142 of these 184 genes (77.2%) are organized in 36 clusters ranging from two to 18 adjacent genes. Figure 3c illustrates the genomic organization of the two largest clusters on super-contigs ctg217 and ctg202 harbouring 18 and 16 adjacent genes affected by CNVs, respectively. Altogether, these data indicate that the distribution of genes affected by CNVs is not uniform in the nematode genome.

3.4 | Cross-validation of CNVs based on aCGH and qPCR experiments

When applying further pruning in the pipeline selection based on (a) probes varying in the same direction in the two AVIR_vs_VIR

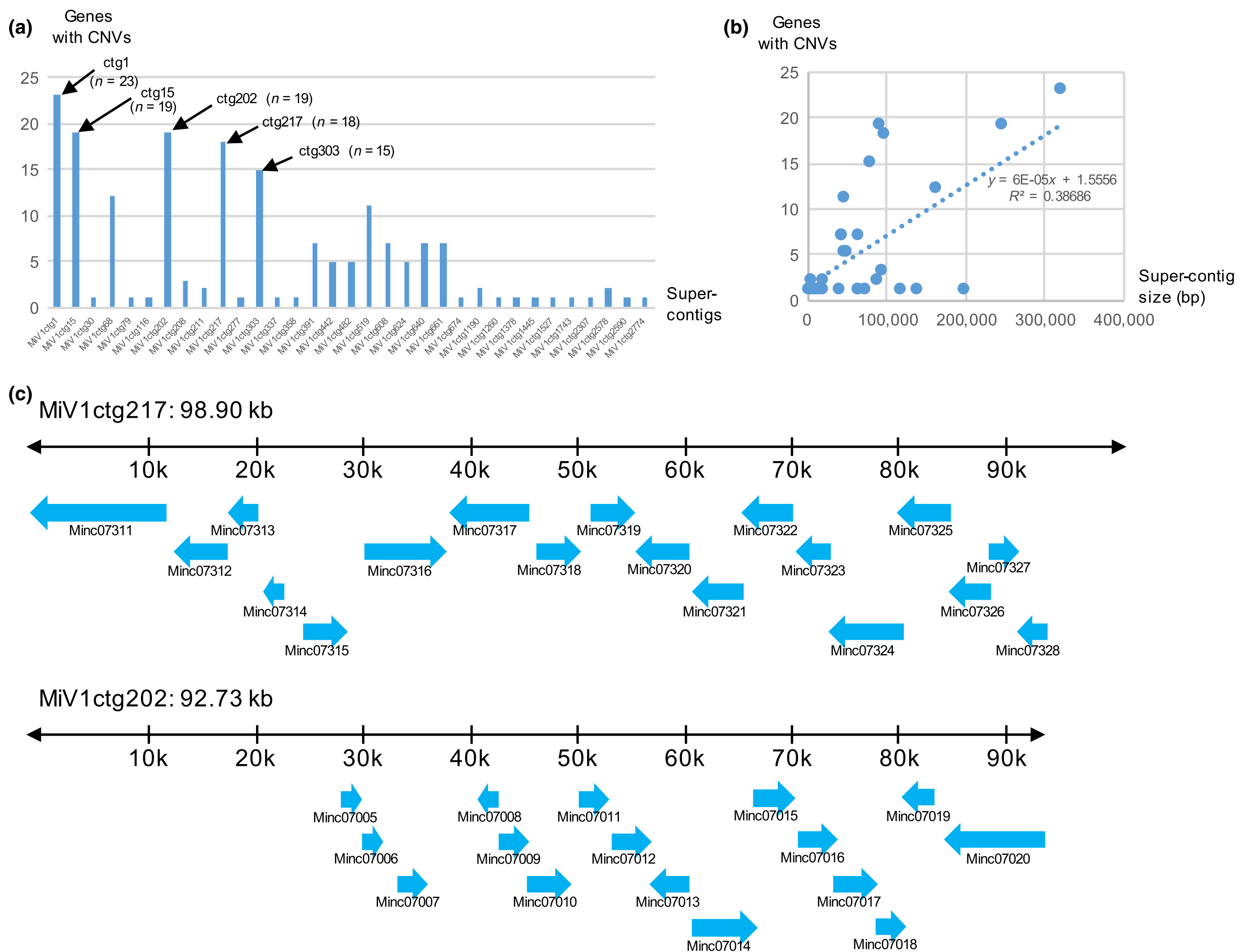

FIGURE 3 Distribution of CNVs and genes exhibiting CNVs in the *Meloidogyne incognita* genome. (a) Number of genes exhibiting CNVs according to their super-contig position. (b) Relationship between the number of genes exhibiting CNVs and super-contig size. (c) Genomic organization of genes exhibiting CNVs in two super-contigs of *Meloidogyne incognita* genome, super-contig 217 (MiV1ctg217) and super-contig 202 (MiV1ctg202)

comparisons and with fold change > 2 and (b) genes tagged by at least two probes exhibiting these features, a short list of 33 highly supported genes affected by CNV was retrieved from the previous list of 184 genes differential between avirulent and virulent NILs (Table 1). Notably, sequence variation in probes targeting these 33 genes was detected for one single gene (Minc07328; Table S5). In both pairs of NILs, fold change values indicated a reduced copy number in virulent nematodes compared to their avirulent counterpart for all the 33 genes considered (Figure 4). In addition to eliminate false positives that may have resulted from poor hybridization conditions, the 33 CNVs were further cross-validated by qPCR on genomic DNA from the two pairs of avirulent/virulent NILs. For 28 of these genes, unambiguous amplification signals were observed, while we were unable to define suitable amplification conditions for the remaining genes. Overall, a clear correlation was observed between CNVs obtained by aCGH and qPCR, with 20/28 genes (i.e., 71%) exhibiting the same pattern of variation in the two pairs of NILs (Table 1; Figure S3). For five additional genes (i.e., 18%), qPCR validation was confirmed for one pair of NILs only. Among these 20 gene families supported by both aCGH and qPCR analyses, pioneer genes, genes with housekeeping functions (carbonic anhydrase, chaperone, GPCR family) as well as genes encoding protease activity (peptidase S16, Lon protease and peptidase C1A) and gene with strong homology with a transposase were identified as significantly affected by CNV.

4 | DISCUSSION

In this study, we used a genomewide aCGH strategy to evaluate variations in gene copy numbers between genotypes of the parthenogenetic plant-parasitic nematode *M. incognita* resulting from experimental evolution on a susceptible or resistant host plant, respectively. In our experimental evolution protocol, the selection environment was highly controlled, and thus, we expected that similar molecular evolution mechanisms would take place to generate

TABLE 1 The 33 *Meloidogyne incognita* genes most affected by copy number variations between avirulent and virulent near-isolines from Kursk and Morelos, as identified by comparative genomic hybridization (CGH), and their qPCR validation

			CGH average fold change[b]		qPCR validation[c]				
Gene	Contig	Mi1V1 annotation/BLASTP at NCBI[a]	Morelos	Kursk	Morelos $2^{-\Delta\Delta Ct}$	Sign.[d]	Kursk $2^{-\Delta\Delta Ct}$	Sign.	Congruence between CGH and qPCR[e]
Minc07002	MiV1ctg202	Pioneer/pioneer	45.95	32.53	355.88	**	151.87	***	Yes
Minc07003	MiV1ctg202	Pioneer/putative helicase MOV-10 [Acropora digitifera]	30.28	23.84	385.12	**	152.66	***	Yes
Minc07007	MiV1ctg202	HSP20-like chaperone	59.63	45.20	1658.86	***	2301.32	***	Yes
Minc07009	MiV1ctg202	Pioneer/hypothetical protein LOAG_14083 [Loa loa]	51.62	33.85	1.38	n.s.	1.93	**	Partly
Minc07011	MiV1ctg202	Pioneer/pioneer	21.78	32.00	139.29	***	288.02	***	Yes
Minc07012	MiV1ctg202	Peptidase S16; Lon protease	52.69	45.53	377.91	***	30.53	n.s.	Partly
Minc07013	MiV1ctg202	Pioneer/hypothetical protein, variant [Loa loa]	32.33	25.30	86.55	***	233.03	***	Yes
Minc07016	MiV1ctg202	Phosphoglycerate mutase	40.94	25.67	1019.03	**	612.23	**	Yes
Minc07017	MiV1ctg202	Phosphoglycerate mutase	30.87	25.04	1.39	**	1.58	*	Yes
Minc07018	MiV1ctg202	Ras GTPase	21.66	16.71	n.d.		n.d.		n.d.
Minc07019	MiV1ctg202	TonB box, N-terminal; achaete-scute transcription factor related	35.28	23.86	n.d.	n.d.	n.d.		n.d.
Minc07319	MiV1ctg217	Sodium neurotransmitter symporter	7.42	6.16	n.d.	n.d.	n.d.		n.d.
Minc07321	MiV1ctg217	Carbonic anhydrase	59.22	52.51	549.57	***	1512.54	**	Yes
Minc07322	MiV1ctg217	Carbonic anhydrase; gonadotropin, beta chain	35.46	30.25	458.76	***	473.8	**	Yes
Minc07328	MiV1ctg217	Peptidase S16; Lon protease	81.34	42.52	76.46	n.s.	206.94	***	Partly
Minc08915	MiV1ctg303	Peptidase S16; Lon protease	80.57	58.91	381.32	***	227.65	***	Yes
Minc08917	MiV1ctg303	HAT dimerization	29.96	22.99	1.97	***	1.95	***	Yes
Minc08919	MiV1ctg303	ATPase, F1 complex, delta/epsilon subunit	49.93	35.97	4.89	***	6.77	**	Yes
Minc09496	MiV1ctg337	Peptidase C1A, papain	80.47	59.24	230.25	***	187.71	***	Yes
Minc11497	MiV1ctg482	Laminin G; concavalin A-like lectin/glucanase; EGF-like region; neurexin	10.76	7.69	1.49	n.s.	3.35	n.s.	No
Minc11500	MiV1ctg482	Cell surface antigen; protein of unknown function GLTT	13.96	13.02	n.d.	n.d.	n.d.		n.d.
Minc11908	MiV1ctg519	Pioneer/hypothetical protein [Absidia glauca]	18.71	15.64	1.28	n.s.	2.33	**	Partly
Minc12829	MiV1ctg608	Peptidase S16; Lon protease	53.38	35.77	279.34	***	248.27	***	Yes
Minc12832	MiV1ctg608	K homology	35.52	28.05	1513.27	**	23.44	n.s.	Partly
Minc12833	MiV1ctg608	Striatin, N-terminal	23.36	21.32	1265.30	*	189.53	***	Yes
Minc12980	MiV1ctg624	Zinc finger, FLYWCH-type/transposase [Meloidogyne javanica]	27.77	17.97	1152.96	**	150.53	***	Yes
Minc12981	MiV1ctg624	Nucleotide-binding, alpha-beta plait	77.12	59.29	135.52	***	134.22	**	Yes
Minc13121	MiV1ctg640	Tubulin	50.63	31.84	1.54	n.s.	1.73	**	Yes
Minc13287	MiV1ctg661	Pioneer/hypothetical protein CAEBREN_24273 [Caenorhabditis brenneri]	24.93	21.41	1.10	n.s.	1.1	n.s.	No
Minc13288	MiV1ctg661	Synaptotagmin; C2 calcium/lipid binding region, CalB	31.87	26.02	234.37	n.s.	314.31	***	Yes
Minc13293	MiV1ctg661	Rhodopsin-like GPCR superfamily	22.41	15.68	143.74	**	138.85	***	Yes
Minc13392	MiV1ctg674	Zinc finger, FLYWCH-type/transposase [Meloidogyne javanica]	67.45	41.20	0.90	n.s.	1.34	n.s.	No
Minc19125	MiV1ctg2774	Pioneer/hypothetical protein SRAE_X000216100 [Strongyloides ratti]	34.09	21.08	n.d.		n.d.		n.d.

Abbreviations: n.d.: not determined; n.s.: not significant.

[a]A BLASTP search against NCBI database was performed for Mi1V1 pioneer genes. [b]CGH fold change between avirulent vs. virulent nematodes. [c]According to the $2^{-\Delta\Delta Ct}$ method Livak and Schmittgen (2001). [d]*, **, ***: significance at $p < 0.05$, 0.01 and 0.001, respectively. [e]Congruence was considered total when qPCR validation was significant for both Kursk and Morelos near-isogenic lines.

similar phenotypes (in terms of virulence) in independent lineages (Bailey & Bataillon, 2016). In that respect, we were primarily interested in identifying CNVs differentiated between avirulent vs. virulent nematodes that were shared by the two pairs of NILs analysed, that is, that resulted from the adaptive evolution of the same trait (i.e., virulence) in independent lineages according to parallel evolution. Indeed, this could provide evidence of positive selection for the trait of interest, considering that genetic drift alone is unlikely to produce such concerted changes in independent lineages (Hirase, Ozaki, & Iwasaki, 2014; Rundle, Nagel, Boughman, & Schluter, 2000).

4.1 | Gene copy number variation is associated with nematode adaptation to plant resistance

The comprehensive genomewide landscape of gene CNVs associated with the virulence of the nematode resulted in a set of 184 genes differentially affected by CNV between avirulent vs. virulent NILs. Previous observations highlighted the occurrence of massive duplications that led to the formation of large multigene families in the genome of *M. incognita*, as illustrated for, for example, genes encoding cell wall-degrading enzymes (Danchin et al., 2010) or proteases (Castagnone-Sereno, Deleury, Danchin, Perfus-Barbeoch, & Abad, 2011). Indeed, it is commonly accepted that the genome of parthenogenetic RKNs can tolerate drastic structural variations, for example, various states of aneuploidy (Triantaphyllou, 1985), or series of synteny breakpoints within the different scaffolds of the genome assembly (Blanc-Mathieu et al., 2017; Castagnone-Sereno & Danchin, 2014), that may be favoured by a relaxed selection pressure for homologous chromosome pairing in the absence of meiosis. More generally, high rates of gene copy number variations have been documented in various other asexual organisms such as trypanosoma, the ciliate *Chilodonella uncinta*, the water flea *Daphnia pulex* or aphids. These observations support the hypothesis that the genomes of asexual species could presumably accumulate more CNVs than sexual ones (Colbourne et al., 2011; Duvaux et al., 2015; Minning, Weatherly, Flibotte, & Tarleton, 2011; Spring, Pham, & Zufall, 2013; The International Aphid Genomics Consortium 2010). The new finding here is that the same CNVs can occur de novo in parallel in the genome of independent *M. incognita* NILs exhibiting virulence against the *Mi-1.2* tomato resistance gene, that is, are associated with the adaptive response to the same environmental challenge.

4.2 | Genes affected by CNVs are not uniformly distributed in the nematode genome

More than half (51.1%) of the genes affected by CNVs in both couples of avirulent vs. virulent NILs were distributed in a limited number of super-contigs of the genome (33 out of 2,995). In addition, most of these genes are organized in clusters, some of them containing up to 18 adjacent genes. This CNV distribution throughout the *M. incognita* genome in a nonrandom manner suggests the occurrence of genomic regions more prone to evolve in response to the selection pressure of the host resistance. Such highly plastic genomic regions where mutations leading to copy number differences between individuals occur more frequently than expected, known as CNV hotspots, have already been documented in various eukaryotes, from either the animal or plant kingdom (Gokcumen et al., 2011; Jiang et al., 2014; Zmienko, Samelak, Kozlowski, & Figlerowicz, 2014).

Gene Ontology analysis of genes affected by CNVs identified several overrepresented functions playing a putative role in RKN parasitism. These functions include cell redox homeostasis (involved in detoxification of the environment), signal transduction, binding, peptidase activity (involved in the degradation of host tissues) and transport activity (Gahoi & Gautam, 2017; Petitot et al., 2016; Shukla et al., 2018). The overabundance of these functional categories may suggest a direct role of many of the identified CNVs in the ability of *M. incognita* to infect resistant plants. However, it could also indicate that the corresponding genes are simply under relaxed conditions of purifying selection (i.e., that additional copies of these genes are not deleterious, and their selective removal by purifying selection is not very active) and thus can better tolerate copy number fluctuations compared to other functions.

4.3 | Gene copy loss events in virulent genotypes

CNVs can result from different types of structural variations, such as deletions, translocations, inversions, tandem duplications and novel insertions (Hastings, Lupski, Rosenberg, & Ira, 2009). Among the 776 CGH probes exhibiting CNVs 92.8% had positive FC ratio indicating a higher copy number in the avirulent isolate vs. the virulent isolate (i.e., loss in the derived virulent isolates). From an evolutionary point of view, such a prevalence of gene copy losses may seem quite surprising, as deletions are expected to be less frequent than duplications, because they should be eliminated by purifying selection when they result in a loss of function (Locke et al., 2006).

Several genes involved in pathogenicity in various plant pathosystems were shown to vary significantly in copy numbers between avirulent and virulent NILs. Three independent genes encoding peptidase S16 (Lon protease) were identified as significantly affected by CNV, and this enzyme is involved in pathogenicity in the bacteria *Pseudomonas syringae* (Lan, Deng, Xiao, Zhou, & Tang, 2007; Zhou et al., 2016) and the fungus *Magnaporthe oryzae* (Li et al., 2015). A gene encoding a peptidase belonging to the C1 family (i.e., C1A; papain) also exhibited CNV supported by both aCGH and qPCR analyses, and this protease family is thought to be directly related to the parasitic aspects of the plant–nematode relationship, for example, pathogenicity and/or evasion of primary host plant defence systems, in the burrowing nematode *Radopholus similis* (Wang et al., 2016) and in *M. incognita* (Neveu, Abad, & Castagnone-Sereno, 2003a; Shingles, Lilley, Atkinson, & Urwin, 2007). A third gene of particular interest showed strong homology with a transposase of *M. javanica*, a RKN species closely related to *M. incognita*. Noticeably, cDNA-AFLP analysis of two *M. javanica* NILs, avirulent or virulent to *Mi-1.2* resistant tomatoes, allowed the characterization of *Cg-1*, a member of a small gene family with one or more copies missing in the virulent isolate compared with the avirulent isolate, and required for nematode virulence (Gleason, Liu, & Williamson, 2008). Further work

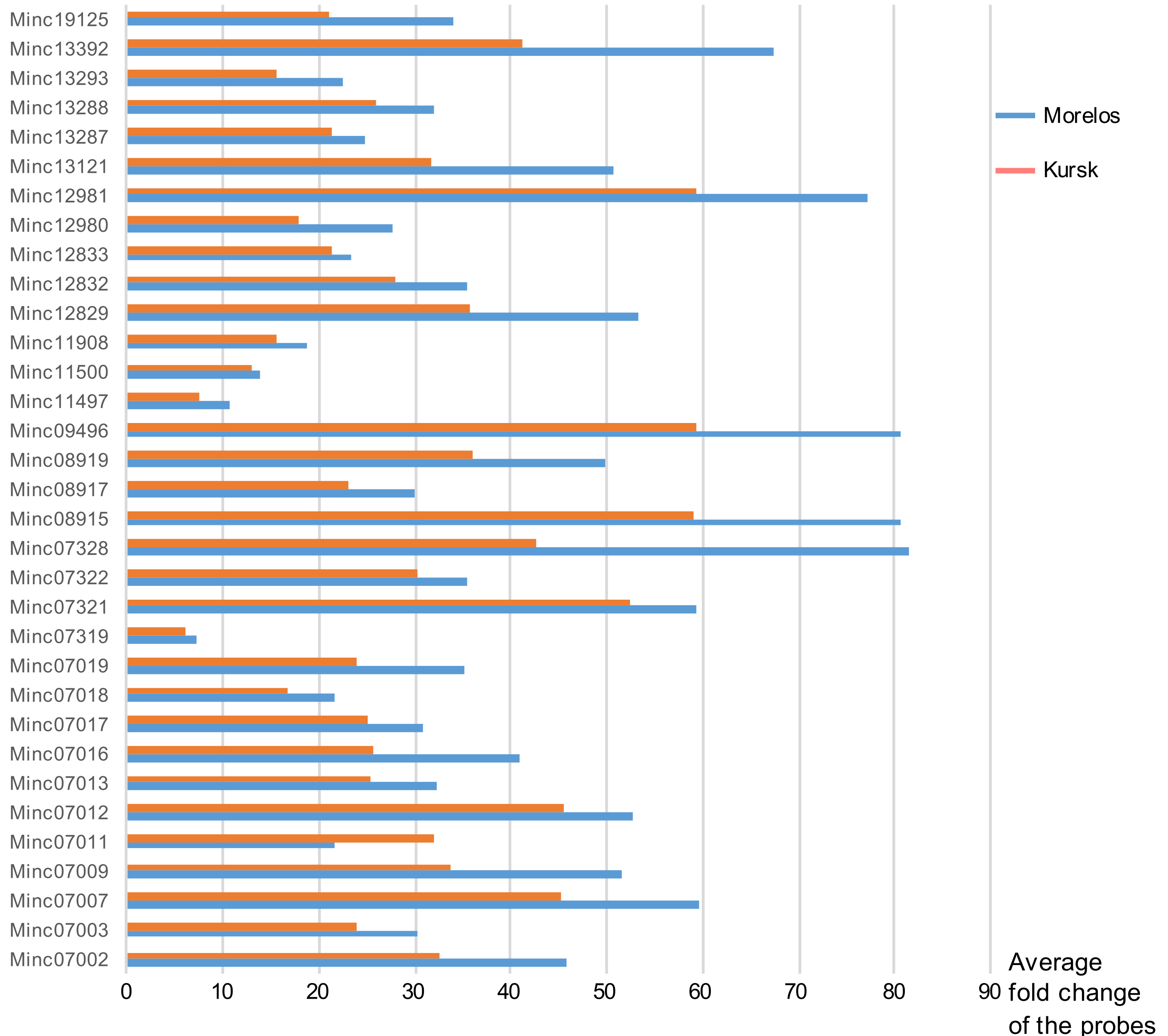

FIGURE 4 Average fold changes of the 33 genes exhibiting differential copy number variations (CNVs) in *Meloidogyne incognita* avirulent and virulent near-isogenic lines from Kursk and Morelos, as selected by a custom, highly stringent CNV-calling pipeline (see Materials and methods for details)

demonstrated that the *Cg-1* gene lies within a member of a novel transposable element family, Tm1, from the *Mutator* transposon superfamily (Gross & Williamson, 2011).

The qPCR validation of a subset of genes of interest largely confirmed the aCGH initial result, that is, gene copies losses in the virulent lines. This outcome is coherent with previous experimental data obtained at the individual gene level in some plant–nematode interactions. For example, in a previous comparative AFLP study, the *map-1* gene family, encoding expansin-like proteins, presented CNVs between *M. incognita* avirulent and virulent NILs (Castagnone-Sereno, Semblat, & Castagnone, 2009; Semblat et al., 2001). In particular, some members of the family (i.e., *map-1.2* and *map-1.3*) were deleted in the virulent NILs (Castagnone-Sereno et al., 2009; Semblat et al., 2001). In the same way, the *HgSLP-1* gene encoding a SNARE-like protein exhibited reduced copy number in virulent populations of the soybean cyst nematode, *Heterodera glycines*, that were able to overcome the *Rhg1* resistance gene (Bekal et al., 2015). In line with these observations, the present work, conducted at the whole-genome scale, revealed an unprecedented level of magnitude of gene copy loss events in virulent genotypes and thus supports the view that gene loss could be a common class of adaptive genetic change in response to the stress generated by host resistance in plant-parasitic nematodes. Recent studies have highlighted the importance of adaptive gene losses as a prevalent evolutionary force that affects organisms from all life kingdoms and contributes to morphological, physiological and metabolic adaptations to changes in

environmental conditions (e.g., Casewell, 2016; Sharma et al., 2018). From a mechanistic point of view, the loss of a given gene may result from either the drastic loss of a DNA fragment (e.g., following the mobilization of a transposable element), or the slow, iterative accumulation of mutations leading to a final loss of function (e.g., during pseudogenization). Although very documented in unicellular organisms (Hottes et al., 2013), the role of gene loss as a major mechanism of adaptive evolution might have been underestimated, especially in multicellular organisms (Albalat & Cañestro, 2016). From this point of view, our results here further illustrate this hypothesis in the case of a metazoan parasite.

5 | CONCLUSION

In conclusion, our findings allowed the characterization of genes exhibiting CNV that reflect genomic variations in the parthenogenetic nematode *M. incognita* in response to the breaking down of host resistance. These variations in gene copy numbers (mainly gene losses) might explain the differential capacity of virulent and avirulent *M. incognita* NILs to parasitize resistant plants, respectively. However, further functional investigation of these candidate genes will be needed to better understand the impact of CNV on *M. incognita* adaptation to plant resistance. This study provides a new catalogue of genes to be functionally tested in the future for their implication in the recognition of nematodes by plant defence systems. More generally, this suggests an important role of CNV in the adaptive evolution of parthenogenetic animals that would deserve to be investigated in other models.

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AUTHOR CONTRIBUTION

P.C.S. designed the experiment with inputs from E.G.J.D., L.P.B. and P.A. K.M., M.K. and L.P. performed the laboratory work. M.D.R., G.D.K, EG.J.D and M.B.B. performed the bioinformatics statistical analyses. P.C.S. wrote the original draft of the manuscript, which was further implemented by P.A., E.G.J.D., M.B.B. and L.P.B. All co-authors read and approved the final version of the manuscript.

DATA ACCESSIBILITY

The raw aCGH data sets were compressed in one file available from the Dryad Digital Repository (DOI: https://doi.org/10.5061/dryad.r0s4 ft2). The *Meloidogyne incognita* genome sequence used in this analysis is available at the NCBI under accession number ASM18041v1 (BioSample: SAMEA2272339; BioProject: PRJEA28837; WGS Project: ABB01). The genome annotation and browser are available at https://meloidogyne.inra.fr/Downloads/Meloidogyne-incognita-V1-2008. In addition, the sequence data of *M. incognita* avirulent and avirulent strains from Kursk and Morelos isolates have been deposited in SRA under BioProject PRJNA526433 and are available at https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA526433.

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SUPPORTING INFORMATION

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