Towards enhanced shelf life and performance: Genetic improvement and quality control of the entomopathogenic nematode *Heterorhabditis bacteriophora*

Dissertation

submitted for the Doctoral Degree

awarded by the Faculty of Agricultural and Nutritional Sciences

of the Christian-Albrechts-Universität zu Kiel

submitted by

M.Sc. Christopher Ogaya

born in Mulago, Kampala, Uganda

Kiel, 2023

Dean: Prof. Dr. Georg Thaller

1. Examiner: Prof. Dr. Ralf-Udo Ehlers

2. Examiner: Prof. Dr. Daguang Cai

Day of oral examination: 24th January, 2024

„Gedruckt mit der Genehmigung der Agrar-und Ernährungswissenschaftlichen Fakultät der Christian-Albrechts-Universität zu Kiel“

Table of contents

1. Introduction……....1
2. Results and discussion…….....7
   2.1. Optimal cold storage conditions of formulated *H. bacteriophora* nematodes defined…....7
   2.2. *Photorhabdus laumondii* single copy genes identified and used for bacteria quantification.....8
   2.3. SNP molecular markers associated with traits relevant for global DJ quality identified……11
   2.4. *H. bacteriophora* SNP marker assisted breeding feasible……..12
   2.5. Traits relevant to *H. bacteriophora* global DJ quality improved by marker assisted breeding…….14
3. Summary……...18
4. Zusammenfassung…… 21
5. References…….....24

Annex 1: Publication 1……...33

Annex 2: Publication 2……...46

Annex 3: Publication 3……...57

Acknowledgements……74

1. General introduction

Heterorhabditis bacteriophora Poinar 1976 (Strongyloidea: Heterorhabditidae) is a biocontrol entomopathogenic nematode (EPN) effective against various soil-dwelling insect pests with world-wide distribution (Grewal *et al*., 2005; Dhakal *et al*., 2020). Their symbiotically associated bacteria of the genus *Photorhabdus* spp. Boemare, Akhurst and Mourant 1993 (Enterobacterales, Morganellaceae) are carried in the intestines of its dauer juveniles (DJ) (Endo & Nickle, 1991). The dauer is the only free living and infective stage responsible for host finding and invasion . For successful insect control and subsequent reproduction in the dead insect, the nematode must transfer viable cells of its symbiotic bacterium into the insect´s hemolymph (Han & Ehlers, 2000). Once inside the insect host, DJs exit the developmentally arrested dauer stage, a process termed DJ recovery, and regurgitate the symbiont cells into the hemocoel (Ciche *et al*., 2008). In tandem, both partners overcome the defense mechanisms of the insect, with bacteria multiplication resulting in insect death by septicemia within 24 – 72 hrs (Eliáš *et al*., 2020). The bacteria then metabolize the insect´s hemolymph providing essential food supply for growth and multiplication of the nematode. Depleting food reserves induce pre-dauer J2d stages during the J1 stage. The J2d acquire symbiotic bacteria followed by DJ formation prior to insect cadaver exit to seek for new hosts (Poinar, 1993). Without an immediate new host location, the DJ can survive in soil for several weeks (Susurluk & Ehlers, 2008). The bacterial symbiont must equally persist inside the DJ during this duration for successful subsequent insect control (Han & Ehlers, 2000).

Mirroring the nematode life cycle inside the insect, *H. bacteriophora* DJ are produced in industrial scale bioreactors (> 10,000 L) in *in vitro* monoxenic *Photorhabdus* spp. cultures for commercial use. These advances in the monoxenic liquid culturing techniques have made EPN production efficient, consequentially lowering production costs (Strauch *et al*., 1998; Ehlers *et al*., 2000; Ehlers, 2001). Bioreactor runs are optimized to ensure a high reproductive potential, a trait

characterized by two factors: i) DJ recovery (% of DJs leaving the arrested stage after inoculation), and ii) the final DJ yield (DJs ml^{-1} at the end of the culture process) (Johnigk *et al*., 2002; Strauch *et al*., 1998; Wang *et al*., 2023). At the end of the culture process, DJs are harvested by centrifugation and filtering, stored in tanks suspended in water and later formulated in an inert carrier prior delivery to the end users. The formulation step under low temperatures (< 10°C) and light desiccation conditions are crucial to guarantee DJ storage stability during transport and until application (Grewal & Peters, 2005). However, under formulation DJ mortality is accelerated with formulation type, nematode species and storage temperature all affecting DJ survival (Grewal, 2002). A reduction of DJ metabolism is attempted by storage at low temperature and a light desiccation inducing a quiescence resulting in a longer shelf life. A possible approach to further prolong shelf life is to enhance tolerance to even lower storage temperature of formulated *Heterorhabditis*. Negative effects of extreme storage temperatures (5 and 25°C) on formulated heterorhabditid nematodes are well elucidated (Strauch *et al*.,2000; Guo *et al*., 2017; Kagimu & Malan, 2019). Cooler storage conditions are, however, preferred, because nematode metabolism is reduced conserving DJ fat reserves, the primary source of energy (Grewal & Peters, 2005). This preferred cold storage temperature still requires optimization because the investigated storage temperature range is still very wide. Focusing on storage temperatures between 5 to 9°C could pinpoint accurate optimal temperature for storage of this species in formulation. Diatomaceous earth, for example, is a broadly used carrier for DJ formulation due to affordability, and capacity to provide a low water activity (aw-value) environment to the formulated DJs (Peters, 2016). These properties make it superior to alternate formulation types like alginate beads developed by Kagimu & Malan (2019), where DJ are not readily released from the beads. Regardless of the formulation type, the environment inside a formulation comprises of a combination of environmental stresses most times acting simultaneously like; cold stress, oxidative stress and desiccation stress, all of

which the DJ must contend with. Additionally, DJs must maintain high infectivity levels against target insect pests during the product's shelf life (Gaugler & Han, 2002). The ability to withstand these stresses can be considered as global DJ quality, hence each trait requires special attention.

H. bacteriophora DJ have accordingly been phenotyped for traits related to this global DJ quality. Reproductive potential in monoxenic liquid cultures (Johnigk *et al*., 2002), desiccation stress (Mukuka *et al*., 2010a), tolerance to temperature extremes (Ehlers *et al*., 2005; Shapiro-Ilan *et al*., 2006; Mukuka *et al*., 2010b), virulence (Godina *et al*., 2022) and oxidative stress (Sumaya *et al*., 2017) are some of the extensively phenotyped important traits in *H. bacteriophora*. Further, DJ tolerance to oxidative stress has been proposed as an accurate predictor for DJ longevity and persistence in soil, highlighting how crucial correlation studies are as substitutes to time consuming assays. This trait is also highly heritable ($h^2 = 0.9$) in *H. bacteriophora* indicating its malleability through conventional breeding (Sumaya *et al*., 2017).

H. bacteriophora has a short generation time, can be cultured *in vitro* easily, and reproduction is either by automixis or amphimixis enabling production of both inbred lines and hybrids (Lunau *et al*., 1993; Strauch *et al*., 1994; Johnigk *et al*., 1999). These characteristics make this species attractive for classical breeding. However, focusing on single traits for genetic improvement can elicit negative trade-off effect on other beneficial traits (Mukuka *et al*., 2010c). To address this drawback, pools of homozygous inbred lines with stable traits, comprising of a collection of strains with desirable traits can be generated (Bai *et al*., 2005; Sumaya *et al*., 2018). Improvement of these beneficial traits focuses primarily on nematode genetics. DJ virulence, however, is a trait greatly influenced by the nematode associated bacterial symbiont. Without the associated *Photorhabdus* spp. symbiont, this trait is lost in *H. bacteriophora* (Gerritsen & Smits, 1993; Han & Ehlers, 2000). Stress exposure is also detrimental to this trait (Shapiro-Ilan *et al*., 2015). However, to what extent virulence detriment is a direct consequence of symbiotic bacteria

deterioration is still unknown. Thus, methods are necessary to directly monitor the persistence and quality of the symbiotic bacteria inside the DJ during storage. Such techniques can be part of the routine quality control for EPN used as commercial biocontrol agents.

The most elaborate approaches to monitor and quantify *Photorhabdus* cells inside the DJ involved tracking GFP labelled *Photorhabdus* spp. cells retained inside the nematode intestine (Ciche *et al*., 2008). This very precise technique is, however, skill-demanding, requires handling of genetically modified organisms (GMO), and is cost-intensive and time-consuming. Viable bacteria plate counts from crushed DJs is not a reliable approach as one cannot be certain that all cells were obtained from the intestine. A quick method that can be applied on commercial nematode material is therefore needed. Quantitative PCR (qPCR), a robust and sensitive technique to quantify minute amounts of nucleic acids (Higuchi *et al*., 1992; Larionov *et al*., 2005), can alleviate these challenges as the technique has already been used as a tool for bacterial quantification (Maier *et al*., 2021). However, quantification of the *Photorhabdus* cells inside *H. bacteriophora* is still a major challenge owing to the confinement of the symbiotic bacteria inside the digestive track of the nematode DJ. This obstacle implies that whenever nematode DNA is extracted, small amounts of the bacterial DNA should be accessible and amplifiable through PCR reactions. The availability of the complete genome of *P. luminescens* subspecies *laumondii* published by Duchaud *et al*. (2003) with more than 4 million predicted genes can facilitate this task. These predicted genes are all potential candidates for primer design to quantify *Photorhabdus* spp. Additional draft genomes of *Photorhabdus* species are also available (Ghazal *et al*., 2016; Palma *et al*., 2016; Somvanshi *et al*., 2019). Conventionally, highly conserved house-keeping genes like the 16S rDNA gene are the default choice for molecular identification of *Photorhabdus* spp. (Machado *et al*., 2018). Unfortunately, the duplicity of this house-keeping gene in the bacteria genome makes it majorly

ideal for detection but may be erroneous for quantification (Větrovský *et al*., 2013). Highly conserved single copy genes are thus desired for robust quantification.

Concerning the partner nematode, the genome sequence of the *H. bacteriophora* TT01 strain has been published (Bai *et al*., 2013), and its gene model annotation has been revised (McLean *et al*., 2018). This nematode genomic information can be exploited for improvement of beneficial traits. Thousands of single nucleotide polymorphisms (SNPs), possible trait molecular markers, have been discovered in *H. bacteriophora* (Levy *et al*., 2020; Fu *et al*., 2021; Godina *et al*., 2022; Godina *et al*., 2023; Wang *et al*., 2023). Levy *et al*. (2020) identified up to 8,810 SNPs in 3 different *H. bacteriophora* strains with varying stress tolerance levels. Almost half of these SNPs were located within or 1,000 bp in the vicinity of predicted genes, providing the possibility that some of them may have a physiologic function directly related to DJ survival. Later, Godina *et al*. (2022) identified another 4,894 SNPs among a collection of 48 *H. bacteriophora* highly homozygous wild type inbred lines (WT ILs). By correlating phenotypic and genotypic data, SNP markers associated to virulence at low temperatures (Godina *et al*., 2022), stress tolerance (Godina *et al*., 2023) and DJ-recovery (Wang *et al*., 2023), have been discovered through association analysis. The application of these SNP markers as tools for initial screening of large numbers of nematode materials prior to laborious phenotyping has been shown (Godina *et al*., 2023). These identified SNP molecular markers can be precision tools in *H. bacteriophora* marker assisted breeding.

To achieve and guarantee high *H. bacteriophora* global DJ quality, a multi-faceted approach is required. Tweaking of technical factors relevant to improved shelf life through optimization of DJ cold storage conditions in formulation is crucial. To complement standard sand bioassays conventionally used for EPN quality control (Grewal & Peters, 2005), robust qPCR assays are required for monitoring the *Photorhabdus* spp. bacteria load in *H. bacteriophora* DJs.

Finally, available *H. bacteriophora* genomic information needs to be exploited to identify additional SNP markers associated with beneficial traits and combine beneficial traits in hybrids leveraging SNP markers for precision.

The objectives of this study were therefore:

- Optimize cold storage conditions of diatomaceous earth formulated *H. bacteriophora* DJs
- Optimize a qPCR technique for quantification and monitoring the *Photorhabdus* spp. symbiotic bacteria load during storage of *H. bacteriophora* DJs
- Characterize *H. bacteriophora* WT ILs for traits relevant to global DJ quality
- Identify SNP molecular markers associated with these beneficial traits
- Use SNP marker assisted breeding to combine traits relevant to global DJ quality in *H. bacteriophora* hybrids
- Phenotype new *H. bacteriophora* hybrids for respective improved traits

2. Results and discussion

2.1 Optimal cold storage conditions of formulated *H. bacteriophora* nematodes defined

The optimal cold storage conditions in diatomaceous earth formulation of two *H. bacteriophora* strains, a commercial strain HB4 and a hybrid strain D2D6, was determined by storing the respective nematode material at temperatures of 5, 6, 7, 8, and 9°C for 12 weeks and the time to 75% survival (MTS_{75}) was predicted. For both strains, better survival was recorded at higher storage temperatures, 28 days at 8°C for D2D6 and 25 days at 9°C for HB4. In contrast, the lowest survival for both strains was recorded in DJs stored at 5°C, 6 days for HB4 and 16 days for D2D6 respectively (Publication 1, Fig. 1). In congruence with the DJ survival data, the lowest virulence was recorded in DJs stored at 5°C, 6% and 46% for HB4 and D2D6, respectively, after 9 weeks of storage (Publication 1, Fig. 2). In industrial EPN production, several factors related to the growth conditions (temperature, medium composition, bacterial strain) and downstream processing (sieving, cleaning) may have effects on the shelf life of the final product. However, these previously mentioned factors may have subordinate effects when storage temperature is considered. Temperature is the primary factor driving DJ survival in formulation (Grewal, 2002). Kagimu & Malan (2019) showed remarkable difference in DJ survival between three storage temperatures of 6, 14 and 25°C of DJs stored in diatomaceous earth formulation. Almost no survival was recorded for DJ stored at 6°C after 4 weeks in comparison to survival above 80% for DJ stored at 14 and 25°C, albeit the survival being the average of three EPN species (*H. bacteriophora*, *Steinernema yirgalemense* and *S. jeffreyense*). Guo *et al*. (2017) also reported higher DJ survival of diatomaceous earth formulated *H. bacteriophora* after 30 days storage at 15°C (> 40%) in comparison to storage at 5°C (30%). In the present study, the lowest survival was recorded for both strains of *H. bacteriophora* DJs stored at 5°C . Grewal (2000) argues that the combination of cold

and desiccation stress could be detrimental to DJ due to a high metabolic cost of the latter that is not adequately compensated at low temperatures.

The associated symbiotic bacteria seem to also be detrimentally affected by low storage temperatures, possibly explaining the low virulence at 5°C. It is known that bacteria-free *Heterorhabditis* nematodes are unable to kill insects without their associated bacterial symbionts (Gerritsen *et al*., 1998). Kagimu and Malan (2019) likewise observed higher virulence in diatomaceous earth formulated DJ stored at 14°C and 25°C when compared to those stored at 6°C after four weeks. In fact, deterioration of DJ virulence is not only limited to cold temperature exposure but extends to other stress factors as shown to by Shapiro-Ilan *et al*. (2015) after DJ exposure to UV-light. qPCR tools to precisely track the bacteria load of *H. bacteriophora* were therefore developed.

2.2 *Photorhabdus laumondii* single copy genes identified and used for bacteria quantification

From the initial 2,036 predicted *Photorhabdus laumondii* DE2 gene models taken for BLAST analysis against NCBI bacterial accessions, a total of ten single copy genes with no homology to any other bacteria accessions were identified, seven of which were used for primer design (Publication 2, Table 3). Single copy genes are ubiquitous in bacteria (Wang *et al*., 2022), and this study yields ten such novel genes in *P. laumondii* employable for both quantification and detection of this species. However, Machado *et al*. (2018) highlights the complexity of *Photorhabdus* spp. species differentiation by using more stringent parameters for species delineation, consequentially his elevation of many former *P. luminescens* subspecies to species level. Two candidate single copy genes, CG2 and CG8 further highlighted the ambiguity of bacteria in this genus by amplifying DNA from *P. kayaii* and *P. thracensis*, both symbiotically associated with *H. bacteriophora*, but were negative for DNA from *P. temperata* and *Xenerhabdus*

nematophila symbiotically associated with *H. downsei* and *S. carpocapsae*, respectively. This hints at the specificity of these primers to only *H. bacteriophora* associated symbionts, however, a larger set of *Photorhabdus* spp. isolates can be tested to further clarify their specificity.

For *P. laumondii* quantification, a primer efficiency of 83% (slope -3.78, intercept 17.41) was determined for candidate gene CG2 after a qPCR assay with 5-point 1/10 genomic DNA dilution with a starting conc. of 32 ng (Publication 2, Fig. 2 A). Accurate absolute quantification of nucleic acids requires highly validated calibration curves with recommended primer efficiencies ranging between 90 – 100% (Bustin *et al*., 2009). We obtained a primer efficiency of 83% for the CG2 gene model, that we deemed sufficient for the purpose of monitoring *H. bacteriophora* DJ bacteria load.

In commercial strain HB4 DJs stored at 6°C in Ringer's solution for up to 300 days, the detected bacterial target gene CG2 copy number was 750-fold lower than in 25-day old fresh DJs (Publication 2, Fig. 3 A). We further proved the concept that detected bacteria amounts were directly proportional to the proportion of bacteria carrying DJs in a mixed artificial HB4 DJ population comprising of bacteria-free DJ. The detected bacterial CG2 target gene copy number decreased with the decreasing proportion of bacteria-carrying DJs (Publication 2, Fig. 3 B). Further, among *H. bacteriophora* WT strains IT4, IT6, IR2, cultured *in vivo* in *Galleria mellonella*, thereby carrying their native bacteria and WT ILs AU11, HU21, IR11, cultured *in vitro* on commercial symbiont *P. laumondii* strain DE2, larger CG2 target gene copy number was detected in WT strains than the WT ILs after > 300 days storage at 6°C (Publication 2, Fig. 3 C). Insect infectivity bioassays were also carried out with the respective materials: Commercial strain HB4 DJs stored at 6°C in Ringer´s solution for > 300 days (Publication 1, Fig. 1 A), WT strains and WT ILs stored for > 300 days (Publication 2, Fig. 1 B), and artificial populations of HB4 DJs with different proportions of bacteria carrying and bacteria-free DJs stored at 6°C for 1 week (Publication 2, Fig.

1 C). In the respective nematode material, insect infectivity was positively correlated with detected bacteria CG2 target gene copy number: Commercial HB4 DJ stored at 6°C in Ringer´s solution for > 300 ($R = 0.79$, $P = 0.06$), WT strains and WT ILs stored for > 300 days ($R = 0.88$, $P = 0.008$) and different artificial HB4 DJ populations with proportions of bacteria free- and bacteria-carrying DJs stored at 6°C for 1 week ($R = 0.96$, $P = 0.009$). This study avails direct evidence proving that decline in *H. bacteriophora* virulence is a consequence of either or both reduced symbiotic bacteria load and reduced proportion of bacteria carrying DJ in DJ populations. This further confounds the hypothesis of insect death being primarily due to the associated bacterial symbiont in *Heterorhabditis* spp. (Han *et al*., 1991; Gerritsen & Smits, 1993; Han & Ehlers, 2000). With storage duration, the infectivity of the DJ decreased. Consequently, the number of cells inside a DJ is an important quality parameter therefore we deployed this qPCR technique as a quality control tool on formulated EPN products.

A 4.5 and 1.8-fold decline in the bacteria CG2 target gene copy number was detected in formulated HB4 DJ stored at 2°C and 4°C, respectively after two weeks and by week 6 an 8-fold decline was recorded in DJ stored at both temperatures. In comparison, a slight increase in bacterial target gene copy number was detected in DJ stored at 7.5°C after 6 weeks (Publication 2, Fig. 5). Moreover, the insect infectivity and detected bacteria DNA copy numbers were also significantly positively correlated ($R = 0.68$, $P = 0.02$). Low storage temperatures are attractive for preservation of the nematode fat reserves, however, the effect on the bacterial symbiont should always be considered. Consistently apparent throughout this study were the negative effects of cold storage temperature on the virulence of diatomaceous earth formulated *H. bacteriophora* DJs (Publication 1, Fig. 2, Publication 3, Fig. 5). We can now partially attribute this to probably declined symbiotic bacterial load. Successful prolonged storage of nematodes in these storage conditions could require new combinations of *H. bacteriophora* DJ with cold adapted *Photorhabdus* spp. strains. Ehlers *et*

al. (2005) reported the availability of eight such *Photorhabdus* strains reproducing at lower temperature conditions. These could be perfect candidates for improved new combinations.

2.3 SNP molecular markers associated with traits relevant for global DJ quality identified

Just like in other eukaryotes, SNPs are the most abundant genetic markers in *H. bacteriophora* (Levy *et al*., 2020; Godina *et al*., 2022). After characterizing *H. bacteriophora* WT ILs for traits relevant to global DJ quality; oxidative stress tolerance at 2°C (predictor of cold tolerance) (Publication 1, Fig. 3), reproductive potential in liquid culture, oxidative stress at 25°C (predictor of DJ longevity), virulence against *Tenebrio mollitor* and against *Diabrotica virgifera* (Publication 3, Table 2), through association analysis, this phenotypic data were correlated with the WT ILs SNP genotypic data availed by the works of Godina *et al*. (2022) and respective beneficial trait associated SNP markers identified. Four of these SNP markers were significantly associated with cold tolerance (Publication 1, Table 2), four with reproductive potential in liquid culture, two with both, longevity and virulence against *T. mollitor*, four associated exclusively with virulence against *T. mollitor* and one with virulence against *D. virgifera* (Publication 3, Table 3). This study thus yielded fourteen novel trait associated SNP markers in this collection of WT ILs complementary to the two associated with virulence at low temperature identified by Godina *et al*. (2022).

To determine if these SNP markers were in the vicinity of relevant genes from the SNP marker location, the *H. bacteriophora* TTO1 genome deposited at NCBI by Bai *et al*. (2013) was screened up- and down-1000 bp for presence of any genes. Among the four SNP markers associated with cold tolerance, only one (SNP3) was located inside transcript g9820 reported to code for *H. bacteriophora* cell surface receptor (*daf-4*) gene, a homologue to the *Ceanorhabditis elegans daf-4* gene. Among the functions of the *daf-4* gene in *C. elegans* (a cell surface receptor) are inhibition

of dauer formation (Estevez *et al*., 1993), and regulating quiescence entry that is triggered by satiety (You *et al*., 2008). To date there is no report linking this gene with survival related traits.

Surprisingly none of the SNP markers in this study were identical to the 20 identified by Godina *et al*. (2023) even for similar traits like oxidative stress, though both SNP marker SNP1 (Publication 1, Table 2) and SNP marker OS9 (Godina *et al*., 2023) associated with oxidative stress tolerance were localized on scaffold1330. What was predominant was localization of SNPs associated with different traits on the same scaffold. SNP markers RP2 and RP3 associated with reproductive potential (Publication 3, Table 3) shared scaffold1351 with SNP marker LH3 associated with longevity from Godina *et al*. (2023). SNP marker RP1 (reproductive potential) (Publication 3, Table 3) and HT1 (Godina *et al*., 2023) associated with heat tolerance were localized on scaffold1357. Scaffold1080 localized SNP marker RP4 (reproductive potential) (Publication 3, Table 3) and SNP marker LH1 associated with longevity (Godina *et al*., 2023). Finally, virulence SNP marker V1 (Publication 3, Table 3) and oxidative stress tolerance SNP marker OS8 (Godina *et al*., 2023) were both localized on scaffold1351. Without extensive functional gene studies in *H. bacteriophora* like done in *C. elegans* (Evans *et al*., 2021), we can only speculate that this localization of these SNP markers on the same scaffold could be due to pleiotropy. However, they are excellent molecular markers for breeding purposes and the uncharacterized genes on scaffolds of interest can be considered important for further investigation. The discovered beneficial trait associated SNP markers were therefore used for marker assisted breeding.

2.4 *H. bacteriophora* SNP marker assisted breeding feasible

A global genotype screening of all analyzed WT ILs for presence of SNP markers associated with global DJ quality identified WT IL XX21 possessing a combination of two SNP markers associated with high cold tolerance (CT1, CT2), two SNP markers associated with high

reproductive potential (RP1, RP2), one SNP marker associated with both longevity and virulence (L/V1) plus two SNP markers associated exclusively with virulence against *T. mollitor* (V1, V2) (Publication 3, Table 4). Phenotypically XX21 also had a high longevity at 25°C (11.0 ± 2.9 days), high reproductive potential (180 ± 28 x 10^3 DJ ml^{-1}), high virulence against *T. mollitor* (71.6 ± 4.2 %), moderate virulence against *D. virgifera* (57.0 ± 10.6 %) (Publication 3, Table 3) and high cold tolerance at 2°C (16.7 ± 0.7 days) (Publication 1, Fig. 3), therefore was selected as a candidate for a genetic cross with a commercial line IL3. The progeny from the genetic cross between these two lines were expected to possess two additional SNP markers associated with virulence against *T. mollitor* donated by the commercial line IL3 (V3, V4) (Publication 3, Table 4).

Genetic improvement of EPN is predominantly through classical breeding (Glazer, 2015), first involving phenotypic characterization of a diverse collection of nematode material followed by hybridization and or artificial selection for the desirable trait (Strauch *et al*., 2004; Mukuka *et al*., 2010c). We forewent phenotyping the 55 homozygous recombinant inbred lines (RILs) resulting from the XX21 x IL3 cross herein called X21L3 for traits relevant to global DJ quality. We instead genotyped them with the seqSNP-platform reported by Godina *et al*. (2023) yielding robust genotypes of which 33 SNPs (17,1%) with minor allele frequency (MAF) ≥ 0,1 showed polymorphisms between both parents and segregation among the progeny RILs. This verified the success of this cross. We leveraged available SNP marker information to rapidly identify 22 promissory X21L3 RILs possessing nine beneficial SNP markers thus omitting the need for prior phenotyping of individual RILs. A single genotypic pool comprising of these 22 X21L3 RILs was consequentially formed (Publication 3, Table 4). Phenotyping was thereby limited to this pool, its parents (XX2 and IL3) and another commercial line HB4.

2.5 Traits relevant to *H. bacteriophora* global DJ quality improved by marker assisted breeding

Reproductive potential, characterized by DJ recovery (%) and final DJ yield (ml^{-1}) of the X21L3 pool was compared to the parental lines and the commercial line HB4. The highest DJ recovery was recorded in the commercial line HB4 (74 ± 2.1 %), while XX21 had the lowest recovery (51 ± 1.99 %). However, for final DJ yield, the highest DJ yield was observed for IL3 (236 ± 4.77 × 10^3 DJ ml^{-1}), while XX21 had the lowest DJ yield (193 ± 2.88 × 10^3 DJ ml^{-1}). Interestingly DJ recovery (58 ± 1.7 %) and DJ yield (203 ± 71.4 × 10^3 DJ ml^{-1}) for the X21L3 pool were higher than for the parental line XX21 hinting at trait improvement. It is noteworthy that even though the X21L3 pool had a lower recovery than the commercial line HB4, no significant difference was observed between the two in final DJ yield (Publication 3, Fig. 2) showing the potential of this X21L3 pool. RILs included in this pool possessed two DJ yield associated SNP markers (RP1 and RP2) and this is reflected in the phenotype of this trait, which is comparable to the commercial line HB4. Owing to the high number of RILs in this pool (22), further increase in reproductive potential through selection is envisioned because subsequent sub-culturing will always favor the high reproducing RILs resulting in further trait stability. Specifically, DJ yield is a highly heritable trait (Johnigk *et al*., 2002).

To mitigate time required for DJ longevity and cold tolerance evaluation, DJ were exposed to oxidative stress conditions at 2ºC and 25ºC. Exposure to 70 mM H_2O_2 is an accurate predictor of DJ longevity in *H. bacteriophora* (Sumaya *et al*., 2017). Under cold storage temperature (2ºC), the X21L3 pool had the longest survival with a mean time to 50% survival (MTS_{50}) of 8.1 ± 0.48 days, whereas the parental line IL3 had the shortest survival time (5.7 ± 0.51 days) (Publication 3, Fig. 3 b). This heterosis effect was also observed by Sumaya *et al*. (2018) with the hybrid pool living 2.6 days longer than the parents, further confirming the heritability of this trait. Oxidative

stress at 25ºC storage was similarly improved in the X21L3 pool (MTS_{50} 5.6 ± 0.54 days) compared to the less stress tolerant parental line IL3 (MTS_{50} 4.5 ± 0.42 days) (Publication 3, Fig. 3 c). Phenotypic results from reproductive potential and oxidative stress tolerance unrefutably show the precision of SNP marker assisted breeding in EPN trait improvement.

It was crucial to determine whether this enhanced stress tolerance of the X21L3 hybrid pool would translate into improved cold storage stability in diatomaceous earth formulation. Thus, the X21L3 pool, the respective parents and commercial line HB4 were characterized for this trait in diatomaceous earth formulation at 2 and 7.5°C storage temperature. At 2ºC, the commercial line HB4 survived longest, MTS_{75} of 38 ± 3.6 days, while the oxidative stress tolerant XX21 had the shortest survival time, MTS_{75} of 14 ± 1.8 days. Regardless, minimal trait improvement, MTS_{75} 16 ± 1.8 days of the X21L3 pool was recorded (Publication 3, Fig. 4 a). At 7.5ºC storage, the commercial line HB4 still survived longest with an MTS_{75} of 52 ± 2.6 days whereas the parental line XX21 survived the shortest (26 ± 1.5 days). Similarly, the X21L3 pool also had a higher MTS_{75} of 31 ± 1.6 days than the parental line XX21 (Publication 3, Fig. 4 b). A marginal nematode genotype contribution to cold storage stability in formulation at 2°C was also evidenced by the better survival of cold tolerant WT IL IR11 (MTS_{50} 9.9 ± 0.3 days) in comparison to the less cold tolerant line PT11 (MTS_{50} 6.5 ± 1.4 days) (Publication 1, Fig. 5). These two WT ILs had extreme cold tolerance phenotypes (Publication 1, Fig. 3). These results hint at *H. bacteriophora* survival in diatomaceous earth formulation not being dictated only by the nematode genotype. Of note though is that SNP markers for desiccation tolerance, a very important trait in diatomaceous earth formulated nematodes, were absent in the X21L3 pool, thus require consideration in future. However, it is plausible that in formulation the symbiotic bacteria could be playing an outsized role in nematode survival possibly explaining why even at the optimal storage temperature of 7.5°C,

X21L3 and the oxidative stress tolerant parent XX21 had a lower survival. IL3 and the commercial line HB4 have been sub-cultured for years in the commercial DE2 bacteria hence might have developed better adaptations with this commercial strain. Further, HB4 is a cross between the stress tolerant HU21 and commercial line IL3 probably partly explaining its better storage stability in formulation. In contrast, XX21 and X21L3 are so far cultured only minimally in the commercial DE2 bacteria. Evidence exists linking symbiotic bacteria quality to *H. bacteriophora* stress tolerance (Bilgrami *et al*., 2006). Perennial lab sub-culturing induced selection pressure on quantitative nematode traits in *C. elegans* is also well elucidated (McGrath *et al*., 2009; Sterken *et al*., 2015). This bacteria-nematode relationship in formulation thus requires deeper scrutiny.

Respective DJs previously stored at 2ºC and 7.5ºC in formulation were evaluated for virulence along storage time by monitoring mortality (%) of *T. mollitor* larvae. For DJ virulence (%) at 2ºC, despite minimal survival in formulation, initial virulence of the virulent parental line XX21 was the highest (96.9 ± 1.81 %) and remained highest after two weeks (92.5 ± 1.76 %) and four weeks (74 ± 1.93 %), respectively. In comparison, the hybrid pool X21L3 had the lowest virulence initially (78.8 ± 2.28 %) and after two weeks (59.2 ± 2.38 %). Only after four weeks was the lowest virulence recorded in IL3 (48 ± 2.7 %) (Publication 3, Fig. 5 a). After 2 weeks, the virulence (%) at 7.5ºC of parent XX21 again was the highest (95 ± 1.95 %), while the X21L3 pool was the lowest (76.46 ± 2.18 %). After 4 weeks (78.75 ± 2.53 %) and 6 weeks (76. 04 ± 1.82 %) X21L3 still had the lowest virulence in comparison to the parents (Publication 3, Fig. 5 b). Virulence is a complex trait requiring contribution of both nematode and bacterium partners for successful infection (Han & Ehlers, 2000). Despite lower survival in formulation, virulent parent XX21 still maintains this trait in diatomaceous earth formulation. However, this was the only trait that was not significantly improved in the X21L3 pool after storage in formulation further highlighting its complexity. Regardless, hybrid pool X21L3 virulence was above the quality control

threshold of 50% insect mortality after 6 weeks of storage. We expect improvement in storage stability in the X21L3 pool with subsequent sub-culturing, as the same phenomena was observed for the commercial line HB4. Moreover, this study has provided robust qPCR tools employable in quantifying the symbiotic bacteria load in the respective materials as demonstrated for bioreactor produced and formulated HB4 DJs stored at different temperatures (Publication 2, Fig. 5).

In conclusion, this study fast-tracked the *H. bacteriophora* breeding program by first identifying 14 novel SNP markers relevant to global DJ quality and leveraged them for SNP marker assisted breeding thereby providing a stress tolerant hybrid X21L3 pool. We also avail a robust qPCR tool deployable in the *Photorhabdus* spp. nematode bacterial load monitoring during storage. Optimal cold storage conditions of diatomaceous earth formulated *H. bacteriophora* DJs have been defined to between 7 - 9°C.

3. Summary

Limited shelf-life is a major constraint to successful commercialization of the entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* that is symbiotically associated with bacteria from the genus *Photorhabdus* spp. Together, this nematode-bacterium partner is used to control insect pests. To extend shelf-life, infective dauer juveniles (DJ) of *H. bacteriophora* are formulated and stored at low storage temperatures (<10°C). Optimization of storage conditions and improvement of quality control parameters during storage can enhance *H. bacteriophora* shelf-life. Further, the available genetic diversity of different *H. bacteriophora* strains can be exploited to identify better performing strains.

To determine optimal cold storage conditions of *H. bacteriophora* DJs formulated in diatomaceous earth, the cold storage potential of two strains was evaluated at temperatures between 5 - 9°C. When assessing DJ decline to reach 75% survival (MTS_{75}) at the respective storage temperatures, *H. bacteriophora* strain HB4 had the longest survival of 25 days at 9°C while strain D2D6 survived longest at 8°C for 28 days. Highlighting the negative effects of low storage temperatures, the lowest virulence was recorded in DJ stored at 5°C, 6% and 46% for HB4 and D2D6, respectively, after 9 weeks of storage. Given that *H. bacteriophora* DJs are stored in water prior to formulations, the cold tolerance at 2°C of 22 wild type inbred lines (WT ILs) stressed with 70 mM H_2O_2, an accurate predictor of DJ longevity, was of interest. The MTS_{50} (time to 50% survival) ranged from 12 to 24 days. The survival of the two WT ILs (PT11 and IR11) with opposite extreme cold tolerance phenotypes was further evaluated in diatomaceous earth formulation at 2°C with the cold tolerant IR11 surviving 3 days longer than PT11.

During storage, *H. bacteriophora* based biological control products can vary in infectivity (quality). However, the influence of storage conditions on their symbiotic bacteria *Photorhabdus* spp. inside the DJ has not received much attention despite the crucial bacteria role in insect

infectivity. We therefore optimized a method to quantify the bacterial load inside the DJ based on a qPCR technique. Information from the genome of *P. laumondii* strain DE2 was used to identify single copy genes with no homology to any other bacterial accessions. One gene was then selected for primer pairs design. Cross-amplification tests with *P. thracensis* and *P. kayaii*, also symbionts of *H. bacteriophora*, were positive, whereas no amplicons were produced for *P. temperata* or *Xenorhabdus nematophila*. To quantify *Photorhabdus* spp. cells inside the DJ and investigate the effect of storage temperature on diatomaceous earth formulated *H. bacteriophora* DJs, the bacterial load of nematodes stored at 2, 4 and 7.5°C for 6 weeks was quantified. A 4.5 and 1.8-fold decline in the bacteria DNA copy number was detected in DJs stored at 2°C and 4°C, respectively, after two weeks and by week 6, an 8-fold decline was recorded in DJs stored at both temperatures. In comparison, a slight increase in bacterial target gene copy number was detected in DJs stored at 7.5°C after 6 weeks. Moreover, the insect infectivity and detected bacteria DNA copy numbers were also significantly correlated ($R = 0.68$, $P = 0.02$). This qPCR approach is complementary to standard bioassays for quality control of *H. bacteriophora* biological control products.

To enhance *H. bacteriophora* shelf life, its genetic diversity can be exploited. A collection of *H. bacteriophora* WT ILs was therefore phenotyped for additional traits relevant to global DJ quality, reproductive potential, stress tolerance and virulence. However, these traits are rarely combined in a single line. Respective genotypic and phenotypic data were thereafter correlated, and fourteen single nucleotide polymorphisms (SNPs) molecular markers associated with these traits identified. Thus, unifying these traits in commercial strains was of high priority through marker-assisted breeding. Recombinant inbred lines (RILs) from a cross between a stress tolerant WT inbred line (XX21) and a commercial line (IL3) were genotyped via SeqSNP and screened for SNP markers associated to beneficial traits. Thereafter a genotypic pool (X21L3) comprising of 22 WT ILs was formed. The X21L3 pool was subsequently evaluated for the target traits in

comparison to the cross parents and a commercial strain HB4. A remarkable improvement in oxidative stress tolerance at 2ºC (cold tolerance) was recorded with X21L3 surviving 1 day longer than the best performing parent (XX21). This new hybrid pool also survived 1 day longer than the least performing parent IL3 for the trait of oxidative stress tolerance at 25ºC (longevity) and recorded a higher DJ recovery (58%) and DJ yield (209,000 DJ ml^{-1}) than the least performing parent XX21. The storage stability in diatomaceous earth formulation at 2ºC and 7.5ºC was also improved by 2 days and 5 days, respectively, in comparison to the least performing parent XX21. This study optimized both technical and genetic aspects towards enhancement of *H. bacteriophora* shelf-life.

4. Zusammenfassung

Die begrenzte Haltbarkeit ist ein wesentliches Hindernis für die erfolgreiche Vermarktung des entomopathogenen Nematoden (EPN) *Heterorhabdistis bacteriophora*, der in Symbiose mit Bakterien der Gattung *Photorhabdus* spp. lebt. Dieser Nematoden-Bakterien-Partner wird zur Bekämpfung von Insektenschädlingen eingesetzt. Für eine möglichst lange Haltbarkeit werden infektiöse Dauerjuvenile (DJ) von *H. bacteriophora* formuliert und bei niedrigen Temperaturen (<10°C) gelagert. Eine weitere Optimierung der Lagerungsbedingungen und eine Verbesserung der Qualitätskontrollparameter während der Lagerung können die Haltbarkeit von *H. bacteriophora* weiterhin verlängern. Außerdem kann die vorhandene genetische Vielfalt der verschiedenen *H. bacteriophora*-Stämme genutzt werden, um leistungsfähigere Stämme zu identifizieren.

Um die optimalen Bedingungen für die Kühllagerung von in Kieselgur formulierten *H. bacteriophora* DJ zu ermitteln, wurde das Kühllagerungspotenzial von zwei Stämmen bei Temperaturen zwischen 5 und 9 °C bewertet. Bei der Bewertung des Rückgangs der DJ bis zum Erreichen einer Überlebensrate von 75 % (MTS_{75}) bei den jeweiligen Lagertemperaturen wies der *H. bacteriophora*-Stamm HB4 die höchste Überlebensrate von 25 Tagen bei 9 °C auf, während der Stamm D2D6 bei 8 °C für 28 Tage am längsten überlebte. Die negativen Auswirkungen niedriger Lagertemperaturen wurden verdeutlicht durch die Feststellung der geringsten Virulenz bei DJ, die bei 5°C gelagert wurden: 6% Mortalität der Insekten für HB4 und 46% für D2D6 nach 9 Wochen Lagerung. Da *H. bacteriophora* DJ vor der Formulierung in Wasser gelagert werden, war die Kältetoleranz bei 2°C von 22 Wildtyp-Inzuchtlinien (WT-ILs), die mit 70 mM H_2O_2 gestresst wurden, ein zuverlässiger Prädiktor für die Langlebigkeit von DJ von Interesse. Die MTS_{50} (Zeit bei der noch 50% der Tiere leben) lag zwischen 11 und 23 Tagen. Das Überleben der beiden WT-ILs (PT11 und IR11) mit entgegengesetzten extremen Kältetoleranzphänotypen wurde weiter in

einer Kieselgurformulierung bei 2°C bewertet, wobei die kältetolerante IR11 3 Tage länger überlebte als PT11.

Während der Lagerung können biologische Bekämpfungsmittel auf der Basis von *H. bacteriophora* in ihrer Infektiosität (Qualität) variieren. Der Einfluss der Lagerungsbedingungen auf die symbiontischen Bakterien *Photorhabdus* spp. im Inneren der DJ wurde bisher jedoch kaum beachtet, obwohl die Bakterien eine entscheidende Rolle für die Infektiosität gegen Insekten spielen. Wir haben daher eine Methode zur Quantifizierung der Bakterien im Inneren der DJ auf der Grundlage einer qPCR-Technik optimiert. Informationen aus dem Genom des *P. laumondii*-Stammes DE2 wurden verwendet, um Gene mit einer einzigen Kopie zu identifizieren, die keine Homologie zu anderen bakteriellen Akzessionen aufweisen. Ein Gen wurde schließlich für die Entwicklung von Primerpaaren ausgewählt. Kreuzamplifikationstests mit *P. thracensis* und *P. kayaii*, ebenfalls Symbionten von *H. bacteriophora*, verliefen positiv, während für *P. temperata* oder *Xenorhabdus nematophila* keine Amplikons erzeugt wurden. Um die Zellen von *Photorhabdus* spp. innerhalb der DJ zu quantifizieren und die Auswirkungen der Lagertemperatur auf die mit Kieselgur formulierten *H. bacteriophora* DJ zu untersuchen, wurde die bakterielle Beladung der Nematoden, die 6 Wochen lang bei 2, 4 und 7,5 °C gelagert wurden, quantifiziert. Bei DJ, die bei 2°C bzw. 4°C gelagert wurden, wurde nach zwei Wochen ein 4,5- bzw. 1,8-facher Rückgang der Bakterien-DNA-Kopienzahl festgestellt, und nach Woche 6 wurde bei DJ, die bei beiden Temperaturen gelagert wurden, ein 8-facher Rückgang verzeichnet. Im Vergleich dazu wurde bei DJ, die bei 7,5 °C gelagert wurden, nach 6 Wochen ein leichter Anstieg der Kopienzahl des bakteriellen Zielgens festgestellt. Außerdem war die Infektiosität gegen Insekten und die nachgewiesene Bakterien-DNA-Kopienzahl signifikant korreliert (R = 0,68, P = 0,02). Dieser qPCR-Ansatz ergänzt die Standard-Bioassays zur Qualitätskontrolle von biologischen Pflanzenschutzmitteln auf Basis von *H. bacteriophora*.

Um die Haltbarkeit von *H. bacteriophora* zu verbessern, kann seine genetische Vielfalt genutzt werden. Eine Sammlung von *H. bacteriophora* WT-ILs wurde daher auf zusätzliche Merkmale hin phänotypisiert, die für die globale DJ-Qualität, das Fortpflanzungspotenzial, die Stresstoleranz und die Virulenz relevant sind. Diese Merkmale sind jedoch nur selten in einer einzigen Linie kombiniert. Die entsprechenden genotypischen und phänotypischen Daten wurden anschließend korreliert und 14 molekulare Marker mit Einzelnukleotid-Polymorphismen (SNPs) identifiziert, die mit diesen Merkmalen in Verbindung stehen. Die Vereinigung dieser Merkmale in kommerziellen Stämmen durch markergestützte Züchtung hatte daher hohe Priorität. Rekombinante Inzuchtlinien (RILs) aus einer Kreuzung zwischen einer stresstoleranten WT-IL XX21 und einer kommerziellen Linie IL3 wurden mittels SeqSNP genotypisiert und auf SNP-Marker untersucht, die mit vorteilhaften Merkmalen assoziiert sind. Danach wurde ein genotypischer Pool (X21L3) gebildet, der 22 WT-ILs umfasst. Der X21L3-Pool wurde anschließend im Vergleich zu den Kreuzungseltern und einem kommerziellen Stamm HB4 auf die Zielmerkmale untersucht. Es wurde eine bemerkenswerte Verbesserung der Toleranz gegenüber oxidativem Stress bei 2ºC (Kältetoleranz) festgestellt, wobei X21L3 einen Tag länger überlebte als der leistungsstärkste Elternteil XX21. Dieser neue Hybridpool überlebte auch einen Tag länger als der am wenigsten leistungsfähige Elternteil IL3 in Bezug auf das Merkmal der Toleranz gegenüber oxidativem Stress bei 25 ºC (Langlebigkeit) und verzeichnete eine höhere DJ-Recovery (58 %) und DJ-Ausbeute (209.000 DJ ml^{-1}) in Flüssigkultur als der am wenigsten leistungsfähige Elternteil XX21. Die Lagerstabilität in Kieselgurformulierung bei 2 ºC und 7,5 ºC wurde ebenfalls um 2 Tage bzw. 5 Tage im Vergleich zum leistungsschwächsten Elternteil XX21 verbessert. In dieser Studie wurden sowohl technische als auch genetische Aspekte optimiert, um die Haltbarkeit von *H. bacteriophora* zu verbessern.

5. References

Bai, C., Shapiro-Ilan, D. I., Gaugler, R., & Hopper, K. R. (2005). Stabilization of beneficial traits in *Heterorhabditis bacteriophora* through creation of inbred lines. *Biological Control* 32, 220–227. DOI: 10.1016/j.biocontrol.2004.09.011

Bai, X., Adams, B. J., Ciche, T. A., Clifton, S., Gaugler, R., Kim, K., Spieth, J., Sternberg, P. W., Wilson, R. K., & Grewal, P. S. (2013). A lover and a fighter: The genome sequence of an entomopathogenic nematode *Heterorhabditis bacteriophora*. *PloS One* 8, e69618. DOI: 10.1371/journal.pone.0069618

Bilgrami, A. L., Gaugler, R., Shapiro-Ilan, D. I., & Adams, B. J. (2006). Source of trait deterioration in entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* during *in vivo* culture. *Nematology* 8, 397–409. DOI: 10.1163/156854106778493394

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* 55, 611–622. DOI: 10.1373/clinchem.2008.112797

Ciche, T. A., Kim, K., Kaufmann-Daszczuk, B., Nguyen, K. C., & Hall, D. H. (2008). Cell invasion and matricide during *Photorhabdus luminescens* transmission by *Heterorhabditis bacteriophora* nematodes. *Applied and Environmental Microbiology* 74, 2275–2287. DOI: 10.1128/AEM.69.4.1890-1897.2003

Dhakal, M., Nguyen, K. B., Hunt, D. J., Ehlers, R.-U., Spiridonov, S. E., & Subbotin, S. A. (2020). Molecular identification, phylogeny and phylogeography of the entomopathogenic nematodes of the genus *Heterorhabditis* Poinar, 1976: A multigene approach. *Nematology* 23, 451–466. DOI: 10.1163/15685411-bja10052

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., Bocs, S., Boursaux-Eude, C., Chandler, M., Charles, J.-F., Dassa, E., Derose, R., Derzelle, S., Freyssinet, G., Gaudriault, S., Médigue, C., Lanois, A., Powell, K., Siguier, P., Kunst, F. (2003). The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nature Biotechnology* 21, 1307–1313. DOI: 10.1038/nbt886

Ehlers, R.-U. (2001). Mass production of entomopathogenic nematodes for plant protection. *Applied Microbiology and Biotechnology* 56, 623–633. DOI: 10.1007/s002530100711

Ehlers, R.-U., Oestergaard, J., Hollmer, S., Wingen, M., & Strauch, O. (2005). Genetic selection for heat tolerance and low temperature activity of the entomopathogenic nematode–bacterium complex *Heterorhabditis bacteriophora–Photorhabdus luminescens*. *Biocontrol* 50, 699–716. DOI: 10.1007/s10526-005-5079-z

Eliáš, S., Hurychová, J., Toubarro, D., Frias, J., Kunc, M., Dobeš, P., Simões, N., & Hyršl, P. (2020). Bioactive excreted/secreted products of entomopathogenic nematode *Heterorhabditis bacteriophora* inhibit the phenoloxidase activity during the infection. *Insects* 11, 353. DOI: 10.3390/insects11060353

Endo, B. Y., & Nickle, W. R. (1991). Ultrastructure of the intestinal epithelium, lumen and associated bacteria in *Heterorhabditis bacteriophora*. *Helminthological Society of Washington* 58, 202–212.

Estevez, M., Attisano, L., Wrana, J. L., Albert, P. S., Massagué, J., & Riddle, D. L. (1993). The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. *Nature* 365, 644–649. DOI: 10.1038/365644a0

Evans, K. S., van Wijk, M. H., McGrath, P. T., Andersen, E. C., & Sterken, M. G. (2021). From QTL to gene: *C. elegans* facilitates discoveries of the genetic mechanisms underlying natural variation. *Trends in Genetics* 37, 933–947. DOI: 10.1016/j.tig.2021.06.005

Fu, Z., Crossley, M. S., Epstein, B., Bates, C., Crowder, D. W., Elling, A. A., Hohenlohe, P. A., Jabbour, R., Ramirez, R. A., & Snyder, W. E. (2021). Using fine-scale relatedness to infer natural enemy movement. *Biological Control* 160, 104662. DOI: 10.1016/j.biocontrol.2021.104662

Gaugler, R., & Han RiChou, H. R. (2002). Production technology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. CABI International, Wallingford, UK, pp. 289-310. DOI: 10.1079/9780851995670.0289

Gerritsen, L. J. M., & Smits, P. H. (1993). Variation in pathogenicity of recombinations of *Heterorhabditis* and *Xenorhabdus luminescens* strains. *Fundamental and Applied Nematology* 4, 367–373.

Gerritsen, L. J., Wiegers, G. L., & Smits, P. H. (1998). Pathogenicity of new combinations of *Heterorhabditis* spp. and *Photorhabdus luminescens* against *Galleria mellonella* and *Tipula oleracea*. *Biological Control* 13, 9–15.

Ghazal, S., Oshone, R., Simpson, S., Morris, K., Abebe-Akele, F., Thomas, W. K., Khalil, K. M., & Tisa, L. S. (2016). Draft genome sequence of *Photorhabdus luminescens* subsp. *laumondii* HP88, an entomopathogenic bacterium isolated from nematodes. *Genome Announcements* 4, e00154-16. DOI: 10.1128/genomeA.00154-16

Godina, G., Kirsch, C., Dörfler, V., Barg, M., Singh, P. R., Vandenbossche, B., Strauch, O., Ehlers, R.-U., & Molina, C. (2022). Single nucleotide polymorphism markers in *Heterorhabditis bacteriophora* associated with virulence at low temperature. *Nematology* 1, 1–14. DOI: 10.1163/15685411-bja10181

Godina, G., Vandenbossche, B., Centurión, A., Dörfler, V., Barg, M., Ehlers, R.-U., & Molina, C. (2023). New genotyping rescues old phenotypes: Beneficial traits in *Heterorhabditis*

bacteriophora wild type material and association to single nucleotide polymorphisms. *Nematology* 25, 761–773. DOI: 10.1163/15685411-bja10255

Grewal, P. S. (2000). Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. *Pest Management Science* 56, 401–406. DOI: 10.1002/(SICI)1526-4998(200005)56:5<401::AID-PS137>3.0.CO;2-4

Grewal, P. S. (2002). Formulation and application technology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI pp. 265-288.

Grewal, P. S., & Peters, A. (2005). Formulation and quality. In: Grewal, P. S., Ehlers, R.-U., & Shapiro-Ilan, D. I. (Ed.). *Nematodes as biocontrol agents*. Wallingford, UK, CAB International pp. 79-90.

Guo, W., Yan, X., & Han, R. (2017). Adapted formulations for entomopathogenic nematodes, *Steinernema* and *Heterorhabditis* spp. *Nematology* 19, 587–596. DOI: 10.1163/15685411-00003072

Han, R., & Ehlers, R.-U. (2000). Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic *in vivo* conditions. *Journal of Invertebrate Pathology* 75, 55–58. DOI: 10.1006/jipa.1999.4900

Han, R., Wouts, W. M., & Li, L. (1991). Development and virulence of *Heterorhabditis* spp. strains associated with different *Xenorhabdus luminescens* isolates. *Journal of Invertebrate Pathology* 58, 27–32. DOI: 10.1016/0022-2011(91)90158-M

Higuchi, R., Dollinger, G., Walsh, P. S., & Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. *Nature Biotechnology*, 10, 413–417. DOI: 10.1038/nbt0492-413

Johnigk, S.-A., Hollmer, S., Strauch, O., Wyss, U., & Ehlers, R.-U. (2002). Heritability of the liquid culture mass production potential of the entomopathogenic nematode *Heterorhabditis*

bacteriophora. *Biocontrol Science and Technology* 12, 267–276. DOI: 10.1080/09583150120124504

Johnigk, S.-A., & Ehlers, R.-U. (1999). *Endotokia matricida* in hermaphrodites of *Heterorhabditis* spp. and the effect of the food supply. *Nematology* 1, 717–726. DOI: 10.1163/156854199508748

Kagimu, N., & Malan, A. P. (2019). Formulation of South African entomopathogenic nematodes using alginate beads and diatomaceous earth. *BioControl* 64, 413–422. DOI: 10.1007/s10526-019-09945-1

Larionov, A., Krause, A., & Miller, W. (2005). A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* 6, 62. DOI: 10.1186/1471-2105-6-62

Levy, N., Faigenboim, A., Salame, L., Molina, C., Ehlers, R.-U., Glazer, I., & Ment, D. (2020). Characterization of the phenotypic and genotypic tolerance to abiotic stresses of natural populations of *Heterorhabditis bacteriophora*. *Scientific Reports* 10, 1–16. DOI: 10.1038/s41598-020-67097-0

Lunau, S., Ehlers, R.-U., Stoessel, S., & Schmidt-Peisker, A. J. (1993). Establishment of monoxenic inocula for scaling up *in vitro* cultures of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis* spp. *Nematologica* 39, 385–399. DOI: 10.1163/187529293X00330

Machado, R. A. R., Wüthrich, D., Kuhnert, P., Arce, C. C. M., Thönen, L., Ruiz, C., Zhang, X., Robert, C. A. M., Karimi, J., Kamali, S., Ma, J., Bruggmann, R., & Erb, M. (2018). Whole-genome-based revisit of *Photorhabdus* phylogeny: Proposal for the elevation of most *Photorhabdus* subspecies to the species level and description of one novel species *Photorhabdus bodei* sp. *nov*., and one novel subspecies *Photorhabdus laumondii* subsp. *clarkei* subsp. *nov*. *International Journal of Systematic and Evolutionary Microbiology* 68, 2664–2681. DOI: 10.1099/ijsem.0.002820

Maier, C., Hofmann, K., Huptas, C., Scherer, S., Wenning, M., & Lücking, G. (2021). Simultaneous quantification of the most common and proteolytic *Pseudomonas* species in raw milk by multiplex qPCR. *Applied Microbiology and Biotechnology* 105, 1693–1708. DOI: 10.1007/s00253-021-11109-0

McGrath, P. T., Rockman, M. V., Zimmer, M., Jang, H., Macosko, E. Z., Kruglyak, L., & Bargmann, C. I. (2009). Quantitative mapping of a digenic behavioral trait implicates globin variation in *C. elegans* sensory behaviors. *Neuron* 61, 692-699. DOI: 10.1016/j.neuron.2009.02.012

McLean, F., Berger, D., Laetsch, D. R., Schwartz, H. T., & Blaxter, M. (2018). Improving the annotation of the *Heterorhabditis bacteriophora* genome. *GigaScience* 7. DOI: 10.1093/gigascience/giy034

Mukuka, J., Strauch, O., & Ehlers, R.-U. (2010a). Variability in desiccation tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology* 12, 711–720. DOI: 10.1163/138855409X12607871174454

Mukuka, J., Strauch, O., Waeyenberge, L., Viaene, N., Moens, M., & Ehlers, R.-U. (2010b). Heat tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *BioControl* 55, 423–434. DOI: 10.1007/s10526-009-9255-4

Mukuka, J., Strauch, O., Hoppe, C., & Ehlers, R.-U. (2010c). Improvement of heat and desiccation tolerance in *Heterorhabditis bacteriophora* through cross-breeding of tolerant strains and successive genetic selection. *BioControl* 55, 511–521. DOI: 10.1007/s10526-010-9271-4

Palma, L., Del Valle, E. E., Frizzo, L., Berry, C., & Caballero, P. (2016). Draft genome sequence of *Photorhabdus luminescens* strain DSPV002N isolated from Santa Fe, Argentina. *Genome Announcements* 4, e00744-16. DOI: 10.1128/genomeA.00744-16

Peters, A. (2016). Formulation of nematodes. In: Glare, T., Moran-Diez, M. (Eds.). Microbial-Based Biopesticides. Methods in Molecular Biology, vol 1477. Humana Press, New York, NY. DOI: 10.1007/978-1-4939-6367-6_10

Poinar, G. O. (1993). Origins and phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and *Steinernema*. *Fundamental and Applied Nematology* 16, 333–338.

Shapiro-Ilan, D. I., Gouge, D. H., Piggott, S. J., & Fife, J. P. (2006). Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biological Control* 38, 124–133. DOI: 10.1016/j.biocontrol.2005.09.005

Shapiro-Ilan, D. I., Hazir, S., Lete, L., (2015). Viability and virulence of entomopathogenic nematodes exposed to ultraviolet radiation. *Journal of Nematology* 47, 184.

Somvanshi, V. S., Dubay, B., Kushwah, J., Ramamoorthy, S., Vishnu, U. S., Sankarasubramanian, J., Rajendhran, J., & Rao, U. (2019). Draft genome sequences for five *Photorhabdus* bacterial symbionts of entomopathogenic *Heterorhabditis* nematodes isolated from India. *Microbiology Resource Announcements* 8, e01404-18. DOI: 10.1128/MRA.01404-18

Sterken, M. G., Snoek, L. B., Kammenga, J. E., & Andersen, E. C. (2015). The laboratory domestication of *Caenorhabditis elegans*. *Trends in Genetics* 31, 224-231. DOI: 10.1016/j.tig.2015.02.009

Strauch, O., & Ehlers, R.-U. (1998). Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. *Applied Microbiology and Biotechnology* 50, 369-374. DOI: 10.1007/s002530051306

Strauch, O., Niemann, I., Neumann, A., Schmidt, A. J., Peters, A., & Ehlers, R.-U. (2000). Storage and formulation of the entomopathogenic nematodes *Heterorhabditis indica* and *H. bacteriophora*. *BioControl* 45, 483-500. DOI: 10.1023/A:1026528727365

Strauch, O., Oestergaard, J., Hollmer, S., & Ehlers, R.-U. (2004). Genetic improvement of the desiccation tolerance of the entomopathogenic nematode *Heterorhabditis bacteriophora* through selective breeding. *Biological Control* 31, 218–226. DOI: 10.1016/j.biocontrol.2004.03.009

Strauch, O., Stoessel, S., & Ehlers, R.-U. (1994). Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis*. *Fundamental and Applied Nematology* 17, 575–582.

Sumaya, N. H., Aryal, S., Vandenbossche, B., Barg, M., Doerfler, V., Strauch, O., Molina, C., & Ehlers, R.-U. (2017). Phenotyping dauer juvenile oxidative stress tolerance, longevity and persistence within wild type and inbred lines of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology,* 19 971–986. DOI: 10.1163/15685411-00003100

Sumaya, N. H., Gohil, R., Okolo, C., Addis, T., Dörfler, V., Ehlers, R.-U., & Molina, C. (2018). Applying inbreeding, hybridization and mutagenesis to improve oxidative stress tolerance and longevity of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology* 151, 50-58. DOI: 10.1016/j.jip.2017.11.001

Susurluk, A., & Ehlers, R.-U. (2008). Field persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* in different crops. *BioControl* 53, 627–641. DOI: 10.1007/s10526-007-9104-2

Větrovský, T., & Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS ONE* 8, e57923. DOI: 10.1371/journal.pone.0057923

Wang, S., Ventolero, M., Hu, H., & Li, X. (2022). A revisit to universal single-copy genes in bacterial genomes. *Scientific Reports* 12, 14550. DOI: 10.1038/s41598-022-18762-z

Wang, Z., Ogaya, C., Dörfler, V., Barg, M., Ehlers, R.-U., & Molina, C. (2023). Pheno- and genotyping *in vitro* dauer juvenile recovery in the nematode *Heterorhabditis bacteriophora*. *Applied Microbiology and Biotechnology*. DOI: 10.1007/s00253-023-12775-y

You, Y., Kim, J., Raizen, D. M., & Avery, L. (2008). Insulin, cGMP, and TGF-β signals regulate food intake and quiescence in *C. elegans*: A model for satiety. *Cell Metabolism* 7, 249–257.

Annex 1: Publication 1

Genotypic markers associated with cold storage survival of the entomopathogenic nematode *Heterorhabditis bacteriophora*

Christopher OGAYA[1,2], Yohannes MOGES[1,3], Verena DÖRFLER[1], Mike BARG[1], Ralf-Udo EHLERS[1,2,3], and Carlos MOLINA[1*]

[1]*e–nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany*

[2]*Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts-University Kiel, Hermann-Rodewald-Str. 4, 24118 Kiel, Germany*

[3]*Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*

Nematology 0 (2023) 1–12 DOI: 10.1163/15685411-bja10295

"Printed with the approval of the Faculty for Agricultural and Nutritional Sciences"

BRILL

Nematology 0 (2023) 1-12

Nematology
brill.com/nemy

Genotypic markers associated with cold storage survival of the entomopathogenic nematode *Heterorhabditis bacteriophora*

Christopher OGAYA [1,2], Yohannes MOGES [1,3], Verena DÖRFLER [1], Mike BARG [1], Ralf-Udo EHLERS [1,2,3] and Carlos MOLINA [1,*]

[1] *e–nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany*
[2] *Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts-University Kiel, Hermann-Rodewald-Str. 4, 24118 Kiel, Germany*
[3] *Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*
ORCID iDs: Ogaya: 0009-0002-7745-6949; Moges: 0000-0002-0598-6265; Dörfler: 0000-0001-6849-7429; Ehlers: 0000-0002-6938-2284; Molina: 0000-0003-4919-5120

Received: 28 June 2023; revised: 2 October 2023
Accepted for publication: 2 October 2023; published online: 5 December 2023

Summary – Limited shelf life is a major constraint to successful commercialisation of entomopathogenic nematodes (EPN), and to extend shelf life, dauer juveniles (DJ) are formulated and stored at low temperatures (4-8°C). We evaluated the cold storage potential of strains of *Heterorhabditis bacteriophora* formulated in diatomaceous earth at storage temperatures between 5 and 9°C. When assessing DJ decline to reach 75% survival (MT_{75}) in the formulation for the respective temperatures, *H. bacteriophora* strain HB4 had the highest survival of 25 days at 9°C, while strain D2D6 survived longest at 8°C for 28 days. A set of 22 *H. bacteriophora* wild type inbred lines was then phenotyped for cold tolerance in water under oxidative stress in 70 mM H_2O_2 at 2°C. The MT_{50} (time to 50% survival) ranged from 11 to 23 days. The phenotypic data were correlated with the respective genotypic data, identifying four single nucleotide polymorphic (SNP) markers associated with cold tolerance. The survival of two lines (PT11 and IR11) with opposite extreme cold tolerance pheno- and genotypes was evaluated in diatomaceous earth formulation at 2°C with the cold tolerant IR11 surviving 3 days longer than PT11. Our study yields a set of valuable SNP markers employable in rapid genotyping of cold tolerance and tracking this trait during the breeding process.

Keywords – cold tolerance, diatomaceous earth, formulation, shelf life, SNP markers, wild type inbred lines.

Two unrelated nematode genera, *Steinernema* Travassos (1927) and *Heterorhabditis* Poinar (1976) (Blaxter *et al*., 1998), converge in life habit having insect parasitism as a central point, building the group of the so called entomopathogenic nematodes (EPN) (Poinar, 1993; Adams & Nguyen, 2002). Nematodes of both genera have a tight symbiotic relationship with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively (Shi *et al*., 2022, and references therein). In both cases, the free living, non-feeding, developmentally-arrested dauer juvenile (DJ) stage is tasked with seeking target insects and delivering a few cells of its symbiotic bacteria into the insect host. The DJ only regain development (DJ recovery) upon insect infection. Once established in the insect's haemolymph, the nematode and bacterial cells overrun the insect's immune system leading to septicaemia within *ca* 48 h, and nematodes develop until reproductive maturity, producing a new generation of DJ once the nutritional sources of the insect cadaver are depleted (bacterial biomass) (Han & Ehlers, 2000).

For the commercialisation of biological control agents, the industrial production of DJ in large volumes has become a major milestone. Advances in monoxenic liquid culturing techniques have made it possible to produce EPN efficiently at low cost (Strauch *et al*., 1998; Ehlers *et al*., 2000; Ehlers, 2001). Under standard industrial production conditions, DJ are produced in large bioreactors, harvested by centrifugation and filtering, stored in tanks suspended in water, and later formulated in an inert carrier prior to delivery to the end users. The formulation step

* Corresponding author, e-mail: c.molina@e-nema.de

 | DOI: 10.1163/15685411-bja10295
 | ISSN: 1388-5545 (print) 1568-5411 (online)

under low temperatures (<10°C) and light desiccation conditions should guarantee that DJ are stably stored during transport and until applied (Grewal & Peters, 2005). For trading EPN products, formulated DJ storage extends for several weeks.

Physiologically, during this cold storage, the energy reserves of the DJ are expected to remain high due to their reduced metabolism at low temperatures. This fact is crucial for later infectivity of the DJ once applied for insect control. However, despite the reduced metabolism, the DJ mortality under formulation increases with storage time (Kagimu & Malan, 2019). The type of formulation, nematode species and storage temperature play a major role in DJ survival. The negative effects of extreme storage temperatures (5 and 25°C) on formulated heterorhabditid nematodes are well elucidated (Strauch *et al*., 2000; Guo *et al*., 2017). Diatomaceous earth is broadly used as a carrier for DJ formulation due to affordability and the capacity to provide a low water activity (aw-value) environment to the formulated DJ (Peters, 2016). To address some of the constraints in shelf life related to the *H. bacteriophora* formulated in diatomaceous earth, Kagimu & Malan (2019) explored the use of alginate beads formulation, reporting a rise in the number of DJ that escaped the beads with increasing storage temperature. Generally, the investigated temperature range in all these reports is very wide. A fine-tuned storage temperatures of 5-9°C could reveal more optimal storage conditions for different heterorhabditid strains. Phenotyping of traits that may be related to improved shelf life have identified stress tolerant nematode strains. Desiccation stress (Mukuka *et al*., 2010a), tolerance to temperature extremes (Ehlers *et al*., 2005; Shapiro-Ilan *et al*., 2006; Mukuka *et al*., 2010b) and oxidative stress (Sumaya *et al*., 2017) are some of the extensively phenotyped traits in *H. bacteriophora*. Moreover, the DJ tolerance to oxidative stress has been proposed as predictor for DJ longevity and phenotypic selection in combination with breeding have improved DJ survival (Sumaya *et al*., 2018).

Besides extensive phenotypic characterisation, nematode genomic information can be exploited for improvement of beneficial traits. Levy *et al*. (2020) identified up to 8810 homozygous single nucleotide polymorphisms (SNPs) in three different *H. bacteriophora* strains with varying stress tolerance levels. Almost half of these SNPs were located within, or 1000 bp in the vicinity of, predicted genes, providing the possibility that some of them may have a physiological function directly related to DJ survival. Later, Godina *et al*. (2022) identified 4894 SNPs among a collection of 48 *H. bacteriophora* highly homozygous wild type inbred lines (WT ILs). A subsequent direct geno-phenotype correlation yielded two SNP molecular markers associated with virulence at low temperatures. The SNPs reported by Godina and co-authors were found in the immediate vicinity of two gene models coding for uncharacterised proteins (g14099, g10389).

Despite these advances concerning pheno- and genotyping, knowledge gaps persist concerning the understanding of the storage potential of different *H. bacteriophora* strains. To date, no detailed information has been provided about the effect of fine storage temperature adjustments on DJ survival rate. Also, predictive storage assays for *H. bacteriophora* must be further improved to yield a more precise correlation between DJ survival and the potential of the DJ to be cold-stored for long time periods under formulation. Further on, correlation between phenotype and genotype data to find genetic markers for cold storage potential in this species is a priority.

In the present study: *i*) we performed a fine-tuned phenotyping of two *H. bacteriophora* strains to determine the changes of DJ survival in relation to specific cold storage temperatures; *ii*) we assessed the virulence of the stored DJ over storage time; *iii*) we used oxidative stress as a predictor assay for cold storage potential in *H. bacteriophora* and phenotyped 22 wild type inbred lines; *iv*) we used the genotypic information developed by Godina *et al*. (2022) to determine SNP markers associated with cold storage potential; and finally, *v*) we selected WT ILs with contrasting genotypes and phenotypes and evaluated their performance under formulation. The present results and genetic markers have a large potential to be incorporated in breeding strategies, as well as for the further understanding of physiological mechanisms related to stress-tolerance and DJ longevity in *H. bacteriophora*.

Materials and methods

Nematode material

Initially, DJ survival was monitored in formulated DJ stored in temperatures between 2 and 9°C. For this purpose, nematodes of the commercial strain HB4 of *H. bacteriophora* were produced in liquid culture in a bioreactor. In parallel, the new hybrid strain D2D6 was produced in monoxenic liquid culture in flasks as described by Hirao & Ehlers (2009). Subsequently, phenotyping for cold tolerance under oxidative stress (70 mM H_2O_2) was carried out in 22 *H. bacteriophora* WT ILs from different geo-

Table 1. *Heterorhabditis bacteriophora* material used in the present study.

Name	Origin
D2D6*	Hybrid
HB4	Commercial
AU11	Australia
DE21	Germany
DE64	Germany
DE81	Germany
HU21	Hungary
IL3	Germany
IR11**	Iran
IR21	Iran
IR22	Iran
IR31	Iran
IT41	Italy
IT42	Italy
MM141	Germany
MM82	Germany
PT11**	Portugal
PT21	Portugal
PT31	Portugal
PT41	Portugal
TR21	Turkey
XX11	South Africa
XX21	South Africa
XX22	South Africa

For evaluation of storage stability under formulation, the commercial strain HB4 and the hybrid D2D6 were used. For the subsequent phenotyping of cold tolerance (2°C) under oxidative stress (70 mM H_2O_2), the commercial strain HB4 was used plus 22 WT-Inbred lines from different origins. For a final confirmation experiment, DJ from WT ILs IR11 and PT11 were further phenotyped under cold storage.

* Hybrid strain not included in the phenotyping for cold tolerance under oxidative stress.

** WT ILs used for final confirmation experiment of cold storage stability.

graphic locations, in addition to the commercial strain HB4. For a final confirmation experiment, the WT ILs IR11 and PT11 were further phenotyped under formulation. An overview of the material used is given in Table 1.

Nematode growth

For phenotyping the storage stability under formulation, HB4 and D2D6 monoxenic cultures were produced by collecting gravid egg-packed hermaphrodites 4 days post-DJ inoculation from cultures on nematode growth gelrite (NGG) (Addis *et al*., 2014). Hermaphrodites were shredded to release eggs and the eggs were separated from the nematode debris by passing the disrupted tissues through a 55 μm sieve. Surface-sterilisation was done by washing the eggs in a solution containing 0.5 ml NaOCl and 1.5 ml of 4 M NaOH for 4 min. Then eggs were collected by centrifugation at 3000 *g* for 2 min. Thereafter, the resulting eggs were washed twice in sterile BSB medium (Bacto tryptic soy broth 10 g, yeast extract 2.5 g, casein peptone 2.5 g, NaCl 2.5 g, KCl 0.18 g, $CaCl_2 \cdot 2H_2O$ 0.11 g, glucose 2 g, distilled water 0.5 l) and incubated in BSB for 48 h to evidence the presence of contaminants. Only sterile wells were transferred to 3 cm diam. Wouts agar plates (16 g Bacto nutrient broth, 12.0 g Bacto agar, 5.0 g sunflower-oil, 1 l distilled water) spotted with drops of *P. luminescens* bacteria. Nematodes were left on Wouts agar for about 11 days and then transferred to Erlenmeyer flasks (250 ml) containing HB liquid medium (Hirao & Ehlers, 2009) pre-incubated with *P. luminescens* (24 h; 25°C). The flasks were incubated on a rotary shaker at 180 rpm (rotation diam. 4 cm) for *ca* 11 days (25°C). Consecutive cultures were carried out using DJ from previous liquid cultures as inoculum. For each experiment a new culture batch was started. A total of three independent nematode batches were produced. The same procedure described above was carried out for a final confirmation experiment with WT ILs IR11 and PT11 in two independent batches.

For phenotyping cold tolerance under oxidative stress, the commercial HB4 strain and the 22 WT ILs of *H. bacteriophora* were cultured on NGG as described by Addis *et al*. (2014). Briefly, *ca* 2000 DJ of each strain were inoculated in individual NGG plates containing a lawn of *P. luminescens* bacteria. The NGG plates were incubated for 7 days at 25°C and after this period mature hermaphrodites at the *endotokia matricida* stage (Johnigk & Ehlers, 2009) were sampled on 250 μm sieves. All mature hermaphrodites were stored in Ringer's solution (NaCl 9 g, KCl 0.42 g, $CaCl_2 \cdot 2\ H_2O$ 0.37 g, $NaHCO_3$ 0.2 g, 1 l distilled water) until all DJ had emerged. For each independent experiment, a new culture batch was started. A total of four independent batches were produced.

Nematode harvest and formulation

Three DJ batches of the commercial strain HB4 were formulated. Nematodes previously produced in bioreactors were concentrated by a separator and further concentrated and cleaned by a centrifugal sifter as described by Ehlers & Shapiro-Ilan (2005). Two batches were stored

in aerated water at 4°C for 15 days and one for 3 days prior to formulation. Three DJ batches of the D2D6 hybrid were formulated. Flask-grown D2D6 DJ were washed by subsequent sieving using 200 and 32 μm mesh screens. For the resulting paste, DJ density was determined by suspending 1 g in 1 l water and counting 500 μl samples in triplicate at 1:50 dilutions. All three batches were stored at 4°C on a rotary shaker (80 rpm) for 3 days prior to harvest and formulation. The DJ paste of both nematode strains was mixed with diatomaceous earth at a ratio of approximately 1:1 (w/w). The DJ densities in the final formulation ranged from 8×10^5 up to 1×10^6 DJ (g formulated nematodes)$^{-1}$. Ten g of formulated DJ were then packaged in plastic bags prior to the start of the cold storage experiments. For a final confirmation experiment, two independent growth batches of WT ILs IR11 and PT11 were formulated as described above.

Nematode survival at different storage temperatures

All three formulated DJ batches from HB4 and D2D6 were stored for 12 weeks at 5, 6, 7, 8 and 9°C in parallel using certified refrigerators (Thermo Scientific Laboratory Refrigerator E473332, Model: TSG505SA). For DJ survival assessment, formulated DJ (*ca* 5×10^6 per bag) were dissolved in 1 l distilled water (dissolving each time the whole bag content). Subsequently, a 1:50 dilution was carried out and a 500 μl aliquot was counted in 24-well cell-counting chambers under the microscope. Three different production batches were tested and for each batch, observation time and storage temperature and three technical counting replicates were carried out. All living DJ per well were counted and along the 12 weeks storage nematode survival was assessed at weeks 2, 5, 9 and 12.

Additional to the above described survival evaluation, two WT ILs with extreme phenotypes for cold tolerance under oxidative stress (IR11 and PT11) were also evaluated for their performance when formulated. Prior to formulation, both lines were produced in liquid culture starting from sterile eggs and formulated as described above. Formulated nematodes of both contrasting WT ILs were stored at 2°C for a period of 2 weeks and DJ survival was monitored every 2 days until death of all nematodes.

Effect of storage temperature on the virulence of formulated nematodes

DJ virulence was evaluated for each formulated HB4 and D2D6 DJ batch, storage temperature and observation time. For this purpose, 15 cm diam. Petri dishes were filled with 150 g sand (7-8% moisture), and in each dish 40 mealworm larvae (*Tenebrio molitor*) were added. To each bioassay plate 800 DJ per treatment (storage temperature, batch and observation time) were added (four replications per treatment). A dose of 800 DJ per bioassay (20 DJ per insect) is used as internal standard at e-nema. Bioassay plates were subsequently incubated at 25°C for 7 days and the number of living and dead insects was assessed. The luminescence of the dead insects was confirmed by a luminometer (Beckmann). Percent mortality for each bioassay was calculated.

Assay for assessment of cold tolerance

A predictive assay for evaluation of DJ longevity (Sumaya *et al*., 2017) was also used to assess the cold storage potential of 22 *H. bacteriophora* WT ILs in addition to the commercial HB4 strain. Nematodes harvested from NGG plates (see sections above) were used for this purpose. For stress induction, approximately 1000 DJ from each line were dispensed in a total vol 400 μl Ringer's solution in 24 cell-well plates and H_2O_2 was added to each cell-well for a final concentration of 70 mM as described by Sumaya *et al*. (2017). For each line, three randomly assigned cell-well positions were selected (technical replicates). Thereafter the 24-well cell plates were incubated at 2°C and DJ survival was recorded every 2-3 days by counting 20 μl aliquots each time (dead and living DJ were counted). Four independent experiments, each with three technical replicates, were carried out. The percentage of DJ survival over time was fitted to logistic models (see Data analysis). Each independent experiment was run for up to 30 days, a time at which most of the WT ILs surpassed 50% survival.

Data analysis

For formulation experiments, DJ survival data (weeks 2, 5, 9 and 12) from HB4 and D2D6 were fitted to logistic models using the percentage of living DJ of each population in relation to the starting DJ amount (start of the formulation storage experiment) using a logit regression function routine in RStudio (https://www.rstudio.com). Fitted curves from each DJ batch

and temperature were obtained and the time for 75% survival was then deduced from each respective model (hereafter called MT_{75}). For the experiments using the 22 WT ILs plus HB4 (predictive assay using oxidative stress), the time for reaching 50% mortality in the DJ populations (MT_{50}) of each line was determined as indicated above. For a final confirmation experiment, 2°C survival data from formulated DJ of WT ILs IR11 and PT11were modelled in the same manner. For each cell-well a fitted model was generated. Differences among storage temperatures on DJ survival (MT_{75} and MT_{50}) and DJ virulence (% insect mortality) in formulation, and differences in cold tolerance among WT ILs (MT_{50}) were analysed by ANOVA. *Post hoc* Tukey's HSD test ($P \leqslant 0.05$) was used to separate means. Independent *t*-tests were used to analyse differences in survival between allele groups ($P \leqslant 0.05$).

Association analysis

As an outcome from the previous report of Godina *et al*. (2022), positions of single nucleotide polymorphisms have been determined and transferred to GTF files masking the *H. bacteriophora* TT01 genome assembly version PRJNA13977.WBPS16 deposited at the wormbase (www.wormbase.org). Subsequently, the same genotypic data matrix analysed by Godina *et al*. (2022) (4897 SNPs) were combined with phenotypic data from four independent experiments derived from DJ survival evaluation of WT ILs under oxidative stress. For association analysis, the Tassel software (downloaded at www.maizegenetics.net) was used with the following parameters: $P \leqslant 0.05$ and 100 permutations, applying the general linear model routine. Thereafter, statistically significant SNP molecular markers associated with cold tolerance were extracted. A SNP molecular marker was only considered significantly associated with cold tolerance when differences between the strong and weak alleles were observed along the individual experiments.

Results

Effect of storage temperature on DJ survival of formulated nematodes

For the storage under formulation for strain HB4, significant differences in time to reach 75% survival (MT_{75}) were determined between different storage temperatures ($F = 21.64$; df = 4, 10; $P < 0.005$). The highest MT_{75} was determined for DJ stored at 9°C (25 ± 5 days) and declined with decreasing storage temperature. Considering all batches, the lowest MT_{75} was determined for DJ stored at 5°C (6 ± 4 days). Significant batch differences in MT_{75} were observed ($F = 13.04$; df = 2, 6; $P < 0.005$). For strain D2D6, significant differences between storage temperatures for the MT_{75} were determined ($F = 4.63$; df = 4, 10; $P < 0.005$). Considering all batches, the lowest MT_{75} was also determined for DJ stored at 5°C (16 ± 6 days), whereas the highest MT_{75} was observed for DJ stored at 8°C (28 ± 11 days). Again, significant batch differences were observed ($F = 8.54$; df = 2, 6; $P < 0.005$). For one batch the performance was MT_{75} 5°C = 26 ± 10 days and MT_{75} 9°C = 32 ± 2 days, whereas for another batch lower survival (MT_{75} 5°C = 15 ± 2 days; MT_{75} 9°C = 14 ± 2 days) was recorded. The mean MT_{75} for all batches of each strain is presented in Figure 1.

Effect of storage temperature on DJ virulence of formulated nematodes

Storage of strain HB4 in formulation for 5 weeks elicited significant differences in DJ virulence between the storage temperatures ($F = 11.34$; df = 4, 15; $P < 0.005$). Average DJ virulence ranged from 10 ± 6% for DJ stored at 5°C to 27 ± 8% for 9°C storage temperature. Significant differences in DJ virulence were also evident after 9 weeks of storage ($F = 21.18$; df = 4, 15; $P < 0.005$) with the highest and lowest DJ virulence recorded in nematodes stored at 9°C (34 ± 8%) and 5°C (6 ± 4%), respectively. Significant batch differences in DJ virulence both after 5 weeks storage ($F = 124.34$; df = 2, 6; $P < 0.005$) and 9 weeks ($F = 257.28$; df = 2, 6; $P < 0.005$) (data not shown) were observed.

For strain D2D6, significant differences in average DJ virulence between storage temperatures were only apparent after 9 weeks storage ($F = 33.92$; df = 4, 15; $P < 0.005$). The lowest DJ virulence was for DJ stored at 5°C (46 ± 6%) at week 9. Significant batch differences in DJ virulence at week 5 ($F = 56.08$; df = 2, 6; $P < 0.005$) and week 9 ($F = 39.65$; df = 2, 6; $P < 0.005$) were also recorded (data not shown). The average DJ virulence for all batches for each strain is presented in Figure 2.

Cold tolerance of wild type inbred lines

Among the 22 WT ILs evaluated for DJ survival at 2°C under oxidative stress conditions (70 mM H_2O_2), significant differences in average time required to reach 50% DJ survival (MT_{50}) were detected ($F = 6.71$; df =

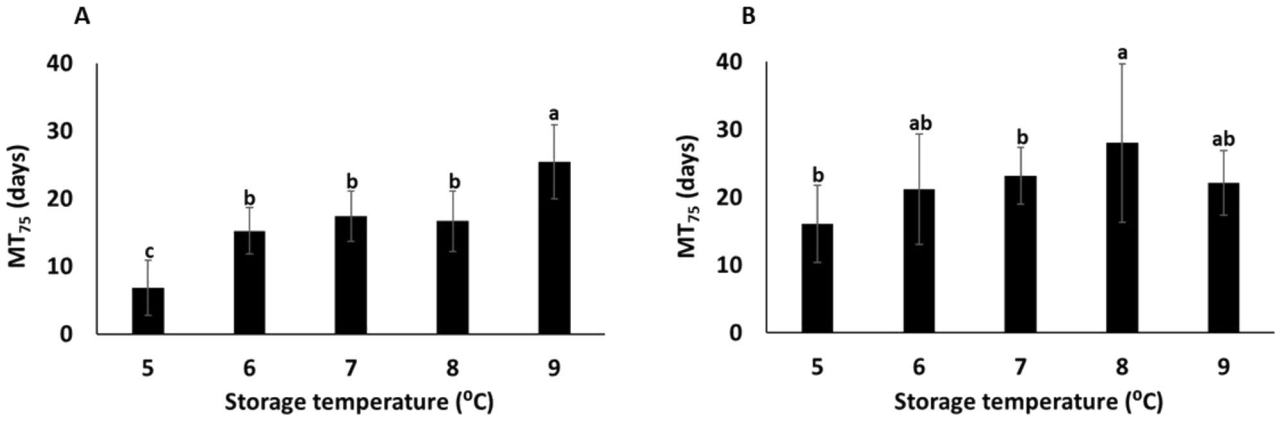

Fig. 1. Time to 75% mean survival (MT_{75}) of *Heterorhabditis bacteriophora* strains HB4 (A) and D2D6 (B) formulated in diatomaceous earth and stored at temperatures between 5 and 9°C. Nematodes of strain HB4 originated from production in liquid culture bioreactors, whereas D2D6 was produced in liquid culture flasks. For each strain three nematode batches were investigated. Different letters above the bars indicate significant differences in the MT_{75} among temperatures (Tukey HSD test, $P < 0.005$). Error bars = SD.

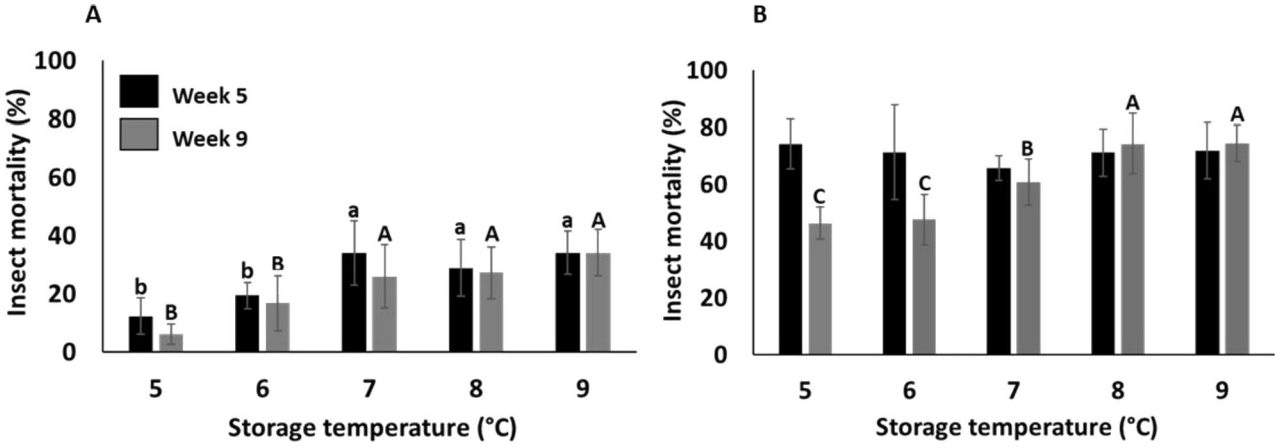

Fig. 2. Average mortality (%) of *Tenebrio molitor* larvae (n = 40) exposed in sand bioassays at 25°C for 1 week to 800 dauer juveniles (DJ) of *Heterorhabditis bacteriophora* strains HB4 (A) and D2D6 (B) that had been stored for 5 and 9 weeks at temperatures of 5-9°C formulated in diatomaceous earth. Error bars = SD. Different lower-case letters above the bars indicate significant differences between storage temperatures after 5 weeks storage and capital letters after 9 weeks (Tukey HSD test, $P \leqslant 0.005$).

22, 46; $P < 0.005$). The Iranian IR11 WT line survived longest with a MT_{50} of 24 ± 1.4, while PT11 from Portugal had the lowest MT_{50} of 12 ± 0.7 days. Strain HB4 reached an average MT_{50} of 15.1 ± 1.21 days and the parents of the hybrid strain D2D6, DE21 reached 14 ± 0.6 and DE64 13 ± 0.6 days. A summary of the WT ILs cold tolerance is depicted in Figure 3.

SNP molecular markers associated with cold tolerance at 2°C

Four SNP molecular markers (Table 2) were identified to be significantly associated with cold tolerance in *H. bacteriophora* ($P < 0.05$). In the *H. bacteriophora* TTO1 genome deposited at NCBI by Bai *et al*. (2013), the respective SNP markers are located on scaffolds 1330-pos.-376614 (SNP1), 1343-pos.-865804 (SNP2), 1251-pos.-72875 (SNP3) and 1296-pos.-110383 (SNP4). SNP3 was located inside transcript g9820 that was reported to code for the *H. bacteriophora* cell surface receptor (*daf-4*) gene. Transcript g6562, that is homologous to an uncharacterised protein (F53A2.1) in *Caenorhabditis elegans*, was 287 bp from SNP3, whilst transcript g2709 homologous to hypothetical protein Y032_0037g3461 *of Ancylostoma ceylanicum* was 168 bp from SNP2. SNP1 was 748 bp from an uncharacterised transcript g4260.

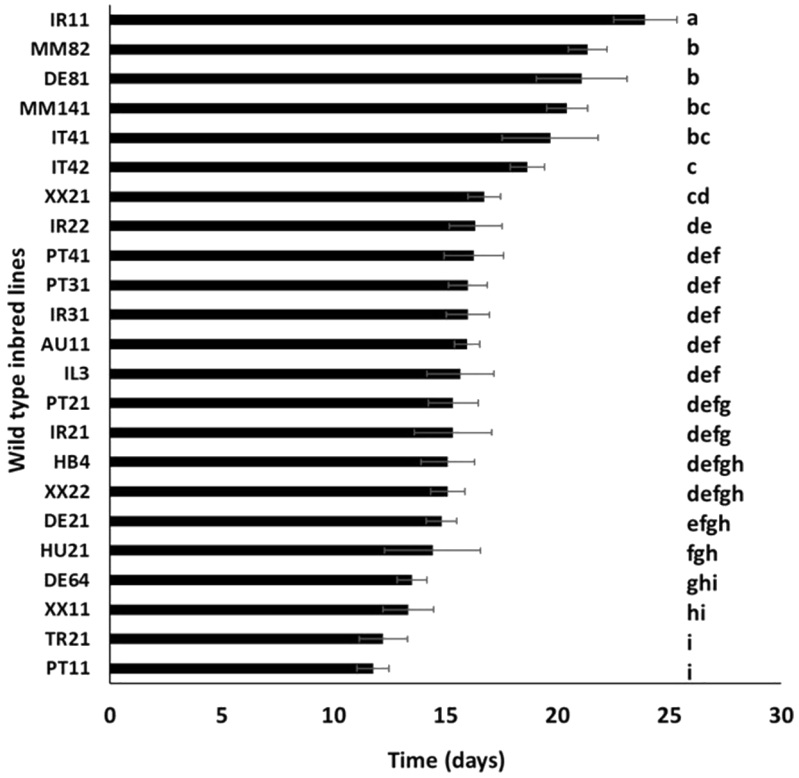

Fig. 3. Mean time to 50% survival (MT_{50}) evaluated for different *Heterorhabditis bacteriophora* wild type inbred lines stressed with 70 mM H_2O_2 and kept at 2°C. Error bars = SD of four independent experiments. Different letters next to bars denote significant difference (Tukey HSD test $P \leqslant 0.05$). The commercial strain HB4 was used as comparative reference.

Table 2. SNP molecular markers associated with cold tolerance at 2°C under oxidative stress conditions of 70 mM H_2O_2 (time to 50% survival loss, MT_{50}) in *Heterorhabditis bacteriophora* determined through association analysis of cold tolerance phenotype with the genotype of 22 wild type inbred lines.

Marker synonym	Scaffold	Position	*P*	Allele	Allele with highest average MT_{50}	Closest transcript (distance in bp)
SNP1	1330	376614	0.001	A/G	G	g4260 (+748)
SNP2	1343	865804	0.002	C/G	C	g2709 (+168)
SNP3	1251	72875	0.008	A/G	A	g9820 (+0)
SNP4	1296	110383	0.002	A/G	G	g6562 (+287)

By grouping WT ILs according to the genotypes they carry for the respective SNP markers (Fig. 4A-D), groups segregate into two distinct phenotypes of high or low cold tolerance. For SNP1, significant differences in MT_{50} ($t = -3.771$; df = 21; $P = 0.001$) were recorded between groups carrying allele G (MT_{50} 18.6 ± 3.2 days) and A (MT_{50} 14.7 ± 1.6 days). For SNP2, WT ILs carrying allele C (MT_{50} 19.2 ± 3.2 days) had significantly higher MT_{50} ($t = 3.482$; df = 21; $P = 0.002$) than those possessing allele G (MT_{50} 15.2 ± 2.1 days). In SNP 3, groups having allele A (17.9 ± 3.4 days) had higher MT_{50} than the groups with the alternate allele G (14.7 ± 1.4 days) and the differences were significant ($t = 2.906$; df = 21; $P = 0.008$). In SNP4, WT ILs carrying G allele (MT_{50} 18.3 ± 3.2 days) also had significantly higher MT_{50} ($t = -3.516$; df = 21; $P = 0.002$) than those carrying allele A (14.6 ± 1.6 days). Allele groups details are provided in Supplementary Table S1.

Seven WT ILs (DE81, IR11, IR21, IR22, IR31, MM141 and MM82) possessed all the alleles associated with high

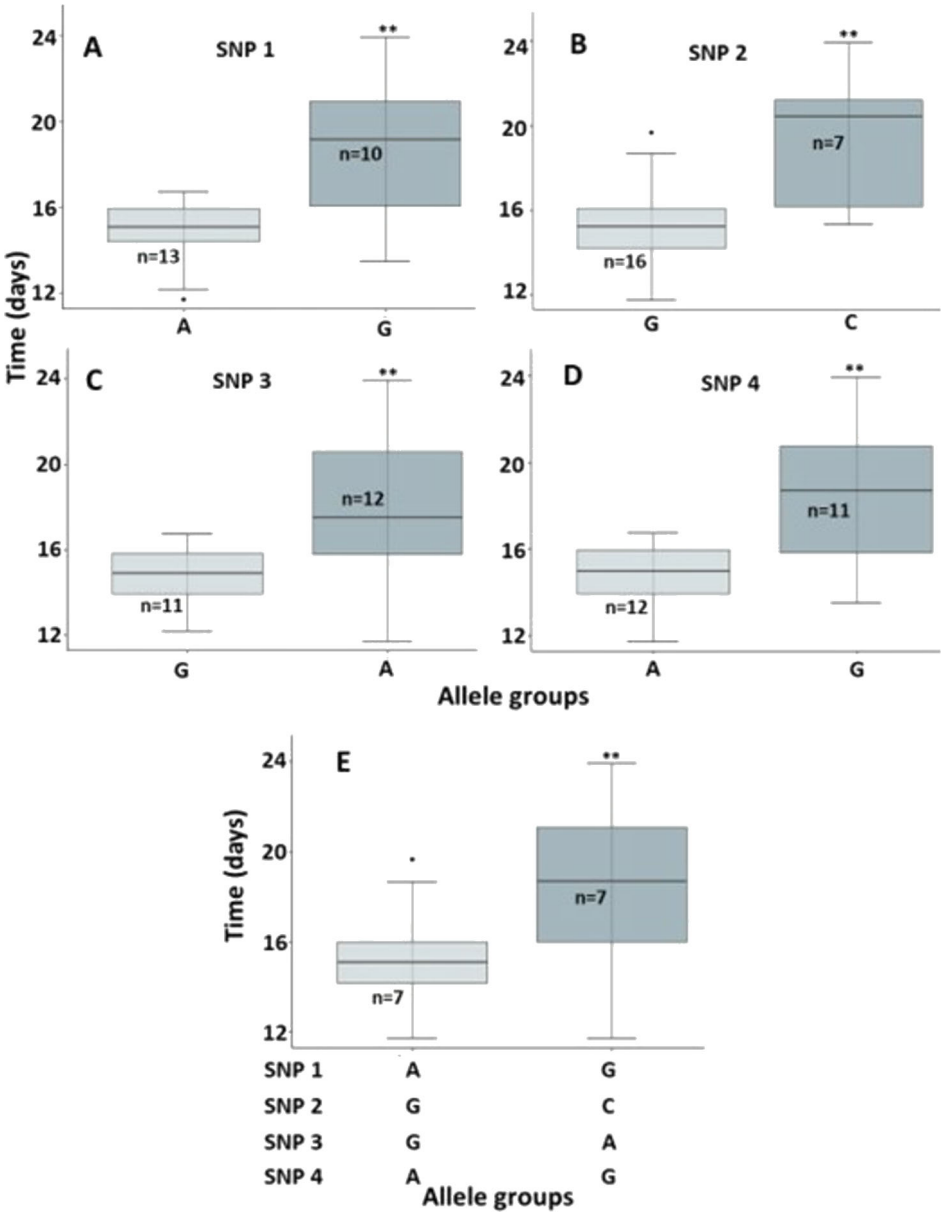

Fig. 4. Mean time to 50% survival (MT_{50}) according to allelic groups of four individual *Heterorhabditis bacteriophora* SNP molecular markers; A: SNP1: Scaffold 1330-pos.-376617 (A/G); B: SNP2: Scaffold 1343-pos.-865804 (C/G); C: SNP3: Scaffold 1251-pos.-72875 (A/G); D: SNP4: Scaffold 1296-pos.-110383 (A/G) with potential association to cold tolerance at 2°C under oxidative stress conditions (70 mM H_2O_2). The allelic groups are based on individual genotypes of the WT ILs. Allelic groups of WT ILs carrying all alleles associated with either high or low average MT_{50}, respectively, for all the SNP markers were also compared (E). Significant differences between allele groups per respective SNP marker are denoted by ** (Welch's two sample *t*-test, $P < 0.05$). The number of WT ILs segregated into respective allele are denoted by n. Information on the allelic groups per SNP is deposited in Supplementary Table S1.

average MT_{50} in all four SNP markers while seven WT ILs (DE21, HU21, PT31, PT41, TR21, XX21, XX22) had the alternate allele associated with low average MT_{50}. A significant difference ($t = 3.018$; df = 12; $P = 0.01$) of 4 days in average MT_{50} existed between the groups (Fig. 4E). Interestingly, some of the WT ILs observed

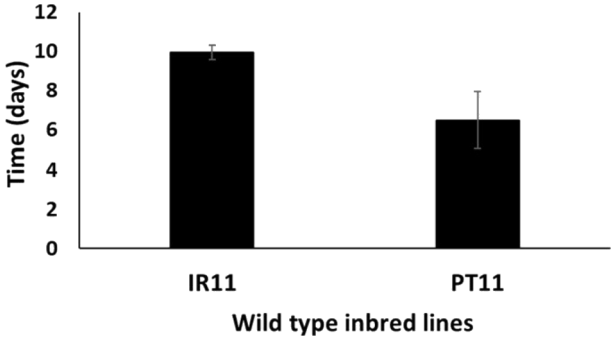

Fig. 5. Mean time to 50% survival (MT_{50}) at 2°C storage temperatures of two diatomaceous earth formulated *Heterorhabditis bacteriophora* wild type inbred lines. The strains were previously phenotyped for cold tolerance at 2°C under oxidative stress conditions (70 mM H_2O_2) and had extreme phenotypes. Mass production was in liquid culture in flasks. Error bars = SD of two independent experiments.

among the cold tolerant lines (*e.g.*, XX21, PT41 and PT3) carry the allele associated with low average MT_{50}. However, IR11, MM82 and DE81 had a significatively higher MT_{75}.

Confirmation experiment for the cold tolerance prediction

At 2°C in diatomaceous earth formulation, the WT IL IR11 that had the highest cold tolerance under oxidative stress conditions (70 mM H_2O_2) at 2°C and had all the alleles associated with high average cold tolerance for all the SNP markers identified (previous section) had a higher MT_{50} of 9.9 ± 0.3 days. The MT_{50} of PT11 was lower at 6.5 ± 1.4 but was not significantly different ($P > 0.05$). PT11 also had alleles associated with low average cold tolerance for the SNP markers (SNP1, SNP2 and SNP4). The performance of the two extreme cold tolerant lines in formulation is summarised in Figure 5.

Discussion

In the industrial EPN production, several factors related to the growth conditions (temperature, medium composition, bacterial strain) and downstream processing (sieving, cleaning) may have effects on the shelf life of the final product. In the first instance, these factors may explain the large variation in shelf life between nematode batches observed in the present work. However, these previously mentioned factors may have a subordinate effect when storage temperature is considered. Temperature is the primary factor driving DJ survival in formulation (Grewal, 2002). Kagimu & Malan (2019) showed remarkable difference in DJ survival between three storage temperatures of 6, 14 and 25°C of DJ stored in diatomaceous earth formulation. Almost no survival was recorded for DJ stored at 6°C after 4 weeks in comparison to survival above 80% for DJ stored at 14 and 25°C, albeit the survival being the average of three EPN species (*H. bacteriophora*, *Steinernema yirgalemense* and *S. jeffreyense*). Guo *et al*. (2017) also reported higher DJ survival of diatomaceous earth formulated *H. bacteriophora* after 30 days DJ storage at 15°C (>40%) than at 5°C (30%). In the present study, DJ stored at 5°C showed the lowest survival for both *H. bacteriophora* strains tested. Grewal (2000) argues that the combination of cold and desiccation stress could be detrimental to DJ due to a high metabolic cost of the latter that is not adequately compensated at low temperatures.

We observed that increase of virulence of formulated nematodes directly correlated with increase of storage temperature. Kagimu & Malan (2019) likewise observed higher virulence in diatomaceous earth formulated DJ stored at 14 and 25°C when compared to those stored at 6°C after 4 weeks. It is plausible that the associated symbiotic bacteria are detrimentally affected by low storage temperatures. Depletion or death of the associated symbiotic bacteria despite survival of the nematodes could be the primary reason for the low virulence. It is known that axenic *Heterorhabditis* nematodes are unable to kill insects without their associated bacterial symbionts (Gerritsen *et al*., 1998). All this evidence points to detrimental effects of low storage temperatures on the associated symbiotic bacteria *Photorhabdus*. It thus points to investigate if the negative effects of low storage temperatures on virulence are due to depletion or inactivation of *Photorhabdus* during cold storage. The study of Shapiro-Ilan *et al*. (2015) already provides evidence that DJ survival after stress exposure (UV-light) and virulence are not necessarily correlated. Also, the exemplary study of Ramakuwela *et al*. (2015) presents evidence that low storage temperatures can be detrimental for the virulence in other EPN (*Steinernema innovationi*).

In this study we elucidate inter-strain variation in *H. bacteriophora* survival in water at 2°C under oxidative stress conditions (70 mM H_2O_2). Fortunately, the inbred lines used in the present study (or their mother strains) have been used for more than a decade in different phenotyping assays, including the two most important factors influencing the shelf life under formulation conditions: *i*) desiccation (Mukuka *et al*., 2010a); and *ii*) cold toler-

ance (Sumaya *et al*., 2017). In our present work, the lines IR11, MM82, DE81, MM141, IT41 and IT42 presented the greatest survival at 2°C under oxidative stress. In the previous phenotyping of Mukuka *et al*. (2010a), strains IR1 (parent to IR11) and IT4 (parent to IT41 and IT42) were not desiccation tolerant. However, phenotyping by Sumaya *et al*. (2017) resulted in IT4 as the most cold tolerant and the offspring IT41 and IT42 were also among the most cold tolerant in our study. IR1 and the offspring IR11 were also both cold tolerant, while PT11 with its parent PT1 were both not cold tolerant in the respective assays, further confirming the robustness of using 70 mM H_2O_2 as an accurate predictor of DJ cold tolerance. However, the marginal differences in performance of the cold tolerant IR11 and non-cold tolerant PT11 in formulation show the limits of this assay for predicting shelf life. In formulation, DJ encounter extra stresses like desiccation from the formulation. A further correlation of the cold tolerance data from our study with the virulence at 15°C data of the same WT ILs phenotyped by Godina *et al*. (2022) showed no correlation between these traits ($R = 0.084$, $P = 0.72$). All this puts into focus the complexity of obtaining a single strain possessing all the desired traits. A possible solution to this challenge could be to pool strains having all the desired traits and complementing it with marker assisted breeding for precise tracking of respective traits during the breeding process.

Just like in other eukaryotes, SNPs are the most abundant genetic markers in *H. bacteriophora* (Levy *et al*., 2020; Godina *et al*., 2022). In this study we used available genotypic information of different *H. bacteriophora* lines to identify potential SNP molecular markers associated with cold tolerance. We found four SNP markers, three of which were in the vicinity of uncharacterised genes. Therefore, we cannot argue where our SNPs stand in relation to specific physiological functions. However, their potential as genetic markers is extremely valuable. Interestingly, SNP3 was found within the gene model g9820, which shows a large homology to the *C. elegans daf-4* gene. Among the functions of the *daf-4* gene in *C. elegans* (a cell surface receptor) are inhibition of dauer formation (Estevez *et al*., 1993), and regulating quiescence entry that is triggered by satiety (You *et al*., 2008). To date, there is no report linking this gene with survival related traits. It is certainly premature to state that this gene has a role in the survival regulation of *H. bacteriophora*. Further approaches, as for example RNAi or CRISPRCas9, are thus needed to close the relationship between the existence of trait-associated SNPs and any possible function.

In conclusion, lower storage temperatures (5 and 6°C) are not optimal for storage of heterorhabditids in diatomaceous earth formulations as survival and virulence is negatively affected. The SNP markers we provide here are a versatile and rapid tool for screening wild type strains of *H. bacteriophora* for cold tolerance and can be added for a more controlled approach of monitoring the breeding process in this species.

Acknowledgements

The scholarship by the German Academic Exchange Service (http://www.daad.de) to the first author and the scholarship by the Flemish University Cooperation for Development (VLIR-UOS, http://www.vliruos.be) to the second author are much appreciated.

References

Adams, B.J. & Nguyen, K.B. (2002). Taxonomy and systematics. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CAB International, pp. 1-33.

Addis, T., Teshome, A., Strauch, O. & Ehlers, R.-U. (2014). Life history trait analysis of the entomopathogenic nematode *Steinernema riobrave*. *Nematology* 16, 929-936. DOI: 10.1163/15685411-00002819

Bai, X., Adams, B.J., Ciche, T.A., Clifton, S., Gaugler, R., Kim, K., Spieth, J., Sternberg, P.W., Wilson, R.K. & Grewal, P.S. (2013). A lover and a fighter: the genome sequence of an entomopathogenic nematode *Heterorhabditis bacteriophora*. *PLoS ONE* 8, e69618. DOI: 10.1371/journal.pone.0069618

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M. *et al.* (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* 392, 71-75. DOI: 10.1038/32160

Ehlers, R.-U. (2001). Mass production of entomopathogenic nematodes for plant protection. *Applied Microbiology & Biotechnology* 56, 623-633. DOI: 10.1007/s002530100711

Ehlers, R.-U. & Shapiro-Ilan, D.I. (2005). Mass production. In: Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (Eds). *Nematodes as biocontrol agents*. Wallingford, UK, CAB International, pp. 65-78.

Ehlers, R.-U., Niemann, I., Hollmer, S., Strauch, O., Jende, D., Shanmugasundaram, M., Mehta, U.K., Easwaramoorthy, S.K. & Burnell, A. (2000). Mass production potential of the bacto-helminthic biocontrol complex *Heterorhabditis indica – Photorhabdus luminescens*. *Biocontrol Science and Technology* 10, 607-616. DOI: 10.1080/095831500750016406

Ehlers, R.-U., Oestergaard, J., Hollmer, S., Wingen, M. & Strauch, O. (2005). Genetic selection for heat tolerance and

low temperature activity of the entomopathogenic nematode–bacterium complex *Heterorhabditis bacteriophora – Photorhabdus luminescens*. *BioControl* 50, 699-716. DOI: 10.1007/s10526-005-5079-z

Estevez, M., Attisano, L., Wrana, J.L., Albert, P.S., Massagué, J. & Riddle, D.L. (1993). The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. *Nature* 365, 644-649. DOI: 10.1038/365644a0

Gerritsen, L.J., Wiegers, G.L. & Smits, P.H. (1998). Pathogenicity of new combinations of *Heterorhabditis* spp. and *Photorhabdus luminescens* against *Galleria mellonella* and *Tipula oleracea*. *Biological Control* 13, 9-15. DOI: 10.1006/bcon.1998.0640

Godina, G., Kirsch, C., Dörfler, V., Barg, M., Singh, P.R., Vandenbossche, B., Strauch, O., Ehlers, R.-U. & Molina, C. (2022). Single nucleotide polymorphism markers in *Heterorhabditis bacteriophora* associated with virulence at low temperature. *Nematology* 24, 925-938. DOI: 10.1163/15685411-bja10181

Grewal, P.S. (2000). Enhanced ambient storage stability of an entomopathogenic nematode through anhydrobiosis. *Pest Management Science* 56, 401-406.

Grewal, P.S. (2002). Formulation and application technology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CAB International, pp. 265-288.

Grewal, P.S. & Peters, A. (2005). Formulation and quality. In: Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (Eds). *Nematodes as biocontrol agents*. Wallingford, UK, CAB International, pp. 79-90.

Guo, W., Yan, X. & Han, R. (2017). Adapted formulations for entomopathogenic nematodes, *Steinernema* and *Heterorhabditis* spp. *Nematology* 19, 587-596. DOI: 10.1163/15685411-00003072

Han, R. & Ehlers, R.-U. (2000). Pathogenicity, development, and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic *in vivo* conditions. *Journal of Invertebrate Pathology* 75, 55-58. DOI: 10.1006/jipa.1999.4900

Hirao, A. & Ehlers, R.-U. (2009). The influence of cell density and phase variants of bacterial symbionts (*Xenorhabdus* spp.) on dauer juvenile recovery and development of biocontrol nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida). *Applied Microbiology and Biotechnology* 84, 77-85. DOI: 10.1007/s00253-009-1961-4

Johnigk, S.-A. & Ehlers, R.-U. (1999). *Endotokia matricida* in hermaphrodites of *Heterorhabditis* spp. and the effect of food supply. *Nematology* 1, 717-726. DOI: 10.1163/156854199508748

Kagimu, N. & Malan, A.P. (2019). Formulation of South African entomopathogenic nematodes using alginate beads and diatomaceous earth. *BioControl* 64, 413-422. DOI: 10.1007/s10526-019-09945-1

Levy, N., Faigenboim, A., Salame, L., Molina, C., Ehlers, R.-U., Glazer, I. & Ment, D. (2020). Characterization of the phenotypic and genotypic tolerance to abiotic stresses of natural populations of *Heterorhabditis bacteriophora*. *Scientific Reports* 10, 1-16. DOI: 10.1038/s41598-020-67097-0

Mukuka, J., Strauch, O. & Ehlers, R.-U. (2010a). Variability in desiccation tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology* 12, 711-720. DOI: 10.1163/138855409X12607871174454

Mukuka, J., Strauch, O., Waeyenberge, L., Viaene, N., Moens, M. & Ehlers, R.-U. (2010b). Heat tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *BioControl* 55, 423-434. DOI: 10.1007/s10526-009-9255-4

Peters, A. (2016). Formulation of nematodes. In: Glare, T. & Moran-Diez, M. (Eds). *Microbial-based biopesticides*. *Methods in Molecular Biology, vol. 1477*. New York, NY, USA, Humana Press. DOI: 10.1007/978-1-4939-6367-6_10

Poinar Jr, G.O. (1993). Origins and phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and *Steinernema*. *Fundamental and Applied Nematology* 16, 333-338.

Ramakuwela, T., Hatting, J., Laing, MD., Hazir, S. & Thiebaut, N. (2015). Effect of storage temperature and duration on survival and infectivity of *Steinernema innovationi* (Rhabditida: Steinernematidae). *Journal of Nematology* 47, 332-336.

Shapiro-Ilan, D.I., Gouge, D.H., Piggott, S.J. & Fife, J.P. (2006). Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biological Control* 38, 124-133. DOI: 10.1016/j.biocontrol.2005.09.005

Shapiro-Ilan, D.I., Hazir, S. & Lete, L. (2015). Viability and virulence of entomopathogenic nematodes exposed to ultraviolet radiation. *Journal of Nematology.* 47, 184-189.

Shi, Y.-M., Hirschmann, M., Shi, Y.-N., Ahmed, S., Abebew, D., Tobias, N.J., Grün, P., Crames, J.J., Pöschel, L., Kuttenlochner, W. *et al.* (2022). Global analysis of biosynthetic gene clusters reveals conserved and unique natural products in entomopathogenic nematode-symbiotic bacteria. *Nature Chemistry* 14, 701-712. DOI: 10.1101/2022.01.21.477171

Strauch, O. & Ehlers, R.-U. (1998). Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. *Applied Microbiology and Biotechnology* 50, 369-374. DOI: 10.1007/s002530051306

Strauch, O., Niemann, I., Neumann, A., Schmidt, A.J., Peters, A. & Ehlers, R.-U. (2000). Storage and formulation of the entomopathogenic nematodes *Heterorhabditis indica* and *H. bacteriophora*. *BioControl* 45, 483-500. DOI: 10.1023/A:1026528727365

Sumaya, N.H., Aryal, S., Vandenbossche, B., Barg, M., Doerfler, V., Strauch, O., Molina, C. & Ehlers, R.-U. (2017). Phenotyping dauer juvenile oxidative stress tolerance, longevity and persistence within wild type and inbred lines of the ento-

mopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology* 19, 971-986. DOI: 10.1163/15685411-00003100

Sumaya, N.H., Gohil, R., Okolo, C., Addis, T., Doerfler, V., Ehlers, R.-U. & Molina, C. (2018). Applying inbreeding, hybridization and mutagenesis to improve oxidative stress tolerance and longevity of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology* 151, 50-58. DOI: 10.1016/j.jip.2017.11.001

You, Y., Kim, J., Raizen, D.M. & Avery, L. (2008). Insulin, cGMP, and TGF-β signals regulate food intake and quiescence in *C. elegans*: a model for satiety. *Cell Metabolism* 7, 249-257. DOI: 10.1016/j.cmet.2008.01.005

Supplementary Table S1. Groups of *Heterorhabditis bacteriophora* WT ILs according to their SNP genotype in four markers associated to cold tolerance.

Marker synonym	Allele	WT inbred lines
SNP1	A	AU11, DE21, HB4, HU21, IL3, PT11, PT21, PT31, PT41, TR21, XX11, XX21, XX22
	G	DE64, DE81, IR11, IR21, IR22, IR31, IT41, IT42, MM82, MM141
SNP2	C	DE81, IR11, IR21, IR22, IR31, MM82, MM141
	G	AU11, DE21, DE64, HB4, HU21, IL3, IT41, IT42, PT11, PT21, PT31, PT41, TR21, XX11, XX21, XX22
SNP3	A	AU11, DE81, IR11, IR21, IR22, IR31, IT41, IT42, MM82, MM141, PT11, PT21
	G	DE21, DE64, HB4, HU21, IL3, PT31, PT41, TR21, XX11, XX21, XX22
SNP4	A	AU11, DE21, HB4, HU21, PT11, PT21, PT31, PT41, TR21, XX11, XX21, XX22
	G	DE64, DE81, IL3, IR11, IR21, IR22, IR31, IT41, IT42, MM82, MM141

The commercial line HB4 was used as reference.

Annex 2: Publication 2

Monitoring the *Photorhabdus* spp. bacterial load in *Heterorhabditis bacteriophora* dauer juveniles over different storage times and temperatures: A molecular approach

Christopher Ogaya[a,c], Nontarak Huong[b], Maria Touceda-González[c], Mike Barg[c], Verena Dörfler[c], Ralf-Udo Ehlers[a,b] and Carlos Molina[c*]

[a]*Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts-University Kiel, Hermann-Rodewald-Str. 4, 24118 Kiel, Germany*

[b]*Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*

[c]*e–nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany*

Journal of Invertebrate Pathology DOI. 10.1016/j.jip.2023.108048

"Printed with the approval of the Faculty for Agricultural and Nutritional Sciences"

Journal of Invertebrate Pathology 203 (2024) 108048

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Monitoring the *Photorhabdus* spp. bacterial load in *Heterorhabditis bacteriophora* dauer juveniles over different storage times and temperatures: A molecular approach

Christopher Ogaya [a,c], Nontarak Huong [b], Maria Touceda-González [c], Mike Barg [c], Verena Dörfler [c], Ralf-Udo Ehlers [a,b], Carlos Molina [c,*]

[a] Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts-University Kiel, Hermann-Rodewald-Str. 4, 24118 Kiel, Germany
[b] Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium
[c] e–nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany

ARTICLE INFO

Keywords:
Entomopathogenic nematodes
qPCR
Bacteria load
Virulence
Single copy gene
Absolute quantification
Relative quantification

ABSTRACT

Biological control products based on the entomopathogenic nematode *Heterorhabditis bacteriophora* can vary in virulence (quality). The influence of their symbiotic bacteria *Photorhabdus* spp. inside the infective dauer juvenile (DJ) on DJ quality has not received much attention in the past. The presence of the bacteria in the DJ is crucial for its biocontrol potential. This investigation provides a method to quantify the bacterial load inside the DJ based on a qPCR technique. Information from the genome of *Photorhabdus laumondii* strain DE2 was used to identify single copy genes with no homology to any other bacterial accessions. One gene (hereby named CG2) was selected for primers design and for further qPCR experiments. Cross-amplification tests with *P. thracensis* and *P. kayaii*, also symbionts of *H. bacteriophora*, were positive, whereas no amplicons were produced for *P. temperata* or *Xenorhabdus nematophila*. We tested our qPCR system in DJ populations carrying defined proportions of bacteria-free (axenic) vs bacteria-carrying nematodes. With an increasing proportion of axenic DJ in a population, virulence declined, and the virulence was proportional to the amount of bacterial DNA detected in the population by qPCR. Along liquid storage over long time, virulence also decreased, and this factor correlated with the reduction of bacterial DNA on the respective DJ population. We observed that stored DJ kept virulent up to 90 days and thereafter the virulence as well as the amount of bacterial DNA drastically decreased. Storage temperature also influenced the bacterial survival. Inside formulated DJ, the loss of bacterial DNA on the DJ population was accelerated under storage temperatures below 7.5 °C, suggesting that reproduction of the bacterial cells takes place when growth temperature is favorable. The role of bacterial survival inside stored DJ can now be adequately addressed using this molecular quality-control technique.

1. Introduction

The entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* (Strongyloidea: Heterorhabditidae) carries cells of its symbiont *Photorhabdus* spp. Boemare, Akhurst and Mourant 1993 (Enterobacterales, Morganellaceae) in its intestine (Endo and Nickle, 1991). For successful insect control and subsequent reproduction in the dead insect, the nematode must transfer viable cells of its symbiotic bacterium into the insecťs hemolymph (Han and Ehlers, 2000). Once inside the insect host, the EPN dauer juveniles (DJ) exit the developmentally arrested dauer stage, a process termed DJ recovery, and regurgitate the symbiont cells into the hemocoel (Ciche et al., 2008). Both partners overcome the defense mechanisms of the insect and the bacteria multiply and kills the host by septicemia within 24 – 72 h (Eliáš et al., 2020). The bacteria metabolize the insecťs hemolymph providing the essential food supply for growth and multiplication of the nematode. Depleting food reserves induces DJ formation. The DJ acquires the symbiotic bacteria before they exit the insect cadaver to seek for new hosts (Poinar, 1993). Without immediate new host location, the DJ can survive in the soil for several weeks (Susurluk and Ehlers, 2008a). During its complete lifecycle the nematode depends on the symbiotic interaction with its symbiotic bacterium. Hence, the bacteria must also

* Corresponding author.
E-mail address: c.molina@e-nema.de (C. Molina).

https://doi.org/10.1016/j.jip.2023.108048
Received 6 October 2023; Received in revised form 22 December 2023; Accepted 27 December 2023
Available online 28 December 2023

survive inside the DJ. Heterorhabditid DJ depend on their symbiotic bacteria to kill the host (Han and Ehlers, 2000).

For decades nematodes have successfully been used to control insect pests, like grubs (Sulistyanto and Ehlers, 1996), the western corn root borer *Diabrotica v. vigifera* (Toepfer et al., 2010) and weevil larvae (Susurluk and Ehlers, 2008b). For commercial use, *H. bacteriophora* nematodes are produced in industrial scale bioreactors (>10,000 l) in monoxenic *Photorhabdus* cultures (Ehlers, 2001). The growth conditions inside the bioreactors are adjusted so that the bacterial density optimally promotes nematode development as food signal for DJ recovery and as nutritional factor for later stages (Strauch and Ehlers, 1998). Optimal bioreactor runs are characterized by a high proportion of *Endotokia matricida* hermaphrodites in the final stages, which produce a new DJ generation loaded with symbiotic bacteria. Upon harvest, DJ are either directly formulated or stored in tanks for some weeks until formulated. Formulated nematodes are then transported to the distributor and end user prior application. The survival and virulence of *H. bacteriophora* DJ is, however, compromised by the interaction between time and environmental factors (Mukuka et al., 2010; Sumaya et al., 2017). Shapiro-Ilan et al. (2015) reported that stress exposure compromises virulence. It is still unsolved to what extent storage and stress exposure negatively affect the bacterial cells inside the DJ. Thus, methods are necessary to monitor the survival and quality of the symbiotic bacteria inside the commercial DJ. Such technique can be part of the routine quality-control for EPN used as commercial biocontrol agents.

The most elaborate approaches to quantify bacterial symbionts in EPN involve tracking GFP labelled *Photorhabdus* spp. cells retained inside the intestine of the DJ (Ciche et al., 2008). This very precise technique is, however, skill-demanding requiring the handling of genetically modified organisms (GMO), and is cost-intensive and time-consuming. Viable plate counts of crushed DJ are not a reliable approach since the quantity of cells obtained from the intestine cannot be precisely determined. A quick method that can be applied on commercial nematode material is therefore needed. Quantitative PCR (qPCR), a robust and sensitive technique to quantify minute amounts of nucleic acids (Higuchi et al., 1992; Larionov et al., 2005), can alleviate these challenges since the technique has already been used as a tool for bacterial quantification (Maier et al., 2021). Quantification of the bacterial DNA inside *H. bacteriophora* is still a major challenge owing to the confinement of the symbiotic bacteria inside the digestive tract of the nematode DJ. This obstacle implies that whenever nematode DNA is extracted, small amounts of the bacterial DNA should be accessible and amplifiable through the PCR reaction.

Concerning available genomic information, the complete genome of *P. laumondii* subsp. *laumondi* (syn.: *P. luminescens* subspecies *laumondii*) published by Duchaud et al. (2003) has more than 4 thousand predicted genes. Additional draft genomes of *Photorhabdus* species have complimented this original genome (Ghazal et al., 2016, Palma et al., 2016, Somvanshi et al., 2019, Machado et al., 2021, Shi et al., 2022). These predicted genes are all potential candidates for primer design to quantify *Photorhabdus* spp. Conventionally, highly conserved house-keeping genes like the 16S rDNA gene, recombinase A (*recA*), DNA polymerase III beta subunit (*gyrB*) and translation initiation factor IF-2 (*infB*) are the default choice for molecular identification of *Photorhabdus* spp. (Machado et al., 2018). Unfortunately, the multiplicity of these house-keeping genes in the bacteria genome make them only ideal for detection but may be erroneous for quantification (Větrovský and Baldrian, 2013). Highly conserved single copy genus-specific genes are thus desired for robust quantification. Concerning the partner nematode, the genome sequence of the *H. bacteriophora* TT01 strain has been published (Bai et al., 2013), and its gene model annotation has been revised (McLean et al., 2018). Highly specific TaqMan 18 s rDNA target gene primers for accurate quantification of this species also exist (Campos-Herrera et al., 2011). However, TaqMan primers and probes imply high costs. Thus, simple cost effective and robust SYBER Green compatible primers as alternative are needed. Complementary to the *H. bacteriophora,* the *H. indica* genome has been also published (Bhat et al., 2022).

This study aimed at: i) identifying single copy genes in the genomes of *P. laumondi* and *H. bacteriophora*, ii) designing suitable qPCR primers, iii) optimizing the qPCR reaction conditions for both, bacterial and nematode DNA amplification, iv) applying the optimized assay to quantify symbiotic bacterial DNA on DJ populations with pre-determined amounts of axenic and bacteria-carrying DJ, v) applying the assay to detect bacterial DNA inside DJ populations of different ages and subjected to different storage environments, vi) correlating the amount of detected bacterial DNA with the DJ virulence of the tested populations.

2. Materials and methods

2.1. Nematode materials

H. bacteriophora wild type (WT) nematode strains, WT inbred lines, and a hybrid strain HB4 were used. The materials had been stored for different periods of time either at 6 °C in Ringeŕs solution or formulated in diatomaceous earth and stored at 2, 4 and 7.5 °C. An overview of all materials is provided in Table 1. WT strains were cultured in the greater wax moth *Galleria mellonella* following Kaya and Stock (1997). WT inbred lines were cultured on nematode growth gelrite (NGG) (3 $g{\cdot}l^{-1}$ Gelrite, 2.5 $g{\cdot}l^{-1}$ peptone from casein, 51 mM NaCl, 1 mM $CaCl_2{\cdot}2\ H_2O$, 1 mM $MgSO_4{\cdot}7\ H_2O$, 1 mM KH_2PO_4, 12 µM filter-sterilized cholesterol in 99 % ethanol) as described by Addis et al. (2014) and coated with *P. laumondii* strain DE2 bacteria**.** The strain HB4 was cultured in HB liquid medium as described by Hirao and Ehlers (2009)**,** either in flasks or in bioreactors incubated with precultures of *P. laumondii* strain DE2. Nematodes from bioreactors were formulated in diatomaceous earth as described by Ehlers et al. (2005). All other DJ were stored in Ringeŕs solution (9 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2{\cdot}2\ H_2O$, 0.2 g $NaHCO_3$, 1 l distilled water) until used for virulence or qPCR assays (see sections below). Before each analysis, DJ suspensions were cleaned from dead DJ by migration through cotton traps, so that only living and active DJ were

Table 1
Nematode strains and inbred lines used in the present study. Nematode WT strains were propagated in *Galleria mellonella* larvae and therefore carried the native Photorhabdus symbiont. For each WT strain, the strain of its *Photorhabdus* symbiont is depicted (*P.k* = *P. kayaii*, *P.l* = *P. laumondii, P.th* = *P. thracensis)*. Commercial HB4 and WT inbred lines were propagated *in vitro* in *P. laumondii* DE2 bacteria. HB4 nematodes were stored in Ringeŕs solution as well as in formulation. For all materials, virulence against mealworm (*Tenebrio mollitor*) as well as qPCR analysis were carried out.

Name	Age (days)	Type	Carried symbiont strain	Storage type
AU11 HU21 IR11	325 325 325	WT Inbred line	*P.l* DE2	Ringer 6 °C
IR2 IT4 IT6	350 300 300	WT Strain	*P.k* native IR2 *P.l* native IT4 *P.th* native IT6	Ringer 6 °C
HB4	10 40 90 150 250 300	Commercial Strain	*P.l* DE2	Ringer 6 °C
HB4	7 14 21 42	Commercial strain	*P.l* DE2	Formulation 2 °C, 4 °C, and 7,5°C

used.

2.2. Production of bacteria-free H. bacteriophora nematodes

To investigate the influence of symbiotic bacteria reduction in *H. bacteriophora* DJ, HB4 nematodes were produced on monoxenic solid cultures on NGG medium (see previous section) coated with *P. temperata*, the symbiont of *H. megidis* according to Han and Ehlers (2000). Briefly, *Heterorhabditis megidis* DJ from strain HSH1 were used to infect third instar *Galleria mellonella* larvae. One day after infection a drop of insect hemolymph was streaked in NBTA agar (10 g Bacto tryptic soy broth, 0.025 g bromothymolblue sodium salt, 14 g Bacto agar, 1 l distilled water, 4 ml of 1 % sterile filtered 2, 3, 5 – triphenyltetrazoliumchloride) to isolate single colonies. Thereafter, liquid culture of single colonies was produced in LM medium (15 g yeast extract, 20 g soy powder, 4 g NaCl, 0.35 g KCl, 0.15 g $CaCl_2$, 0.1 g $MgSO_4$, 6 g lecithin diluted in same volume of rapeseed oil, 40 g rapeseed oil, 1 l distilled water, pH = 7 ± 0.1) and the resulting bacteria was used for NGG coating as previously described. Under these growth conditions, nematodes develop but do not retain the non-specific symbiotic bacteria in the DJ (Gerritsen and Smits, 1997). In parallel HB4 DJ were also produced on NGG medium coated with *P. laumondii* strain DE2. These DJ retain the bacterial symbiont. Both types of DJ were harvested, surface-cleaned, and stored at 6 °C in Ringer's solution for not more than 1 week before use. Thereafter, DJ carrying symbiotic bacteria DE2 were mixed with bacteria-free DJ at different ratios. A summary of the different DJ populations is presented in Table 2. Further, NGG-produced HB4 DJ on *P. laumondii* DE2 strain were used for subsequent virulence and qPCR assays after 25 days of storage (see sections below).

2.3. Virulence assays

Virulence bioassays of all the respective nematode materials used mealworm larvae (*Tenebrio mollitor*). Petri dishes of 150 cm Ø filled with 150 g sand (7 – 10 % moisture content) received 40 *T. mollitor* larvae and 500 DJ. Dishes were incubated at 25 °C in the dark for 7 days. Each sand assay was conducted in triplicate. Thereafter, virulence was evaluated by counting dead and living insects to determine insect mortality (%). A luminometer (Junior LB 9509, Berthhold Technologies, Bad Wildbad, Germany) was used to confirm the *H. bacteriophora* infection due to the *Photorhabdus* bacteria bioluminescence. Virulence assays were applied to: i) long-time stored at 6 °C (up to 300 days) liquid culture-produced HB4 DJ, ii) Long-time stored at 6 °C (350 days) WT strains and WT inbred lines (NGG produced HB4 DJ stored at 6 °C for 25 days were used as a positive control for this assay), iii) DJ populations with different ratios of axenic vs bacteria-carrying DJ, and iv) commercially formulated HB4 DJ (produced in large bioreactors) stored at different temperatures for up to 6 weeks.

Table 2
Heterorhabditis bacteriophora DJ populations comprising of different proportions of DJ originating from monoxenic cultures either with the specific symbiont *P. laumondii* (DE2) or from cultures with *P. temperata* (HNA), symbiont of *H. megidis*. This symbiont is not retained in *H. bacteriophora* DJ and after surface sterilization the DJ are free of symbiotic bacteria.

	Proportions in %	
Name	Bacteria-carrying DJ	Bacteria-free DJ
P100	100	0
P80	80	20
P50	50	50
P20	20	80
P0	0	100

2.4. Sequence analysis and PCR primers design

For all sequence analysis steps, the software CLC Main Workbench 20.0.4 (QIAGEN GmbH Germany) was used. The draft genome of *P. laumondii* DE2 strain (e-nema GmbH, unpublished) was screened for the presence of all predicted gene models. For this purpose, the annotation extraction routine of CLC bio was used to retrieve the individual sequences of all predicted gene models found within the DE2 genome sequence. A simultaneous BLAST analysis of all these gene models against bacterial databases (excluding *Photorhabdus* accessions) at NCBI (https://blast.ncbi.nlm.nih.gov) was done using the CLC BLAST routine. Only gene models with no homology (no high scoring pairs, HSP) to any other bacterial accession were selected for further steps. These gene models were then locally BLASTed against the DE2 genome to determine their copy number. Gene models yielding a single long HSP region were selected for primer design. Regions between 200 and 330 bp for the respective genes were selected for primer design with a primer length of 20–30 bp, annealing temperature ranging between 58—64 °C and GC content < 60 %. Primers were synthetized by LGC genomics GmbH (Berlin, Germany) and annealing temperature confirmed through gradient PCR (data not shown). In a similar way, the *H. bacteriophora* TT01 genome sequence PRJNA13977.WBPS16 deposited at Wormbase (https://wormbase.org) was screened for gene models present as single copy. Prior to this, publicly available *Ceanorhabditis elegans* DJ recovery related gene sequences were gathered from the public databases NCBI (https://www.ncbi.nlm.nih.gov/) and WormBase (https://wormbase.org//#012–34-5) by a simple search using their assigned name and tag. Subsequently, the WormBase IDs were used to retrieve all the related RefSeq_mRNA using the batch ENTREZ (https://www.ncbi.nlm.nih.gov/sites/batchentrez) at NCBI. All retrieved sequences were stored locally as Fasta-formatted files prior to homology screening against the *H. bacteriophora* draft genome. For homology search, a maximum threshold (E-value) of $1e^{-6}$ was accepted. The output of all searches was saved in tabular format comprising the sequence identity, degree of homology, positions of homology hits, and number of HSPs per sequence. Sequences with a single HSP in the genome of *H. bacteriophora* were used to design nematode primers that were used for relative qPCR quantification (see sections below).

2.5. DNA extraction, purification, and standardization of PCR conditions

For PCR amplification, DNA was extracted from 1000 DJ using the DNeasy® Blood & Tissue Kit (QIAGEN GmbH, Germany) according to the manufacturer's instructions. DNA extraction was done in triplicate. To destroy the DJ cuticle, the nematodes were frozen at −20 °C overnight and then crushed with a micro pestle. Bacterial DNA was extracted using E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek lnc, USA) from 1 ml of a bacterial suspension according to the manufacturer's instructions and DNA concentrations were measured using a NANODROP LITE Spectrophotometer (Thermo Fisher Scientific, USA).

PCR reactions were carried out in a Bioer Thermocycler (Bioer, Hangzhou, China). Reactions were done in a final volume of 20 µl containing 1x Dreamtaq Reaction Buffer, 0,4 mM dNTPs, 5 pmol of each primer, 0.5 U Dreamtaq DNA polymerase and ~ 32 ng DNA template. The PCR profile started with initial denaturation at 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 *sec*, 60 °C for 30 *sec* and 72 °C for 45 *sec*. PCR amplicons were separated on 1.5 % agarose gels stained in GelRed™ (Biotuim, USA) and visualized in a gel imaging document (Qauntum Vilber Lourmat, Germany). A cross-amplification test for the primers was carried out with DNA from the following *Photorhabdus* species: *P. laumondii,* strains DE2 and HB1.3, *P. thracensis* strains IT6 and IR2, *P. kayaii*, strains HU1 and HU2, *P. temperata,* strain K122, and one *Xenorhabdus* (*X. nematophila*, strain BUL31).

2.6. Bacterial DNA quantification by qPCR

All qPCR reactions were carried out in a final volume of 15 µl containing 7.5 µl 2x Go Taq® qPCR master mix (Promega GmbH, Germany), 2.5 pmol each primer, and ~ 32 ng DNA template. Reactions were carried out in a StepOnePlus™ real-time PCR machine (Applied Biosystems, Thermo Fisher Scientific, Germany) with the following profile: 95 °C for 10 min, 40 cycles of 95 °C for 15 *sec*, 60 °C for 1 min and a final melt curve analysis with starting temperature of 95 °C. A standard curve was first calibrated from a five-point 1/10 dilution series of pure *P. laumondii* DE2 genomic DNA from a starting concentration of 32 ng μl^{-1}. A linear regression slope from the log of the respective DNA concentrations (ng) and their respective C_t values (minimum cycles for fluorescence detection) was then determined following Rasmussen (2001). To determine the target gene copy number in the respective DNA concentrations, following Whelan et al. (2003), respective DNA concentrations (in ng) were multiplied by Avogadro's constant (6.022×10^{23} g/mol), which is the number of molecules in one mol. This product was then divided by the DNA template length (in bp) confirmed by Sanger sequencing the PCR amplicon, multiplied by the conversion factor to ng (1×10^9) and multiplied by the average mass of one base pair of double stranded DNA (660 g/mol).

$$Targetgenecopynumber = \frac{DNA(ng) \times 6.022 \times 10^{23}}{Length(bp) \times 1 \times 10^{9} \times 660}$$

To determine the bacteria target gene copy number in unknown samples (absolute quantification), obtained C_t values after qPCR were interpolated on the standard curve following Lee et al. (2006). The primer efficiency (*E*) was simultaneously determined from the slope of the same standard curve using the formular:

$$E = 10^{-1/slope} - 1$$

Parallel to absolute quantification, a relative quantification was carried out using a nematode primer as reference. Primers targeting the *H. bacteriophora* gene model homolog to the *Caenorhabditis elegans* zzz-1 gene were used as reference and primer efficiency determined as described above but using nematode DNA. Nematode and bacteria DNA relative quantities were calculated using both primer efficiencies following the formula of Pfaffl (2001).

$$Relativequantity = \frac{(E_{Target})\Delta C_{tTarget}(control - sample)}{(E_{Ref})\Delta C_{tRef}(control - sample)}$$

where E_{Target} is the calculated primer efficiency of the target gene (bacterial candidate primer), E_{Ref} is the primer efficiency of the reference gene (nematode reference primer), and C_t is the cycle at which the fluorescence signal is detected in the reaction. Control: DNA from fresh and virulent DJ; Sample: DNA from stored DJ along different times and conditions.

2.7. Data analysis

The analysis of infected insects (%), the absolute quantity of bacterial DNA amount (target gene copy number), and the relative quantity of the bacterial DNA amount was analyzed through one-way ANOVA and Tukey's HSD tests at $P \leq 0.05$ for means comparisons. The analysis of diatomaceous earth formulated DJ data was performed by two-way ANOVA and Tukey's HSD tests at $P \leq 0.05$. Non-normally distributed data were analyzed using the Kruskal-Walli's test and means were compared by the Dunn's tests at $P \leq 0.05$ without adjusting *P* values. All data analysis was done in RStudio software (https://www.rstudio.com/).

3. Results

3.1. Virulence of Heterorhabditis bacteriophora DJ

Significant differences in insect mortality were recorded among commercial HB4 DJ stored in Ringer solution at 6 °C for up to 300 days ($F_5 = 690.1$; $P < 0.005$). The determined insect mortality along the first 90 days of storage was above 80 %. Thereafter it declined drastically down to 14 ± 4 % after 150 days of storage. In general, DJ of up to 300 days of age had a drastic virulence reduction (Fig. 1A). In counterpart, some *H. bacteriophora* WT strains and inbred lines did not lose virulence after long storage. Interestingly, WT strains carrying their native bacteria caused insect mortality after 300 (IT6, 21 ± 10 %; IT4,7 ± 3 %) and 350 days (IR2, 26 ± 5 %) while no virulence was detected after 325 days in the WT inbred lines AU11, HU21 and IR11 carrying *P. laumondii* DE2 bacteria. The control HB4 DJ caused 36 ± 7 % insect mortality after 25 days of storage. Significant differences in insect mortality ($F_6 = 23.88$; $P < 0.005$) were determined (Fig. 1B).

To check whether a declining load of symbiotic bacteria in a DJ population will result in lower insect mortality, populations comprising axenic and bacteria-carrying DJ were used in virulence tests. Insect mortality (%) declined as the proportion of bacteria-carrying DJ decreased and the differences between the DJ populations were significant ($F_4 = 50.63$; $P < 0.001$). The P100 population containing only DJ with bacteria caused insect mortality of 55 ± 9 % while the population of only bacteria-free DJ was not virulent (Fig. 1C).

3.2. Sequence analysis and primer design

Out of the initial 2,036 predicted *Photorhabdus laumondii* DE2 gene models taken for BLAST analysis against NCBI bacterial accessions, 10 genes showed no homology to any other bacteria. After local BLAST against the DE2 genome it turned out that all 10 gene models had a single full length HSP thus were considered single copy genes. Regions of 200 to 330 bp with minimal overlap with short length HSP regions in the DE2 genome were selected for primer design for the respective candidate genes (CG). After BLAST analysis of the selected regions for primer design against all bacterial accessions at NCBI, only seven genes had less than 3 short length HSPs within the primer design region. The seven candidate bacterial genes selected for primer synthesis are presented in Table 3. No overlap with any other organism was found after BLASTing the sequences of the theoretical amplicons against NCBI. In the case of *H. bacteriophora*, from the initial 1,660*C. elegans* gene sequences checked for homology against *H. bacteriophora* sequences, 197 had a high homology (E value $< 9.75e^{-07}$; sequence identity > 64.5 %). From these, the gene model homologous to the *C. elegans zzz-1* fulfilled the requirement for being single copy and primers were designed as previously described. The region coding for this gene was located on Scaffold1020 between positions 495.704 and 496.899 of the public *H. bacteriophora* TT01 genome draft.

3.3. Primer verification and cross-amplification test

Out of seven primer pairs chosen for further investigation, primers from two candidate genes, candidate gene 2 (CG2) and candidate gene 8 (CG8) yielded unambiguous amplicons. For both genes, the cross-amplification test with other *Photorhabdus* sp. resulted positive for *P. thracensis* (strains IT6 and IR2) and *P. kayaii* (strains HU1 and HU2) whereas negative for *P. temperata (K122)* and *Xenorhabdus nematophila* (BUL31), the symbiotic partner of *H. downesi* and *Steinernema carpocapsae*, respectively (data not shown). BLAST analysis of the primer amplification regions of candidate genes CG2 and CG8 against *Photorhabdus* accessions showed the presence of CG8 in 21 *Photorhabdus* species while CG2 was only present in *P. laumondii*, *P. bodei*, *P. kleinii*, *P. kayaii*, *P. thracencis*, *P. stackebranditii*, and *P. khani*. Results from this *in silico* analysis are summarized in Table 4.

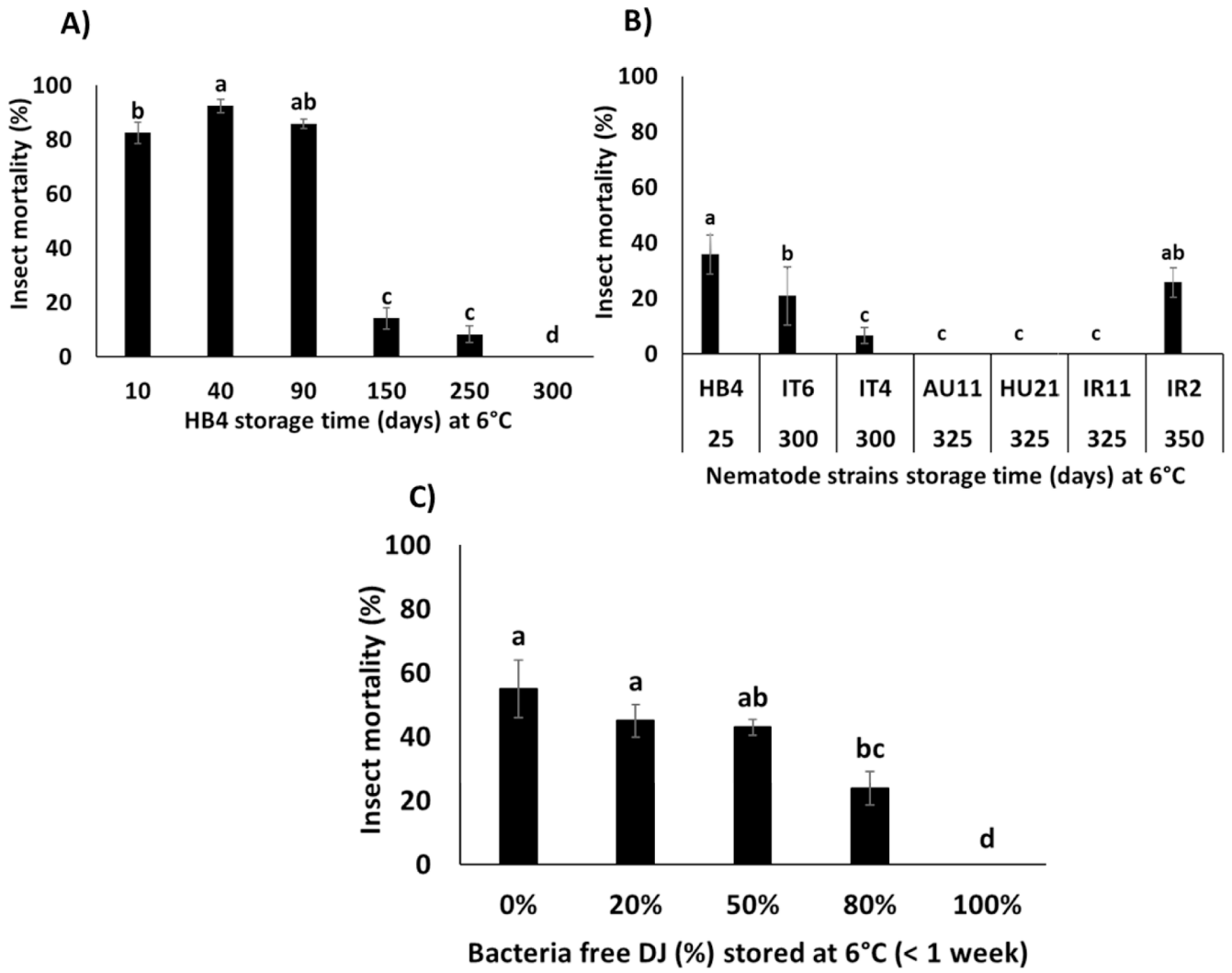

Fig. 1. Average *Tenebrio mollitor* larvae mortality (%) in sand bioassays with *Heterorhabditis bacteriophora* DJ stored at 6 °C up to 300 days. Bioassays were carried out in triplicate with 40 insects each and a DJ dose of 500 DJ per assay. All bioassays were incubated at 25 °C in the dark for 7 days until evaluation. Virulence of strain HB4 DJs stored at 6 °C for up to 300 (A). Virulence of different wild type strains and inbred lines stored at 6 °C for more than 300 days in comparison to fresh HB4 DJ cultured on NGG (25 days storage at 6 °C) (B). virulence of artificial populations from HB4 DJ with different proportions of bacteria-free nematodes stored for less than a week at 6 °C (C). Different letters above bars denote significant differences between treatments (Tukey HSD test $P < 0.05$). Error bars SD.

Thereafter, an efficiency of 83 % for *P. laumondii* CG2 (slope −3.79, intercept 17.41), 74 % for *P. laumondii* CG8 (slope −4.15, intercept 18.13), and 99 % for *H. bacteriophora* zzz-1 derived primers was determined after qPCR of dilution series, as exemplarily shown in Fig. 2A. Considering the higher primer amplification efficiency and that our main goal is to monitor commercially-produced DJ (carrying *P. laumondii*), further optimizations for qPCR were done with CG2. For the amplicon resulting for this primer pair, a single peak at melting temperature of 83.3 °C was contained (Fig. 2B). Sequencing of the purified PCR CG2 amplicon yielded a 286 bp fragment with 99.16 % homology to *P. laumondii* accessions after NCBI BLAST (CP024900 and CP015281). The CG2 primer pairs were therefore chosen for primary quantification of bacterial load in stored *H. bacteriophora* DJ.

3.4. Bacterial DNA amount quantification in Heterorhabditis bacteriophora DJ

In congruence with the insect virulence after sand bioassays against mealworm, the bacteria target gene CG2 DNA copy number detected inside HB4 DJ stored at 6 °C decreased with the DJ age and the differences within time points were significant ($F_5 = 23.88$; $P < 0.005$). In DJ stored up to 300 days, the detected bacterial copy number was 750-fold lower than in fresh DJ (Fig. 3A). Among *H. bacteriophora* WT strains IT4, IT6, IR2, carrying their native bacteria and WT inbred lines AU11, HU21, IR11, carrying *P. laumondii* DE2 symbiont, significant differences in bacterial CG2 gene DNA copy number were detected ($X^2 = 12.80$; df = 6, $P < 0.005$) when comparing with 25-day old HB4 DJ (Fig. 3 B). Larger CG2 gene DNA copy numbers were detected in WT strains carrying their native symbiotic bacteria than the WT inbred lines carrying the *Photorhabdus* DE2 strain, although differences were not significant.

Concerning artificial DJ populations with different ratios of axenic vs bacteria-carrying DJ, the bacterial target gene CG2 DNA copy number decreased with the decreasing proportion of bacteria-carrying DJ (Fig. 4A) and the differences between populations were significant ($F_4 = 2325$; $P < 0.0001$). To exclude the possibility that "axenic" DJ may carry still their symbiont (*P. temperata*) but this remains undetected due to the restricted sequence homology of CG2 (see Table 4), we conducted in parallel the amplification with the CG8 primers (despite the lower efficiency). The detected CG8 DNA copy number was also significantly different ($F_4 = 1640$; $P < 0.0001$) between the populations and decreased with decreasing proportion of bacteria-carrying DJ (Fig. 4B).

Parallel to absolute quantification of bacterial DNA copy number

Table 3
Photorhabdus laumondii strain DE2 candidate genes for primer design, gene model length, PCR fragment size and forward (F) and reverse (R) primer sequences. BLAST results of 2,036 predicted genes at NCBI data base yielded 10 candidate genes with no homology to any other bacteria accessions (0 HSPs), and only a single copy after local BLAST against the DE2 genome (1 major large HSP). Sequences of the *Heterorhabdits bacteriophora* reference gene model (zzz-1) are also provided.

Gene Name	Short Name	Gene length	PCR-fragment size	Orientation	Sequences
Plum_DE2.2_02019	CG1	834	294	F	AGTCCGTCTGGTTTCGTAG
				R	CTTGCTGCCAAAAATGCG
Plum_DE2.2_01026	CG2	723	283	F	CGAGTGGGATAGACGTACAG
				R	AGGATCACCGGAATTAGGA
Plum_DE2.2_01800	CG3	597	249	F	TGATGCAAGGACAACGGT
				R	GCACAACGTATCGGCTAA
Plum_DE2.2_01042	CG4	675	285	F	AATTTTCCAAAGCCGAGAGT
				R	CGTTCACGCTAGATTCCA
Plum_DE2.2_00873	CG5	684	331	F	TGGTGTTTGCTACATGGC
				R	GCAGAAATCAGTGGAAGGAAA
Plum_DE2.2_00192	CG8	624	180	F	GCGGCATTAGAATGGGTTATT
				R	ACGGGCCGCATTATTGGA
Plum_DE2.2_00815	CG10	1011	310	F	CTCAATCAAGATGCCAGCC
				R	ATTTGCCATACATACAGTCACC
GG14650|c5_g1_i4	zzz-1	1196	321	R	GAGGAAGTGGTGGTTGGA
				F	TTGTCACCGTACTTGGCT

Table 4
In silico BLAST analysis of selected primer design regions of candidate genes CG2 and CG8 against *Photorhabdus* spp. accessions at NCBI. CG8 was present in 21 *Photorhabdus* species while CG2 was only present in seven.

Species	Percentage identity to CG2	Accession number	Percentage identity to CG8	Accession number
P. laumondii	99 %	NC_005126.1	100 %	NC_005126.1
P. bodei	94 %	NZ_NSCM010000	98 %	NZ_NSCM010000
P. luminescens			96 %	NZ_JXSK010000
P. noenieputensis			97 %	NZ_JAJQTM0100
P. kleinii	90 %	NZ_JAJAFY0100	96 %	NZ_JAJAFY0100
P. kayaii	89 %	NZ_JAJAFZ0100	96 %	NZ_JAJAFZ0100
P. antumapuensis			96 %	NZ_JAHZMK0100
P. caribbeanensis			95 %	NZ_RCWB010000
P. hindustanensis			95 %	NZ_PUWT010000
P. namnaonensis			95 %	NZ_LOIC010000
P. aegyptia			94 %	NZ_JFGV010000
P. akhurstii			93 %	NZ_CP020335.1
P. aballayi			93 %	NZ_JAPFCD0100
P. hainanensis			93 %	NZ_RCWD010000
P. temperata			91 %	NZ_JGVH010000
P. thracensis	89 %	NZ_CP011104.1	90 %	NZ_CP011104.1
P. cinerea			89 %	NZ_PUJW010000
P. symbiotica			87 %	NC_012962.1
P. stackebrandtii	90 %	NZ_PUJV010000	86 %	NZ_PUJV010000
P. khani	90 %	NZ_AYSJ010000	86 %	NZ_AYSJ010000
P. tasmaniensis			87 %	NZ_PUJU010000

inside the DJ, bacterial DNA was quantified relative to the nematode DNA using *H. bacteriophora* primer zzz-1 as a reference. Bacterial DNA amounts in WT strains and WT inbred lines (Fig. 5A) were compared relative to fresh HB4 DJ (25 days old). Relative bacterial DNA amounts inside the WT strains and WT inbred lines were significantly different (Fig. 5A) ($X^2 = 12.60$; df = 6, $P < 0.05$), and the amounts were significantly correlated to the absolute quantification method (Fig. 5B).

3.5. Monitoring the bacterial load of formulated DJ over storage time

Storage temperature of formulated DJ had a significant effect on insect mortality ($F_2 = 52.56$; $P < 0.001$). The lower the storage temperature the lower was the DJ virulence. The storage time was limited to 6 weeks. Within this time no drastic decline in insect mortality (%) was recorded for DJ stored at 7.5 °C, whereas for lower temperatures a virulence reduction was determined (Fig. 6 A). The detected target CG2 gene copy number was significantly influenced by the storage temperature of the formulated nematodes ($F_2 = 241.19$; $P < 0.001$) and the storage duration ($F_3 = 71.26$; $P < 0.001$). A 4.5-fold decline in the bacteria DNA copy number was recorded in DJ stored at 2 °C after two weeks while the decline was more gradual for DJ stored at 4 °C (1.8-fold decline in DNA copy number). After 6 weeks, DJ stored at both 2 and 4 °C, had an 8-fold decline in copy number. In contrast, bacteria in DJ stored at 7.5 °C slightly decreased significantly from weeks 2–4 and

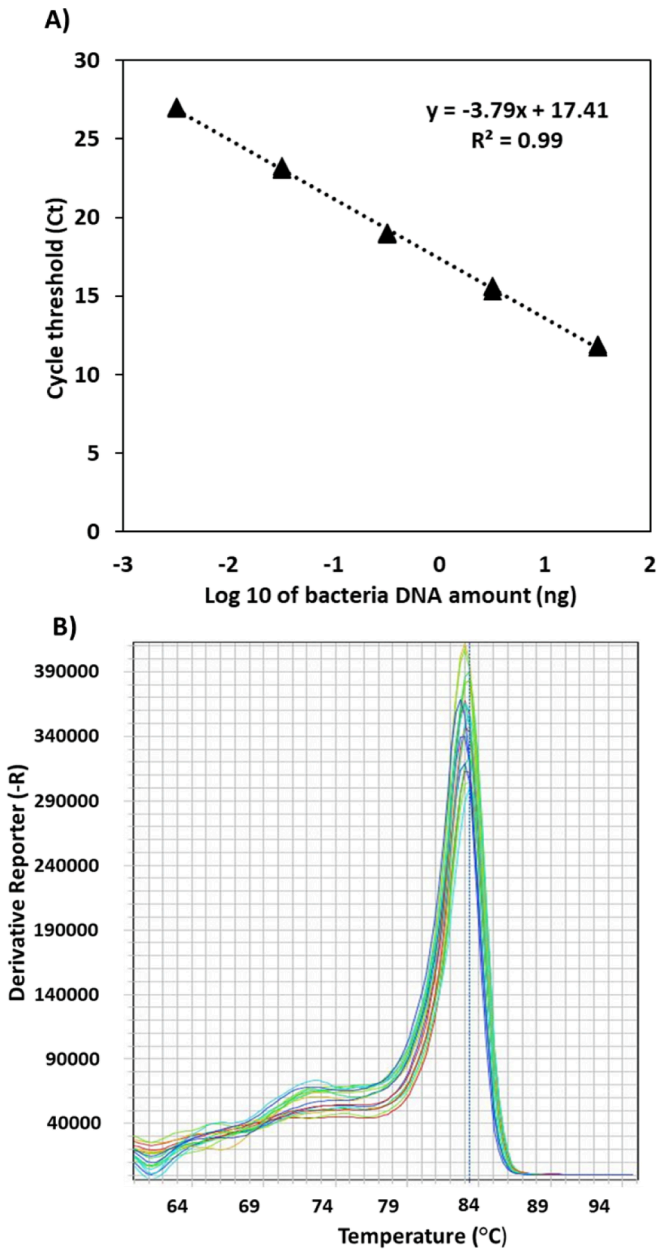

Fig. 2. Primer efficiency calculation for candidate gene CG2 using a DNA five-point dilution series (A). By plotting the C_t values obtained from each dilution series against the log values of corresponding DNA amount (slope = −3.79) a primer efficiency of 83 % was determined. Single product amplification of candidate gene CG2 verified by melting curve analysis. A single peak depicts that a single amplicon is being amplified (B).

increased significantly within the next 2 weeks reaching the highest CG2 copy number after 6 weeks of storage (Fig. 6 B).

3.6. Correlation between insect infectivity and detected bacteria DNA copy number

The correlation between insect mortality and detected bacteria DNA copy number was positive, however not significant ($R = 0.79$, $P = 0.06$) for HB4 DJ stored at 6 °C in Ringeŕs solution for up to 300 days. However, the correlation between these two parameters in the remaining tested nematode materials was positive and significant. For nematode WT strains and inbred lines stored at 6 °C for more than 300 days ($R = 0.88$, $P = 0.008$), for different proportions of bacteria free- and bacteria-

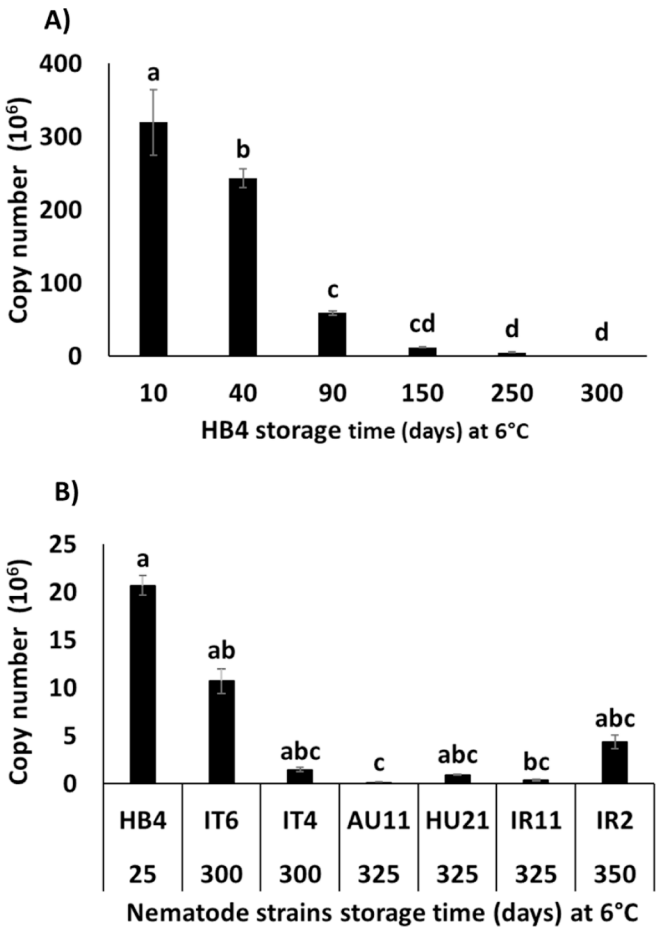

Fig. 3. Estimated bacterial load (*Photorhabdus* gene CG2 copy number) in *Heterorhabditis bacteriophora* DJ deduced by absolute DNA quantification. Copy number estimation in 32 ng of DNA (extracted from 1,000 DJs) of HB4 DJ stored at 6 °C up to 300 days (A). Copy number estimation in 32 ng of DNA (extracted from 1,000 DJ) of WT strains and inbred lines stored at 6 °C for more than 300 days in comparison to fresh HB4 DJ (25 days storage at 6 °C) (B). Different letters above bars denote significant differences between treatments (Tukey HSD test: $P < 0.05$ for normally distributed data and Dunn's test: $P < 0.05$ for non-normally distributed data). Error bars SD.

carrying HB4 DJ stored at 6 °C for 1 week ($R = 0.96$, $P = 0.009$) and for formulated HB4 DJ stored at 2, 4 and 7.5 °C for 6 weeks ($R = 0.68$, $P = 0.02$) a high correlation was recorded.

4. Discussion

Progress resulting from this investigation is the provision of a molecular method to estimate the bacterial load inside DJ material. Two easy and robust qPCR procedures can now complement virulence bioassays in quality control of products containing *H. bacteriophora*. The study also yielded 10 new *P. laumondii* single copy genes with potential application for both detection and quantification of this species. Single copy genes are ubiquitous in bacteria (Wang et al., 2022). The single copy CG2 primers were able to amplify DNA from *P. thracensis* and *P. kayaii*, both symbionts of *H. bacteriophora*, but not from *P. temperata* and *X. nematophila*, highlighting its limits for species identification of the genus *Photorhabdus*. However, *in silico* analysis revealed candidate gene CG8 to be present in 21 *Photorhabdus* species making this gene interesting for evolutionary studies. Through a stringent species delineation within the genus *Photorhabdus*, Machado et al. (2018) have thereby elevated many former *P. luminescens* subspecies to species level. These single copy genes may thus be included in *Photorhabdus* phylogenetic

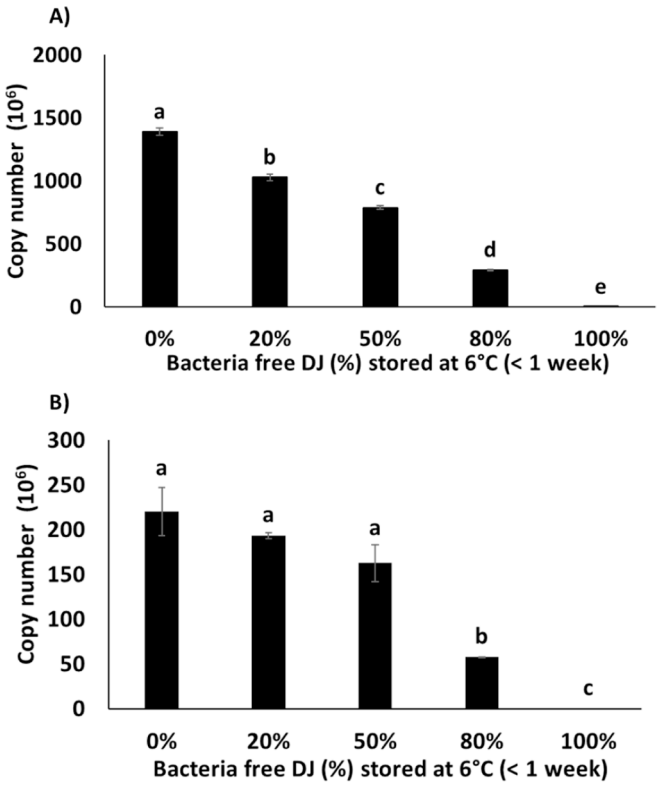

Fig. 4. Estimated bacterial load in different populations of *Heterorhabditis bacteriophora* (strain HB4) with variable proportions of bacteria free DJ stored at 6 °C for less than a week. Copy number estimation of target gene CG2 in 32 ng of DNA extracted from 1,000 DJ (A). Copy number estimation of target gene CG8 in 32 ng of DNA extracted from 1,000 DJ (B). Different letters above bars denote significant differences between treatments (Tukey HSD test: $P < 0.05$).

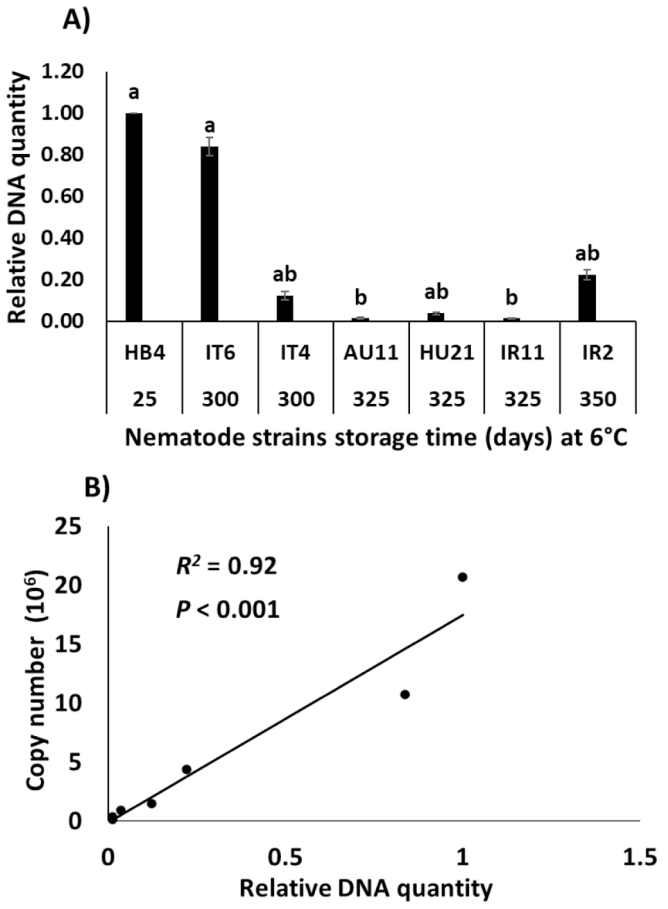

Fig. 5. Estimated bacteria load (*Photorhabdus* gene CG2 copy number) in *Heterorhabditis bacteriophora* DJ deduced by relative DNA quantification. Nematode primer designed from the gene model homolog to the *Caenorhabditis elegans zzz-1* gene were used as reference. Bacteria DNA amount inside *H. bacteriophora* WT strains and lines after more than 300 days storage in Ringer's solution at 6 °C were compared relative to DNA from fresh HB4 DJ (25 days storage at 6 °C) (A). Different letters above the bars indicate significant differences (Dunn's test: $P < 0.05$). Error bars: SD. Correlation between absolute bacterial DNA quantification (target gene copy number) and relative bacterial DNA quantification in *H. bacteriophora* WT strains and inbred lines stored at 6 °C in Ringer's solution for more than 300 days (B).

studies.

For quantification purposes of *P. laumondii* cells carried by DJ, the single copy nature of the candidate gene CG2 makes it ideal for commercial products. Accurate absolute quantification of nucleic acids requires highly validated calibration curves with recommended primer efficiencies ranging between 90 and 100 % (Bustin et al., 2009). We obtained a primer efficiency of 83 % for the CG2 gene, which we deemed sufficient for the purpose of monitoring DJ bacteria load. Moreover, interpretation of our results would not have been significantly different even with higher efficiency. The equation from Pfaffl (2001) that we modified for this study accounts for the primer efficiency in relative bacteria DNA quantification and these results were highly correlated with absolute quantification. We, however, could not determine the exact number of symbiotic bacteria cells carried by a single DJ owing to the technical challenges of extracting DNA from a defined amounts of bacteria cells which is highly variable per assay and the difficulty of DNA extraction from a single DJ. However, standardizing all DNA reactions to start with 32 ng of unknown DNA aided to optimize our assay. Designing Taqman probes for the CG2 primers could increase the accuracy of bacterial quantification as highlighted by Campos-Herrera et al. (2011).

As *Heterorhabditis* spp. DJ not carrying cells of symbiotic *Photorhabdus* are unable to kill insect hosts (Han et al., 1991; Gerritsen and Smits, 1993: Han and Ehlers, 2000), the presence of the bacteria in the DJ is crucial for the biocontrol potential. This investigation showed that with decreasing number of bacteria-carrying DJ the virulence of the DJ populations declines and that the nematodes can survive longer than their bacterial partners. With storage duration, the virulence of the DJ is decreasing and this may correlate with the reduction of cells inside the DJ. Consequently, the number of cells inside a DJ is an important quality parameter. The results also attest that storage temperature influences bacterial survival. Below 7.5 °C, the loss of bacterial cells is accelerated. This matches well with the findings of Strauch et al. (2000), who reported that 7.5 °C is the optimal storage temperature for *H. bacteriophora*. However, whether even higher storage temperature will better protect the bacterial cells from decaying, needs further investigation.

This study indicates that DJ of WT strains produced in *G. mellonella* carried living Photorhabdus cells even after 300 days of storage and that *in vitro* cultured DJ of WT inbred lines fed with DE2 bacteria had lost their symbionts after the same time. However, culture methods have been reported to have a trivial effect on symbiont bacteria retention (Ciche and Ensign, 2003). Hence, further attention is necessary to clarify whether nematode strains differ in promotion of survival of the symbiont cells or whether the WT strains maintained their symbionts for a longer time because they carry their native bacteria. That would be an indication for an environmental adaptation between symbiont and its nematode host. The exchange of the bacterial symbiont is common practice to select the optimal strains to promote *in vitro* production, specifically DJ recovery and yield (Ehlers, 2001). In breeding programs producing novel hybrids between lines, progenies may be combined

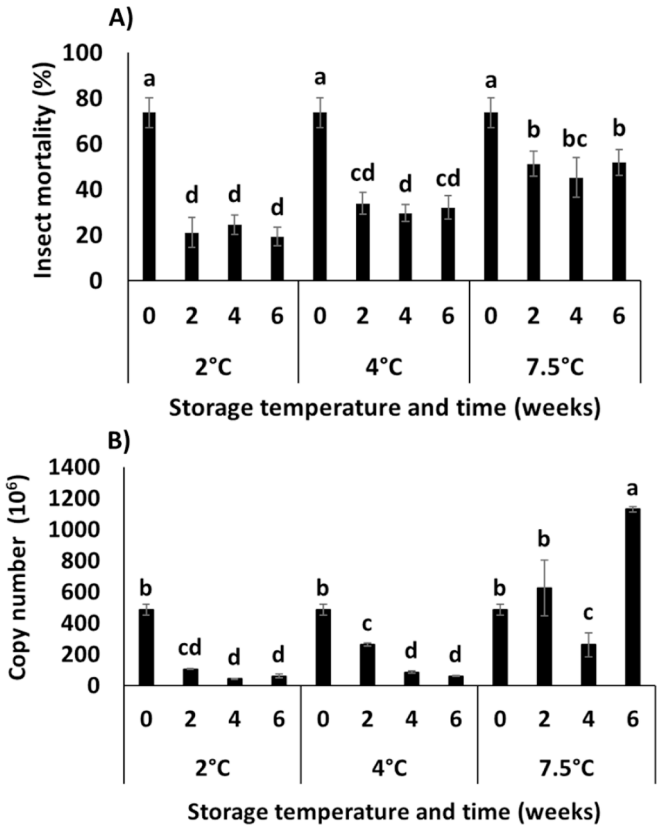

Fig. 6. Average *Tenebrio molitor* larvae mortality (%) in sand bioassays treated with formulated *Heterorhabditis bacteriophora* DJ (strain HB4) stored at 2, 4 and 7.5 °C up to 6 weeks (A). Bioassays were carried out in triplicate with 40 insects each and a DJ dose of 500 DJ per assay. All bioassays were incubated at 25 °C in the dark for 7 days until evaluation. Estimated bacterial load (*Photorhabdus* gene CG2 copy number) in *H. bacteriophora* DJ deduced by absolute DNA quantification for the corresponding storage temperatures and time in 32 ng of DNA extracted from 1,000 DJ (B). Error bars: SD. Different letters above bars denote significant differences among treatments (Tukey HSD test: $P < 0.05$).

with bacterial strains that are not the native ones (Godina et al., 2022). Meta-organisms (nematode-bacterium complex) adaptation can result in increasing fitness like observed for *C. elegans* (Petersen et al., 2023). The combination with a non-specific symbiont can therefore result in a loss of fitness, which might have been the reason for the loss of the symbiont in DJ fed with DE2 bacteria.

In commercial production, nematode DJ are propagated in liquid culture and stored at 4°–7 °C. Under these conditions the symbiotic bacterial load is not drastically reduced for at least 90 days in liquid storage. Ciche and Ensign (2003) reported no fluctuation in bacterial symbiont load inside DJ up to 30 days of storage. It thus appears that an acceptable level of virulence can be maintained for at least 90 days under these conditions. Within this period, DJ should be applied. Storage in the formulation used in this study resulted in accelerated loss of the bacterial load. The detrimental effect of low storage temperatures of 2 and 4 °C and the loss of virulence in *H. bacteriophora* DJ are apparent from this study. Low storage temperatures are attractive for preservation of the nematode fat reserves, however, the effect on the bacterial symbiont should always be considered. Successful prolonged storage of nematodes in these storage conditions could require new combinations of *H. bacteriophora* DJ with cold-adapted *Photorhabdus* spp. strains. Ehlers et al. (2005) reported the availability of 8 such *Photorhabdus* strains reproducing at lower temperature conditions. These could be perfect candidates for improved new combinations. The increasing copy numbers inside formulated DJ stored at 7.5 °C indicates that a slight reproduction of the bacterial cells takes place when growth temperature is favorable.

In conclusion, the role of bacterial survival inside stored DJ has not received adequate attention in the past and this contribution provides a molecular quality-control method that can take this factor into consideration.

CRediT authorship contribution statement

Christopher Ogaya: Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Investigation. **Nontarak Huong:** Data curation, Investigation, Validation. **Maria Touceda-González:** Methodology. **Mike Barg:** Methodology, Validation. **Verena Dörfler:** Methodology, Validation. **Ralf-Udo Ehlers:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Carlos Molina:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The scholarship by the German Academic Exchange Service (http://www.daad.de) to the first author and the scholarship by the Flemish University Cooperation for Development (VLIR-UOS, http://www.vliruos.be) to the second author are much appreciated.

References

Addis, T., Teshome, A., Strauch, O., Ehlers, R.-U., 2014. Life history trait analysis of the entomopathogenic nematode *Steinernema riobrave*. Nematology 16, 929–936. https://doi.org/10.1163/15685411-00002819.

Bai, X., Adams, B.J., Ciche, T.A., Clifton, S., Gaugler, R., Kim, K., Spieth, J., Sternberg, P. W., Wilson, R.K., Grewal, P.S., 2013. A lover and a fighter: The genome sequence of an entomopathogenic nematode *Heterorhabditis bacteriophora*. PLoS One 8, e69618.

Bhat, C.G., Somvanshi, V.S., Budhwar, R., Godwin, J., Rao, U., 2022. Nematode genome announcement: the draft genome sequence of entomopathogenic nematode *Heterorhabditis indica*. J. Nematol. 53, e2021–e2101. https://doi.org/10.21307/jofnem-2021-101.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Campos-Herrera, R., El-Borai, F.E., Stuart, R.J., Graham, J.H., Duncan, L.W., 2011. Entomopathogenic nematodes, phoretic *Paenibacillus* spp., and the use of real time quantitative PCR to explore soil food webs in Florida citrus groves. J. Invertebr. Pathol. 108, 30–39. https://doi.org/10.1016/j.jip.2011.06.005.

Ciche, T.A., Ensign, J.C., 2003. For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? Appl. Environ. Microbiol. 69, 1890–1897. https://doi.org/10.1128/AEM.69.4.1890-1897.2003.

Ciche, T.A., Kim, K., Kaufmann-Daszczuk, B., Nguyen, K.C., Hall, D.H., 2008. Cell invasion and matricide during *Photorhabdus luminescens* transmission by *Heterorhabditis bacteriophora* nematodes. Appl. Environ. Microbiol. 74, 2275–2287. https://doi.org/10.1128/AEM.02646-07.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., Bocs, S., Boursaux-Eude, C., Chandler, M., Charles, J.F., Dassa, E., Derose, R., Derzelle, S., Freyssinet, G., Gaudriault, S., Médigue, C., Lanois, A., Powell, K., Siguier, P., Kunst, F., 2003. The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. Nat. Biotechnol. 21, 1307–1313. https://doi.org/10.1038/nbt886.

Ehlers, R.-U., Oestergaard, J., Hollmer, S., Wingen, M., Strauch, O., 2005. Genetic selection for heat tolerance and low temperature activity of the entomopathogenic nematode–bacterium complex *Heterorhabditis bacteriophora–Photorhabdus luminescens*. Biocontrol 50, 699–716. https://doi.org/10.1007/s10526-005-5079-z.

Ehlers, R.-U., 2001. Mass production of entomopathogenic nematodes for plant protection. Appl. Microbiol. Biotechnol. 56, 623–633. https://doi.org/10.1007/s002530100711.

Eliáš, S., Hurychová, J., Toubarro, J., Frias, J., Kunc, M., Dobeš, P., Simões, N., Hyršl, P., 2020. Bioactive excreted/secreted products of entomopathogenic nematode *Heterorhabditis bacteriophora* inhibit the phenoloxidase activity during the infection. Insects 11, 353. https://doi.org/10.3390/insects11060353.

Endo, B.Y., Nickle, W.R., 1991. Ultrastructure of the intestinal epithelium, lumen, and associated bacteria in *Heterorhabditis bacteriophora*. J. Helminthol. Soc. Wash. 58, 202–212.

Gerritsen, L.J.M., Smits, P.H., 1993. Variation in pathogenicity of recombinations of *Heterorhabditis* and *Xenorhabdus luminescens* strains. Fundam. Appl. Nematol 16, 367–373.

Gerritsen, L.J.M., Smits, P.H., 1997. The influence of *Photorhabdus luminescens* strains and form variants on the reproduction and bacterial retention of *Heterorhabditis megidis*. Fundam. Appl. Nematol. 20, 317–322.

Ghazal, S., Oshone, R., Simpson, S., Morris, K., Abebe-Akele, F., Thomas, W.K., Khalil, K. M., Tisa, L.S., 2016. Draft genome sequence of *Photorhabdus luminescens* subsp. *laumondii* HP88, an entomopathogenic bacterium isolated from nematodes. Genome Announc. 4, e00154–e00216. https://doi.org/10.1128/genomeA.00154-16.

Godina, G., Kirsch, C., Dörfler, V., Barg, M., Singh, P.R., Vandenbossche, B., Strauch, O., Ehlers, R.-U., Molina, C., 2022. Single nucleotide polymorphism markers in Heterorhabditis bacteriophora associated with virulence at low temperature. Nematology 1, 1–14. https://doi.org/10.1163/15685411-bja10181.

Han, R., Ehlers, R.-U., 2000. Pathogenicity, development and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic in vivo conditions. J. Invertebr. Pathol. 75, 55–58. https://doi.org/10.1006/jipa.1999.4900.

Han, R., Wouts, W.M., Li, L., 1991. Development and virulence of *Heterorhabditis* spp. strains associated with different *Xenorhabdus luminescens* isolates. J. Invertebr. Pathol. 58, 27–32. https://doi.org/10.1016/0022-2011(91)90158-M.

Higuchi, R., Dollinger, G., Walsh, P.S., Griffith, R., 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnol. J. 10, 413–417. https://doi.org/10.1038/nbt0492-413.

Hirao, A., Ehlers, R.-U., 2009. Influence of cell density and phase variants of bacterial symbionts (*Xenorhabdus* spp.) on dauer juvenile recovery and development of biocontrol nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida). Appl. Microbiol Biotechnol. 84, 77–85. https://doi.org/10.1007/s00253-009-1961-4.

Kaya, H.K., Stock, P., 1997. Techniques in insect nematology. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, New York, NY, USA, pp. 281–324. https://doi.org/10.1016/B978-012432555-5/50016-6.

Larionov, A., Krause, A., Miller, W., 2005. A standard curve based method for relative real time PCR data processing. BMC Bioinform. 6, 62. https://doi.org/10.1186/1471-2105-6-62.

Lee, C., Kim, J., Shin, S.G., Hwang, S., 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J. Biotechnol. 123, 273–280. https://doi.org/10.1016/j.jbiotec.2005.11.014.

Machado, R.A.R., Muller, A., Ghazal, S.M., Thanwisai, A., Pagès, S., Bode, H.B., Hussein, A.M., Khalil, M.K., Tisa, L.S., 2021. Photorhabdus heterorhabditis subsp. aluminescens subsp. nov., Photorhabdus heterorhabditis subsp. heterorhabditis subsp. nov., Photorhabdus australis subsp. thailandensis subsp. nov., Photorhabdus australis subsp. australis subsp. nov., and Photorhabdus aegyptia sp. Nov. isolated from Heterorhabditis entomopathogenic nematodes. Int. J. Syst. Evol. Microbiol. 71, 004610 https://doi.org/10.1099/ijsem.0.004610.

Machado, R.A.R., Wüthrich, D., Kuhnert, P., Arce, C.C.M., Thönen, L., Ruiz, C., Zhang, X., Robert, C.A.M., Karimi, J., Kamali, S., Ma, J., Bruggmann, R., Erb, M., 2018. Whole-genome-based revisit of *Photorhabdus* phylogeny: Proposal for the elevation of most *Photorhabdus* subspecies to the species level and description of one novel species *Photorhabdus bodei* sp. nov., and one novel subspecies *Photorhabdus laumondii* subsp. *clarkei* subsp. nov. Int. J. Syst. Evol. Microbiol. 68, 2664–2681. https://doi.org/10.1099/ijsem.0.002820.

Maier, C., Hofmann, K., Huptas, C., Scherer, S., Wenning, M., Lücking, G., 2021. Simultaneous quantification of the most common and proteolytic *Pseudomonas* species in raw milk by multiplex qPCR. Appl. Micro. Biotechnol. 105, 1693–1708. https://doi.org/10.1007/s00253-021-11109-0.

McLean, F., Berger, D., Laetsch, D.R., Schwartz, H.T., Blaxter, M., 2018. Improving the annotation of the *Heterorhabditis bacteriophora* genome. GigaScience 7. https://doi.org/10.1093/gigascience/giy034.

Mukuka, J., Strauch, O., Ehlers, R.-U., 2010. Variability in desiccation tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*. Nematology 12, 711–720. https://doi.org/10.1163/138855409X12607871174454.

Palma, L., Del Valle, E.E., Frizzo, L., Berry, C., Caballero, P., 2016. Draft genome sequence of *Photorhabdus luminescens* strain DSPV002N isolated from Santa Fe, Argentina. Genome Announc. 4, e00744–e00816. https://doi.org/10.1128/genomeA.00744-16.

Petersen, C., Hamerich, I.K., Adair, K.L., Griem-Krey, H., Montserrat Torres, O., Hoeppner, M.P., Bohannan, B.J.M., Schulenburg, H., 2023. Host and microbiome jointly contribute to environmental adaptation. ISME J. 17, 1953–1965. https://doi.org/10.1038/s41396-023-01507-9.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 45e–e. https://doi.org/10.1093/nar/29.9.e45.

Poinar Jr, G.O., 1993. Origins and phylogenetic relationships of the entomophilic rhabditids, *Heterorhabditis* and *Steinernema*. Fundam. Appl. Nematol. 16, 333–338.

Rasmussen, R., 2001. Quantification on the lightcycler. In: Meuer, S., Wittwer, C., Nakagawara, K.-.-I. (Eds.), Rapid Cycle Real-Time PCR. Springer, Berlin, Heidelberg, Germany, pp. 21–34. https://doi.org/10.1007/978-3-642-59524-0_3.

Shapiro-Ilan, D.I., Hazir, S., Lete, L., 2015. Viability and virulence of entomopathogenic nematodes exposed to ultraviolet radiation. J. Nematol. 47, 184.

Shi, Y.M., Hirschmann, M., Shi, Y.N., Ahmed, S., Abebew, D., Tobias, N.J., Grün, P., Crames, J.J., Pöschel, L., Kuttenlochner, W., Richter, C., Herrmann, J., Müller, R., Thanwisai, A., Pidot, S.J., Stinear, T.P., Groll, M., Kim, Y., Bode, H.B., 2022. Global analysis of biosynthetic gene clusters reveals conserved and unique natural products in entomopathogenic nematode-symbiotic bacteria. Nat. Chem. https://doi.org/10.1101/2022.01.21.477171.

Somvanshi, V.S., Dubay, B., Kushwah, J., Ramamoorthy, S., Vishnu, U.S., Sankarasubramanian, J., Rajendhran, J., Rao, U., 2019. Draft genome sequences for five *Photorhabdus* bacterial symbionts of entomopathogenic *Heterorhabditis* nematodes isolated from India. Microbiol. Resour. Announc. 8, e01404–e1418. https://doi.org/10.1128/MRA.01404-18.

Strauch, O., Ehlers, R.-U., 1998. Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. Appl. Microbiol. Biotechnol. 50, 369–374. https://doi.org/10.1007/s002530051306.

Sulistyanto, D., Ehlers, R.-U., 1996. Efficacy of the entomopathogenic nematodes *Heterorhabditis megidis* and *Heterorhabditis bacteriophora* for the control of grubs (*Phyllopertha horticola* and *Aphodius contaminatus*) in golf course turf. Biocontrol. Sci. Technol. 6, 247–250. https://doi.org/10.1080/09583159650039430.

Sumaya, N.H., Aryal, S., Vandenbossche, B., Barg, M., Doerfler, V., Strauch, O., Molina, C., Ehlers, R.-U., 2017. Phenotyping dauer juvenile oxidative stress tolerance, longevity and persistence within wild type and inbred lines of the entomopathogenic nematode *Heterorhabditis bacteriophora*. Nematology 19, 971–986. https://doi.org/10.1163/15685411-00003100.

Susurluk, A., Ehlers, R.-U., 2008a. Field persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* in different crops. BioControl 53, 627–641. https://doi.org/10.1007/s10526-007-9104-2.

Susurluk, A., Ehlers, R.-U., 2008b. Sustainable control of black vine weevil larvae, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) with *Heterorhabditis bacteriophora* in strawberry. Biocontrol. Sci. Technol. 18, 635–640. https://doi.org/10.1080/09583150802090026.

Toepfer, S., Burger, R., Ehlers, R.-U., Peters, A., Kuhlmann, U., 2010. Controlling western corn rootworm larvae with entomopathogenic nematodes: Effect of application techniques on plant-scale efficacy. J. Appl. Entomol. 134, 467–480. https://doi.org/10.1111/j.1439-0418.2009.01469.x.

Větrovský, T., Baldrian, P., 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS One 8, e57923.

Wang, S., Ventolero, M., Hu, H., Li, X., 2022. A revisit to universal single-copy genes in bacterial genomes. Sci. Rep. 12, 14550. https://doi.org/10.1038/s41598-022-18762-z.

Whelan, J.A., Russell, N.B., Whelan, M.A., 2003. A method for the absolute quantification of cDNA using real-time PCR. J. Immunol. Methods 278, 261–269. https://doi.org/10.1016/S0022-1759(03)00223-0.

Annex 3: Publication 3

SNP Marker assisted breeding in *Heterorhabditis bacteriophora* for improvement of reproductive potential and stress tolerance.

Christopher OGAYA[1,3], Michelle Ann B. DIANO[2], Innocent HATEGEKIMANA[2], Verena DÖRFLER[3], Ralf-Udo EHLERS[1,2], and Carlos MOLINA[3*]

[1]*Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts-University Kiel, Hermann-Rodewald-Str. 4, 24118 Kiel, Germany*

[2]*Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*

[3]*e–nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany*

Nematology 0 (2024) 1-16 DOI: 10.1163/15685411-bja10307

"Printed with the approval of the Faculty for Agricultural and Nutritional Sciences"

BRILL

Nematology 0 (2024) 1-16

Nematology
brill.com/nemy

Single nucleotide polymorphism (SNP) marker-assisted breeding of *Heterorhabditis bacteriophora* for improvement of reproductive potential and stress tolerance

Christopher OGAYA [1,3], Michelle Ann B. DIANO [2], Innocent HATEGEKIMANA [2], Verena DÖRFLER [3], Carlos MOLINA [3] and Ralf-Udo EHLERS [1,2,*]

[1] *Faculty of Agricultural and Nutritional Sciences, Christian-Albrechts-University Kiel, Hermann-Rodewald-Str. 4, 24118 Kiel, Germany*
[2] *Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium*
[3] *e-nema GmbH, Klausdorfer Str. 28-36, 24223 Schwentinental, Germany*
ORCID iDs: Ogaya: 0009-0002-7745-6949; Diano: 0000-0001-8636-7898; Hategekimana: 0009-0009-6245-5805; Dörfler: 0000-0001-6849-7429; Molina: 0000-0003-4919-5120; Ehlers: 0000-0002-6938-2284

Received: 16 October 2023; revised: 14 December 2023
Accepted for publication: 17 December 2023; published online: 6 February 2024

Summary – The entomopathogenic nematode (EPN), *Heterorhabditis bacteriophora*, is an efficient biological control agent against several economically important insect pests. Recent research has assessed the possibility to correlate desired beneficial traits with genotype data to pave a way for marker-assisted breeding approaches. A collection of *H. bacteriophora* WT inbred lines has been phenotyped in this framework for stress- and virulence-related traits. However, these traits are rarely combined in a single line. Thus, unifying these traits in commercial strains is of high priority. This investigation unified beneficial traits in hybrid pools through marker-assisted breeding using single nucleotide polymorphisms (SNPs) associated with reproductive potential, longevity, virulence and cold tolerance. Recombinant inbred lines (RILs) generated from a cross between a stress tolerant WT inbred line (XX21) and a line high in *in vitro* reproduction potential (IL3) were genotyped *via* SeqSNP and screened for SNP markers associated with beneficial traits. Thereafter, a genotypic pool (X21L3) comprising 22 ILs was formed. The X21L3 pool was subsequently evaluated for the target traits in comparison with the cross parents and a commercial strain HB4. An improvement of oxidative stress tolerance at 2°C (cold tolerance) was recorded with X21L3 surviving 1 day longer than the best performing parent (XX21). The hybrid pool also survived 1 day longer than the least performing parent IL3 for the trait longevity at 25°C under oxidative stress conditions. A higher dauer juvenile (DJ) recovery (58%) and DJ yield (209 000 DJ ml^{-1}) than the least performing parent XX21 was recorded for the pool. The storage stability in diatomaceous earth formulation at 2°C and 7.5°C was also improved by 2 and 5 days, respectively, in comparison to the least performing parent XX21. This study depicts the potential of precision marker-assisted breeding for beneficial trait improvement of *H. bacteriophora*.

Keywords – biological control, cold tolerance, entomopathogenic nematode, hybrid inbred lines, longevity, reproductive potential, SeqSNP, virulence.

Heterorhabditis bacteriophora is a biocontrol entomopathogenic nematode (EPN) effective against various soil-dwelling insect pests with world-wide distribution (Grewal *et al*., 2005; Dhakal *et al*., 2020). The infective dauer juveniles (DJ), the only free-living stage in the soil, search for insects and invade them through natural openings (Griffin, 2012; Stuart *et al*., 2015). Once inside the haemocoel, the DJ release symbiotically associated bacteria of the genus *Photorhabdus* into the host's haemocoel, where the insect immune system is overridden through bacteria proliferation, culminating in insect host death by septicemia within 48-72 h. Nutrient depletion after two generations inside the host's body triggers DJ formation

* Corresponding author, e-mail: ruehlers@aef.uni-kiel.de

Published with license by Koninklijke Brill NV | DOI: 10.1163/15685411-bja10307
 | ISSN: 1388-5545 (print) 1568-5411 (online)

again and exit from the cadaver in search of new hosts (Eleftherianos *et al*., 2010).

Heterorhabditis bacteriophora is cultured *in vitro* in monoxenic liquid cultures with two primary factors determining reproductive potential: *i*) DJ recovery (% of DJ leaving the arrested stage after inoculation), and *ii*) the final DJ yield (DJ ml^{-1}) at the end of the culture process (Strauch *et al*., 1998; Johnigk *et al*., 2002). It is also crucial for EPN strains used in biological control to be stress tolerant (high survival) and to maintain high infectivity levels against target insect pests during the shelf life of the product (Gaugler & Han, 2002). DJ must survive a combination of environmental stresses composed of cold temperatures, oxidative stress and light desiccation stress once they are harvested. The ability to withstand these stresses can be considered as global DJ quality.

Previous studies have demonstrated that the DJ oxidative stress tolerance trait in *H. bacteriophora* shows high heritability (0.9), indicating its potential for improvement through conventional selective breeding (Sumaya *et al*., 2017). Earlier reports have also determined a high variability in desiccation tolerance among *H. bacteriophora* strains (Mukuka *et al*., 2010a) and a recent study has demonstrated that DJ infectivity is prone to decline upon exposure of the DJ to environmental stresses. This decline may also depend on symbiotic bacteria-related factors (data unpubl.).

Several characteristics make *H. bacteriophora* attractive for classical breeding; it has a short generation time, can be cultured *in vitro* easily, and reproduction is either by automixis or amphimixis, enabling production of both homozygous inbred lines (ILs) and hybrids (Lunau *et al*., 1993; Strauch *et al*., 1994; Johnigk *et al*., 1999). However, focusing on single traits for genetic improvement can elicit negative trade-off effects on other beneficial traits, as exemplified by reduced DJ virulence, insect invasion and reproductive potential in improved desiccation tolerant hybrids (Mukuka *et al*., 2010b). To counteract this factor, pools of homozygous inbred lines with stable traits, comprising a collection of nematodes with different desirable traits can be generated (Bai *et al*., 2005; Sumaya *et al*., 2018). However, tools to track the respective traits are necessary.

Recently, thousands of single nucleotide polymorphisms (SNPs) have been discovered in *H. bacteriophora* (Levy *et al*., 2020; Fu *et al*., 2021; Godina *et al*., 2022, 2023; Wang *et al*., 2023). By correlating phenotypic and genotypic data (association analysis), SNP markers associated with virulence at low temperatures (Godina *et al*., 2022), oxidative stress tolerance (Godina *et al*., 2023) and DJ recovery (Wang *et al*., 2023) have been discovered. The application of these SNP markers as tools for initial screening of large numbers of nematode materials prior to laborious phenotyping has been shown (Godina *et al*., 2023). Recently, in a preliminary work to the present study, Ogaya *et al*. (2024) extensively phenotyped 22 *H. bacteriophora* inbred lines deriving from wild type strains (WT) and correlated these data with the available genotypic information to find four SNP markers associated with cold storage potential in *H. bacteriophora*. One of these SNP markers was located inside the gene model g9820 reported to code for the *H. bacteriophora* cell surface reporter (*daf-4*) gene. The authors focused on this single trait and demonstrated that ILs carrying the SNP alleles associated with cold tolerance were phenotypically superior to lines carrying the alternate alleles. All the studies previously mentioned focused on the desired traits individually. Thus, the next logical step would involve the combination of SNP markers associated to different traits in new hybrids and the evaluation of the performance of the new materials.

In the present work we leverage SNP marker-assisted breeding to generate a better performing *H. bacteriophora* hybrid pool, using ILs deriving from WTs reported by Godina *et al*. (2022) and Ogaya *et al*. (2024) as a starting point. We carried out the following steps: *i*) identified SNP markers associated with DJ *in vitro* recovery, DJ yield, oxidative stress tolerance and virulence in the set of ILs; *ii*) identified a candidate IL within this set possessing the highest combination of beneficial SNP markers; *iii*) hybridised this IL with a line high in *in vitro* DJ recovery and yield (IL3); *iv*) generated recombinant hybrid lines (RILs) from the progeny and genotyped them *via* SeqSNP; *v*) screened for RILs possessing the highest combination of beneficial SNP markers and generated a pool with these RILs; and, finally, *vi*) evaluated the hybrid pool for DJ recovery, DJ yield, oxidative stress tolerance, storage stability and virulence. This study accelerated improvement of beneficial traits of *H. bacteriophora* through genetic marker-assisted breeding.

Materials and methods

Nematode strains and culturing

The 48 ILs used in this study (Table 1) were previously derived from natural isolates (wild types) or hybridisation of strains as described by Godina *et al*. (2022).

Table 1. Name and origin of wild type and hybrid (HY) inbred line of *Heterorhabditis bacteriophora*.

Name	Origin	Name	Origin
AU11	Australia	IR11	Iran
CN41	China	IR21	Iran
CN42	China	IR22	Iran
DE21	Germany	IR31	Iran
DE64	Germany	IT41	Italy
DE71	Germany	IT42	Italy
DE81	Germany	IT62	Italy
HU11	Hungary	MM141	Lab Selected
HU12	Hungary	MM142	Lab Selected
HU21	Hungary	MM82	Lab Selected
HY12	Lab Hybrid	MOX16	EMS-mutant
HY22	Lab Hybrid	PT11	Portugal
HY42	Lab Hybrid	PT21	Portugal
HY51	Lab Hybrid	PT31	Portugal
HY52	Lab Hybrid	PT41	Portugal
IL1	Lab Selected	S-CR11	Lab Selected
IL10	Lab Selected	S-DT11	Lab Selected
IL2	Lab Selected	S-DT21	Lab Selected
IL3	Lab Selected	SHT11	Lab Selected
IL4	Lab Selected	TR21	Turkey
IL5	Lab Selected	TR41	Turkey
IL6	Lab Selected	XX11	South Africa
IL7	Lab Selected	XX21	South Africa
IL8	Lab Selected	XX22	South Africa

Inbred lines IL1 to IL8 and IL10 had been produced by inbreeding of a foundation strain, which was the result of several crosses of seven strains from Germany (three), USA (two), Moldavia (one) and the type strain Brecon from Australia as described by Johnigk *et al*. (2002). The phenotype of a sub-section (25 and 23 ILs including an additional commercial line HB4) of this collection was previously determined for DJ recovery in liquid culture (Wang *et al*., 2023) and oxidative stress tolerance at 2°C (Ogaya *et al*., 2024), respectively. In this study we therefore phenotyped all 48 ILs for oxidative stress tolerance at 25°C (indicative for longevity; Sumaya *et al*., 2017), and a sub-section of 25 ILs for DJ yield, DJ virulence against *Diabrotica virgifera* and virulence against *Tenebrio molitor*.

All ILs were grown on solid nematode growth Gelrite (NGG) (3.0 g l^{-1} gelrite, 2.50 g l^{-1} peptone, 51 mM NaCl, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, and 12 μM cholesterol) pre-coated with *P. laumondii* (strain DE2) bacteria suspended in semi-solid NGG (NGG with 1.5 g l^{-1} gelrite and 1.0 g l^{-1} peptone) and adjusted to a density of approx. 20×10^9 cells ml^{-1} as described by Addis *et al*. (2014). NGG plates were inoculated with 2000 DJ per plate. Seven days post inoculation, mature hermaphrodites were collected on a 300 μm sieve by washing off the bacteria, re-suspended in Ringer's solution (9.0 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \cdot 2H_2O$, 0.2 g $NaHCO_3$ dissolved in 1 l H_2O) and stored at 25°C until all DJ were released. The resulting DJ were then harvested using cotton traps over a 55 μm sieve and stored in sterile culture flasks at 4°C for not more than 2 weeks until use in the respective experiments.

Phenotyping of inbred lines

Oxidative stress tolerance at 25°C (results highly correlated with DJ longevity) was phenotyped as described by Sumaya *et al*. (2017). Briefly, DJ suspensions (1000 DJ in 400 μl Ringer solution) from each IL were placed in individual positions in triplicate of a 24-cell well plate. Oxidative stress was induced in each position by addition of 15 μl of 1.94 M H_2O_2. At this concentration DJ are not rapidly killed within hours but their longevity is substantially reduced. The 24-cell well plates were stored at 25°C in parallel for approximately 1 month until all DJ were dead. For determination of the DJ mortality during the storage duration, every 2-3 days *ca* 40 μl of DJ suspension was retrieved from each well and the numbers of living and dead DJ were counted under the microscope. The experiment was carried out three times with three independent growth batches.

Assessment of DJ yield of 25 ILs in liquid culture and of DJ *in vitro* recovery phenotype was done according to Wang *et al*. (2023). In liquid culture, DJ recovery was determined 3 days after DJ inoculation and incubation at 25°C by counting the percentage of developed hermaphrodites in 1 ml aliquots under an inverted microscope. Yield was measured by taking 1 ml aliquots from 250 ml culture flasks (50 ml liquid medium) 12 days post DJ inoculation and a 1/50 dilution was carried out. Subsequently, 50 μl aliquots were used to count the total number of DJ in triplicate. To exclude a negative influence of contaminants on DJ yield, liquid medium from the culture flasks was streaked on NBTA agar (10 g Bacto tryptic soy broth, 0.025 g bromothymol blue sodium salt, 14 g Bacto agar, 1 l distilled water, 4 ml filter-sterilised 2,3,5-triphenyl-tetrazolium chloride). DJ yield phenotyping was carried out in four independent experiments.

For virulence phenotyping, sand bioassays were set up in Petri dishes (14 cm diam.) filled with 150 g of unsterilised sand (0.1-2.0 mm particle size, 7-8% moisture content) as described by Godina *et al*. (2022). Each sand

bioassay contained 40 *T. molitor* larvae or 20 *D. virgifera* larvae and was inoculated with a dose of 800 and 300 DJ, respectively. After DJ inoculation, bioassays were incubated at 25°C in the dark for 7 days. Thereafter, the percentage of dead insect larvae per bioassay was registered. For each treatment, four technical replicates were carried out. The mortality of insect larvae due to *H. bacteriophora* was confirmed through bioluminescence detection produced by the symbiotic bacteria using a luminometer (Junior LB 9509, Berthhold Technologies). The virulence phenotyping experiments were carried out in four independent experiments.

Association analysis

All WT ILs used in this study had previously been genotyped by Godina *et al*. (2022) through genotyping by sequencing (GBS), yielding 4894 and 712 SNPs presenting minor allele frequencies (MAF) $\geqslant$ 0.01 and 0.1, respectively. Genotypic data of the individual lines are available upon request. Positions of SNPs among the set of ILs were determined and transferred to GTF (gene transfer format) files, masking the *H. bacteriophora* TT01 genome assembly version PRJNA13977.WBPS16 deposited at wormbase (www.wormbase.org) (Godina *et al*., 2022). A GTF file is available upon request. To reveal whether any of the 4897 (MAF $\geqslant$ 0.01) discovered SNPs were associated with oxidative stress tolerance at 25°C, DJ recovery, DJ yield or DJ virulence, association analysis was carried out by correlating the respective genotypic data with phenotypic data. Therefore, the Tassel 5.0 software (available online at www.maizegenetics.net) was used with the following parameters: $P \leqslant 0.05$ and 100 permutations, applying the general linear model routine. A SNP molecular marker was only considered significantly associated with a respective trait when differences between groups of ILs separated according to the 'strong' and 'weak' alleles observation along the individual experiments (for each trait). As output of this section, a set of SNPs associated to different beneficial traits was conformed (hereafter called beneficial SNPs).

Selection of inbred line for hybridisation and homozygous RIL generation

To identify candidate ILs for a subsequent genetic cross, we screened all ILs for the desired genotype concerning the previously identified beneficial SNPs. We applied the following conditions for the screening: *i*) the largest number possible of the strong desired alleles for beneficial SNPs must be present in the given IL, or *ii*) the desired allele must be present in the other hybridisation partner line IL3. Those ILs with the largest number of common beneficial SNPs were evidenced using the Venn diagram tool available at Bioinformatics & Evolutionary Genomics UGent (https://bioinformatics.psb.ugent.be/webtools/Venn/) by entering the list of individual phenotypes.

Subsequent genetic crosses with candidate IL and commercial line IL3 were carried out using clearly differentiable males and virgin females at the fourth juvenile stage (J4) according to Iraki *et al*. (2000) with minor modifications. Briefly, after DJ infection of wax moth larvae (*Galleria mellonella*) at a dose of 50 DJ per larva with respective nematode parents and culture at 25°C, virgin J4 females and adult males were recovered from dissected insect cadavers after 5 days for mating experiments. All crosses were performed in 12-cell well plates containing 500 μl of NGG pre-coated with 25 μl *P. laumondii* strain DE2 diluted in semi-solid NGG. In each cell well, *ca* ten males and five females of each parent were placed and the 12-cell well plates incubated at 25°C. A reciprocal cross was done for each cross and the respective crosses replicated twice. Virginity tests were included with five virgin females from respective parents placed in separate wells without males. Resulting progenies were validated as true only when cell wells with virgin females had no progenies. DJ obtained from the mated females were then collected and used for further *in vitro* culturing.

Generation of homozygous RILs from the genetic crosses were done by selfing for 6-7 generations according to Johnigk *et al*. (2002). Gravid hermaphrodites were washed with Ringer's solution and single individuals were transferred into cell wells (Greiner) filled with 50 μl Ringer's solution. Each individual formed the basis for an inbred line. Due to the lack of food, the hermaphrodites do not lay eggs and juveniles develop through *endotokia matricida* inside the uterus (Johnigk & Ehlers, 1999). After 3-4 days DJ had developed, which were then transferred to solid nematode growth Gelrite medium containing *P. laumondii*. Three days later three single hermaphrodites of each line were transferred into Ringer's solution. The offspring of one of the three individuals were chosen for the next propagation step. This procedure was repeated for seven generations, resulting in almost 100% homozygous populations.

RIL GENOTYPING

RILs from genetic crosses were genotyped by SeqSNP as described in detail by Godina *et al*. (2022, 2023). For this purpose, a selection of 712 *H. bacteriophora* GBS-generated SNPs (MAF $\geqslant 0.1$) was previously transferred to the SeqSNP sequencing platform (www.biosearchtech.com). For the present genotyping, the RIL DNA was extracted from DJ in duplicate using the Peqgold® Tissue DNA extraction kit (VWR International). Purity of DNA was checked in 1% agarose (GelRed staining) and the quantity was determined using a NanoDrop (Thermo Fisher Scientific). DNA aliquots were sent for SeqSNP sequencing. After sequencing, genotype output files were quality-filtered at the sequencing source (min. eight reads, MAF > 0.1, consistent technical replicates) and HAP map files (haplotype blocks) were generated for further analyses.

SNP ANALYSIS OF RILS AND GENERATION OF GENETIC POOLS

The obtained homozygous RILs from the cross between the candidate inbred line (with highest combination of beneficial traits) with IL3 were genotyped by SeqSNP (as described above). The genotyped RILs were then screened for the presence of desired alleles of SNPs associated with beneficial traits donated by the parents. To identify groupings of RILs with the highest combination of beneficial SNP markers, the lists of candidate RILs and their genotypes were combined using the Venn diagram tool as previously described. As outcome, a subset of RILs sharing the largest possible number of beneficial SNPs was derived. Subsequently, this subset of RILs was joined in a hybrid pool. The hybrid pool was initiated by inoculating equal amounts of the involved lines in semi solid NGG plates. After growth, the resulting DJ were harvested and used for further genotyping.

MONOXENIC LIQUID CULTURES OF THE HYBRID POOL AND CROSS PARENTS

For phenotyping the DJ recovery and DJ yield of the RILs and respective parents in liquid culture, bacteria-free nematodes were generated by egg surface sterilisation following Lunau *et al*. (1993). Four days after DJ inoculation in NGG plates, gravid hermaphrodites were washed off the medium using a 300 μm sieve. Hermaphrodites were placed in test tubes with 3-4 small (0.5-1.0 cm) pieces of razor blades and vortexed to free the eggs. Eggs were separated from other debris using a 55 μm sieve and were transferred to 2 ml Eppendorf tubes. Isolated eggs were surface-sterilised in sterilisation solution (0.5 ml NaOCl, 1.5 ml of 4 M NaOH in 10 ml distilled water) and centrifuged. Post sterilisation, eggs were washed in BSB medium (Bacto tryptic soy broth 10 g, yeast extract 2.5 g, casein peptone 2.5 g, NaCl 2.5 g, KCl 0.18 g, $CaCl_2 \cdot 2H_2O$ 0.11 g, glucose 2 g, distilled water 0.5 l). Sterile eggs were then transferred into 24-cell well plates filled with BSB medium and incubated for 24 h at 25°C to allow hatching. Uncontaminated hatched J1 nematodes were transferred to Wouts agar (16 g Bacto nutrient broth; 12.0 g Bacto agar; 5.0 g corn oil or sunflower oil, 1 l H_2O) containing a few drops of *P. laumondii* (strain DE2) and stored for 12 days at 25°C.

Contamination-free Wouts plates were transferred to flasks containing pre-cultured *P. laumondii* (DE2) bacteria as described by Hirao & Ehlers (2009). Flasks were stored on a rotary shaker with 180 rpm at 25°C for at least 12 days. Thereafter, cultures were transferred to 4°C to be used as stock inoculum for subsequent experiments. For subsequent growth cycles, *P. laumondii* (DE2) frozen stocks (−80°C) were inoculated in a 250 ml flask with 50 ml liquid medium (LM) and incubated at 25°C for 24 h on a rotary shaker. An aliquot of 2 ml was then subcultured in a 1000 ml flask with 200 ml LM and incubated at 25°C for 24 h on a rotary shaker. After 24 h, sterile DJ were inoculated at a final density of 5000 DJ ml^{-1}.

PERFORMANCE EVALUATION OF THE GENOTYPIC HYBRID POOL

The generated hybrid pool was phenotyped in parallel to the parents of the cross and a commercial strain HB4 for oxidative stress at both 2 and 25°C, reproductive potential in liquid culture and virulence against *T. molitor* as described for the inbred lines (sections above). Further, the storage stability of the respective nematode materials in diatomaceous earth formulation was investigated. For this purpose, DJ obtained from liquid cultures were washed with tap water over a 32 μm sieve. Formulation of the obtained DJ paste was as described by Ogaya *et al*. (2024). Briefly, by diluting 1 g of DJ paste in 1 l tap water and counting 500 μl at 1:5 dilution in triplicate, the DJ density was determined. Diatomaceous earth formulation was then mixed with DJ paste at a ratio of 1:1 (w/w) and bags of 10 g were produced. The bags were then stored at 2 and 7.5°C for up to 9 weeks in refrigerators. For 2°C, the survival of the formulated DJ was evaluated on day 1, day 3, week 2, week 3 and week 4 and for 7.5°C on day

1, week 2, week 4, week 6 and week 9 (each time with an unopened bag). For determination of DJ survival (DJ (g formulation)$^{-1}$), one bag of 10 g of formulated DJ was dissolved in 1 l tap water and 20 ml of the solution was kept in culture bottles at 15°C for 2 h until counting (3 × 500 μl counts from 1:50 dilution).

Data analysis

The Shapiro-Wilkinson test ($P > 0.05$) and homogeneity of variance Levene's test ($P > 0.05$) were used to test data normality prior to analysis. Significant differences were then determined by ANOVA analysis and means with significant differences were separated using a *post-hoc* Tukey's honest significant difference (HSD) test at $P \leqslant 0.05$ for normally distributed data. Non-normally distributed data were analysed by Kruskal-Wallis test and means compared with Dunn's test $P \leqslant 0.05$. For DJ survival data in formulation (living DJ (g formulation)$^{-1}$), prior to ANOVA analysis, data from DJ stored at 2°C were fitted to a logistic model, while those stored at 7.5°C were fitted to a linear model. Using the initial number of living DJ as the initial point, the mean time survived by 75% (MTS_{75}) of the DJ population for each line was then predicted. For survival under oxidative stress, the percentage DJ mortality at both temperatures was fitted to a logistic model and the mean time survived by 50% (MTS_{50}) was predicted for each line and temperature. All data analysis was done in RStudio (https://www.rstudio.com).

Results

Phenotypic traits in inbred lines

The DJ yield in monoxenic liquid culture of 25 ILs assessed during this investigation was significantly different ($F = 25.3$; df = 24; $P \leqslant 0.001$) and ranged between $(81.2 \pm 7.5) \times 10^3$ DJ ml^{-1} (IR22) and $(191.2 \pm 10.6) \times 10^3$ DJ ml^{-1} (TR21). The phenotypic data for 23 of the same ILs for oxidative stress tolerance at 2°C was published by Ogaya *et al*. (2024) and in this study it was assessed at 25°C. Significant differences were recorded among a collection of 48 ILs ($Chi^2 = 173.33$; df = 47; $P \leqslant 0.01$) and the MTS_{50} ranged from 4.4 ± 0.6 days (TR41) to 12.3 ± 1.9 days (IT42). DJ virulence against *D. v. virgifera* among the same sub-set of 25 ILs ranged from 74.4 ± 9% (PT21) to 46 ± 7.1% (XX22) and was significantly different ($F = 1.62$; df = 24; $P \leqslant 0.05$). Virulence against *T. molitor* was generally higher and ranged from 76 ± 6.4% (IT41) to 50.3 ± 4.5% (IL3) with significant differences ($F = 3$; df = 24; $P \leqslant 0.001$). Respective phenotypic data of different traits are summarised in Table 2.

Association analysis with existing genotype data

By correlating phenotypic data with the SNP genotypes, four SNP markers were significantly associated with reproductive potential. Three of these were associated with DJ yield, SNP RP1 on scaffold1357 (TT01 genome draft) and SNPs RP2 and RP3 (scaffold1351). SNP RP4, localised on scaffold1080, was the only SNP marker associated with DJ recovery after correlating the phenotypic DJ recovery data from Wang *et al*. (2023) with genotypic data. Two SNP markers were associated with oxidative stress tolerance at 25°C (DJ longevity), SNP L/V1 and L/V2 localised on scaffold1190 and scaffold1265, respectively. The same SNP markers were also associated with virulence against *T. molitor*. Four SNP markers were exclusively associated with virulence against *T. molitor*: SNP V1, V2, V3 and V4 localised on scaffolds1356, 1336, 1272 and 1236, respectively. Only one SNP marker, SNP V5 on scaffold1294, was associated with *D. virgifera* virulence. The SNP markers associated with oxidative stress tolerance at 2°C are reported in Ogaya *et al*. (2024). Further, five SNP markers were localised inside uncharacterised genes. SNP RP1 in gene model g1270, RP3 in gene model g2992, L/V2 in gene model g6743, V1 in gene model g1268, and V4 in gene model g1921. SNP V2 was localised 476 bp from the uncharacterised gene model g2001, while the remainder of the SNP markers were not localised in the vicinity of any genes (SNP RP2, RP4, L/V1, V3 and V5). Details about the SNP markers are provided in Table 3.

Groupings of phenotypic data according to observed SNP alleles for respective traits are depicted in Figure 1. For the SNP at scaffold1190 associated with both longevity at 25°C and virulence (SNP L/V1), the allele T associated with higher longevity was carried by 11 ILs and their longevity was significantly higher than that of the 27 ILs carrying the alternate allele C ($t = 4.36$ df = 36 $P \leqslant 0.001$) (Fig. 1A). For SNP marker on scaffold1343 associated with cold tolerance (SNP CT2) (Ogaya *et al*., 2024), seven ILs possessed allele C associated with a higher cold tolerance and the MTS_{50} was significantly different in the 16 ILs carrying the alternate allele G ($t = 3.482$; df = 21; $P = 0.002$) (Fig. 1B). Scaffold1294 possessed the SNP marker associated with

Table 2. Mean of independent experiments and their SD of phenotypic data for dauer juvenile (DJ) survival under oxidative stress (MTS_{50}) at 25°C, infectivity (%) against western corn rootworm (*Diabrotica virgifera*) and mealworm (*Tenebrio mollitor*) and DJ yield (DJ ml^{-1}) of *Heterorhabditis bacteriophora* WT and hybrid inbred lines.

Line name	MTS_{50} in H_2O_2		*Diabrotica virgifera* infect.		*Tenebrio mollitor* infect.		DJ yield ($\times 10^3$ DJ ml^{-1})	
	Mean ± SD	Significance	Mean ± SD	Significance	Mean ± SD	Significance	Mean ± SD	Significance
AU11	6.6 ± 0.4	bcdefghijk	62.4 ± 8.0	bc	75.3 ± 4.4	a	115.1 ± 13.4	ij
CN41*	7.5 ± 1.3	fghijklmno	–	–	–	–	–	–
CN42*	6.1 ± 1.1	abcdefgh	–	–	–	–	–	–
DE21	4.4 ± 1.1	abc	50.3 ± 10.3	c	66.7 ± 4.1	abc	149.7 ± 14.0	cdefg
DE64	10.5 ± 0.7	pqrs	51.9 ± 8.6	c	71.2 ± 5.7	ab	145.7 ± 20.2	defgh
DE71	7.9 ± 1.2	fghijklmnop	–	–	–	–	–	–
DE81	9.7 ± 2.6	opqr	58.5 ± 15.8	bc	67.4 ± 8.5	abc	141.3 ± 15.0	efghi
HB4	–	–	–	–	–	–	172.4 ± 16.6	abcd
HU11	8.3 ± 0.8	hijklmnop	61.0 ± 19.0	bc	69.6 ± 3.9	abc	84.4 ± 3.8	k
HU12	8.7 ± 0.8	jklmnopq	62.9 ± 6.6	bc	66.6 ± 12.4	abc	152.9 ± 24.8	cdef
HU21	4.6 ± 1.2	abcd	60.7 ± 12.7	bc	69.5 ± 8.0	abc	–	–
HY12	6.6 ± 0.4	abcdefghijk	–	–	–	–	–	–
HY22	5.6 ± 0.9	abcdefg	–	–	–	–	–	–
HY42	6.2 ± 0.6	abcdefghij	–	–	–	–	–	–
HY51	5.9 ± 0.7	abcdefgh	–	–	–	–	–	–
HY52	4.3 ± 0.5	ab	–	–	–	–	–	–
IL1	6.2 ± 1.7	abcdefghij	–	–	–	–	–	–
IL10	7.4 ± 1.6	efghijklmno	–	–	–	–	–	–
IL2	7.1 ± 0.8	defghijklmn	–	–	–	–	–	–
IL3	4.1 ± 1.0	a	52.2 ± 9.2	c	50.3 ± 4.5	c	185.2 ± 20.0	ab
IL4	6.1 ± 1.1	abcdefghi	–	–	–	–	–	–
IL5	4.5 ± 0.8	abc	–	–	–	–	–	–
IL6	5.4 ± 1.2	abcdef	–	–	–	–	–	–
IL7	7.4 ± 2.1	efghijklmno	–	–	–	–	–	–
IL8	4.6 ± 0.6	abcd	–	–	–	–	–	–
IR11	11.5 ± 1.6	rs	62.8 ± 15.2	bc	70.7 ± 7.5	ab	132.2 ± 16.9	fghij
IR21	7.7 ± 0.6	fghijklmno	67.7 ± 6.4	bc	68.2 ± 7.2	abc	170.5 ± 31.7	abcd
IR22	8.9 ± 1.1	klmnopq	71.9 ± 7.9	ab	63.5 ± 5.4	abc	81.2 ± 7.5	k
IR31	9.2 ± 1.7	klmnopqr	64.7 ± 10.3	bc	75.1 ± 8.6	a	175.4 ± 16.0	abc
IT41	9.2 ± 3.0	lmnopqr	56.2 ± 13.4	bc	76.0 ± 6.4	a	155.5 ± 12.6	cdef
IT42	12.3 ± 1.9	s	60.4 ± 14.7	bc	72.2 ± 4.5	ab	123.4 ± 21.7	ghij
IT62	9.5 ± 2.6	mnopqr	–	–	–	–	–	–
MM141	6.9 ± 1.0	cdefghijklm	61.4 ± 9.4	bc	62.9 ± 13.3	abc	107.0 ± 17.0	j
MM142	9.5 ± 1.5	nopqr	58.4 ± 9.9	bc	60.8 ± 6.9	bc	119.0 ± 11.8	hij
MM8	7.1 ± 0.8	cdefghijklmn	55.6 ± 12.4	bc	75.1 ± 3.5	a	114.7 ± 8.4	ij
MOX1	8.1 ± 1.0	ghijklmnop	–	–	–	–	–	–
PT11	4.8 ± 0.4	abcde	69.1 ± 15.1	bc	70.2 ± 4.6	ab	142.7 ± 14.1	efgh
PT21	6.2 ± 1.0	abcdefghij	74.4 ± 9.0	a	75.3 ± 5.0	a	138.4 ± 15.8	efghi
PT31	8.9 ± 1.6	klmnopq	61.4 ± 13.1	bc	63.9 ± 9.8	abc	170.2 ± 15.7	abcd
PT41	8.6 ± 1.2	ijklmnopq	61.1 ± 10.6	bc	71.6 ± 9.9	ab	137.4 ± 13.5	efghi
S-CR11	5.3 ± 0.9	abcdef	–	–	–	–	–	–
S-DT11	4.1 ± 1.2	a	–	–	–	–	–	–
S-DT21	4.9 ± 0.5	abcde	–	–	–	–	–	–
SHT11	5.8 ± 0.5	abcdefgh	–	–	–	–	–	–
TR21	6.7 ± 1.0	bcdefghijkl	53.5 ± 10.3	bc	66.4 ± 9.9	abc	191.2 ± 10.6	a
TR41	4.4 ± 0.6	ab	–	–	–	–	–	–

Table 2. (Continued.)

Line name	MTS_{50} in H_2O_2		*Diabrotica virgifera* infect.		*Tenebrio mollitor* infect.		DJ yield ($\times 10^3$ DJ ml^{-1})	
	Mean ± SD	Significance	Mean ± SD	Significance	Mean ± SD	Significance	Mean ± SD	Significance
XX11	8.0 ± 1.5	ghijklmnop	52.1 ± 21.2	c	69.5 ± 8.0	abc	132.4 ± 16.7	fghij
XX21	11.0 ± 2.9	qrs	57.0 ± 10.6	bc	71.6 ± 4.2	ab	180.9 ± 28.0	abc
XX22	9.8 ± 1.2	opqrs	46.7 ± 7.1	d	73.9 ± 4.2	a	162.0 ± 10.3	bcde

Significant differences ($P < 0.05$) between lines for each trait are denoted by different letters. For virulence and reproductive potential experiments, a subset of WT inbred lines with reduced origin redundancy was tested. Traits that were not phenotyped in specific lines are depicted by (–).
*CN4 WT ILs were excluded from association and further phenotyping analyses since the mother strain has been identified as *Heterorhabditis becheriana*.

Table 3. SNP markers in *Heterorhabditis bacteriophora* WT inbred lines associated with cold tolerance at 2°C (CT), longevity at 25°C and virulence (L/V), reproductive potential in liquid culture (RP) and virulence against *Tenebrio mollitor* and *Diabrotica virgifera* (V).

Scaffold	SNP ID	Associated trait ($P < 0.05$)	Allele with highest average of related trait	Closest gene (distance up to 1000 bp)	Gene homologue after BLAST analysis
1330	CT1	Cold tolerance	G	g4260 (+748)	Uncharacterised
1343	CT2	Cold tolerance	C	g2709 (+168)	Y032_0037g3461 protein
1251	CT3	Cold tolerance	A	g9820 (+0)	*H. bacteriophora daf-4*
1296	CT4	Cold tolerance	G	g6562 (+287)	*C. elegans* F53A2.1 protein
1357	RP1	DJ Yield	T	g1270 (+0)	Uncharacterised
1351	RP2	DJ Yield	A	–	–
1351	RP3	DJ Yield	T	g2992 (+0)	Uncharacterised
1080	RP4	DJ Recovery	T	–	–
1190	L/V1	Longevity/Virulence	T	–	–
1265	L/V2	Longevity/Virulence	T	g6743 (+0)	Uncharacterised
1356	V1	Virulence *T. molitor*	T	g1268 (+0)	Uncharacterised
1336	V2	Virulence *T. molitor*	T	g2001 (+476)	Uncharacterised
1272	V3	Virulence *T. molitor*	G	–	–
1236	V4	Virulence *T. molitor*	G	g1921 (+0)	Uncharacterised
1294	V5	Virulence *D. vigifera*	C	–	–

For respective SNP markers, the scaffold on which the SNP is localised, and the allele associated with better performance of the respective traits are presented. SNP localisation in reference to genes and homologues to respective genes post BLAST analysis is indicated.

virulence against *D. v. virgifera* (SNP V5) and the six ILs carrying allele C had a significantly higher insect infectivity (%) than the 17 ILs carrying the alternate allele T ($t = 4.19$; df $= 21$; $P \leqslant 0.05$) (Fig. 1C). Seven ILs had a higher DJ yield and possessed allele T associated with a higher DJ yield for SNP marker on scaffold1357 (SNP RP1) associated with this trait and the differences were significantly different ($t = 2.9$; df $= 22$; $P \leqslant 0.05$) (Fig. 1D).

XX21 WAS CONSIDERED A SUITABLE DONOR LINE FOR BENEFICIAL TRAITS

After a global genotype screening of all analysed ILs for presence of SNP markers associated with the above-mentioned beneficial traits, XX21 was found to possess a combination of two SNP markers associated with high cold tolerance (CT1, CT2), two SNP markers associated with high reproductive potential (RP1, RP2), one SNP marker associated with both longevity and virulence

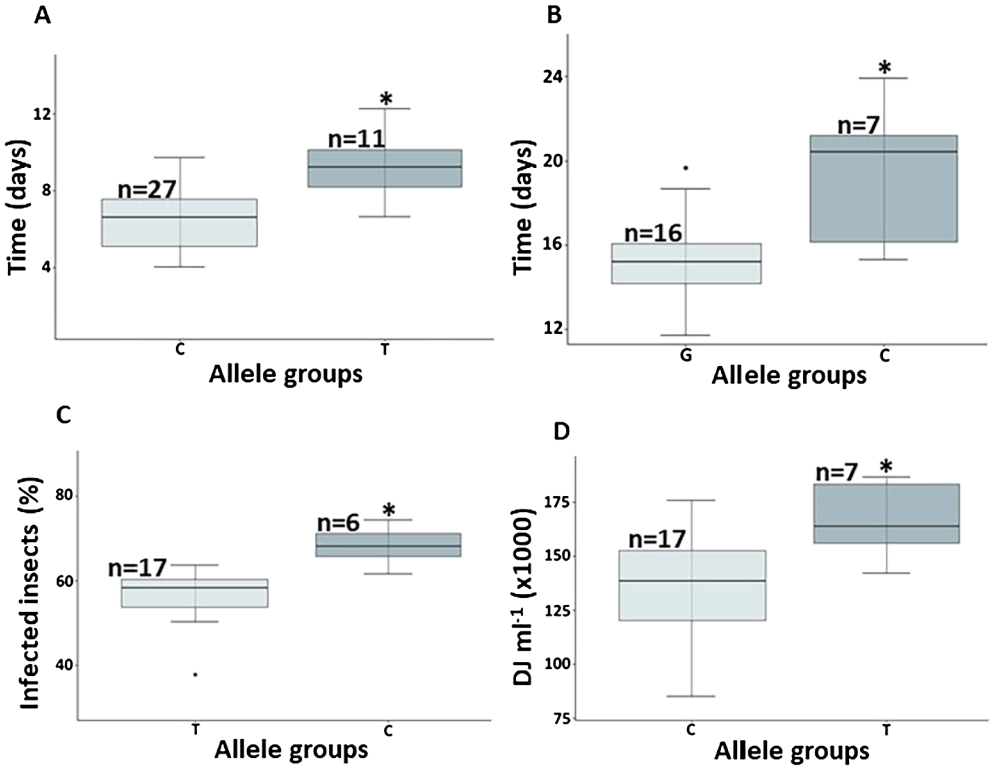

Fig. 1. Allelic discrimination groups in *Heterorhabditis bacteriophora* WT inbred lines according to the genotype of respective SNP markers: Mean time to 50% survival (MTS_{50}) at 25°C according to allelic groups of SNP molecular marker SNP L/V1 (A), MTS_{50} at 2°C according to allelic groups of SNP molecular marker SNP CT1 (B), average infected insects (%) according to allelic groups of SNP molecular marker SNP V5 (C), and average DJ yield (DJ ml^{-1}) according to allelic groups of SNP molecular marker SNP RP1 (D). The number of WT inbred lines carrying the respective allele per SNP marker are denoted by n. Significant differences between the allele groups per respective trait are denoted by a star (*) above the box plot ($P < 0.05$).

(L/V1) plus two SNP markers associated exclusively with virulence against *T. molitor* (V1, V2). Phenotypically XX21 also had a high longevity at 25°C (11.0 ± 2.9 days), high cold tolerance at 2°C (16.7 ± 0.7 days) (Ogaya *et al*., 2024), high reproductive potential (180 ± 28 × 10^3 DJ ml^{-1}), high virulence against *T. molitor* (71.6 ± 4.2%) and moderate virulence against *D. v. virgifera* (57.0 ± 10.6%). We therefore selected XX21 as a candidate for hybridisation with the line IL3. The progeny from the genetic cross between these two lines were expected to possess two additional SNP markers associated with virulence against *T. molitor* donated by line IL3 (V3, V4).

Outcome of cross, RIL production and genotyping by seqSNP

Inbred line XX21 was successfully crossed with line IL3, and all virginity tests were positive (no progeny from wells having only virgin females). After transferring individual hermaphrodites originating from hybrids from this cross in NGG plates for seven selfing cycles, 55 homozygous RILs of the XX21 × IL3 cross herein called X21L3 were obtained. The RILs from this cross were subsequently genotyped with the seqSNP-platform reported by Godina *et al*. (2023). All the 55 RILs yielded robust genotypes and among them 33 SNPs (17.1%) showed polymorphisms between both parents and segregation among the progeny RILs (MAF ⩾ 0.1). In relation to the TT01 genome sequence (PRJNA13977.WBPS16), these polymorphic SNPs were distributed in 15 scaffolds. From them, the scaffolds 1336 and 1346 each contained five SNPs, followed by scaffold 1356 (four SNPs) and 1355 (three SNPs).

Table 4. SNP marker profile of the X21L3 pool and SNP alleles donated by the respective parents.

Pool	SNP ID	Associated trait	Strong allele donated by IL3	Strong allele donated by XX21
X21L3	CT1	Cold tolerance	G	G
	CT2	Cold tolerance	C	C
	RP1	R. potential	T	T
	RP2	R. potential	A	A
	L/V1	Virulence, longevity	–	T
	V1	Virulence	–	T
	V2	Virulence	–	T
	V3	Virulence	G	–
	V4	Virulence	G	–

In total nine SNP markers associated with cold tolerance, reproductive potential, longevity and virulence were aggregated in the hybrid inbred line pool of *Heterorhabditis bacteriophora*.

Pool generation with X21L3 RILs

Filtering X21L3 RILs for presence of RILs with beneficial SNP markers yielded 22 RILs carrying nine beneficial SNP markers hence a single genetic pool was generated with these RILs. The details pertaining to these nine SNP markers and the beneficial traits associated with them and present in the X21L3 pool are depicted in Table 4. The SNP profiles of each individual RIL included in the X21L3 pool are shown in Table 5. Thereafter, the X21L3 pool was transferred into monoxenic liquid culture for subsequent confirmatory phenotyping.

Phenotyping X21L3 pool for reproductive potential

DJ recovery (%) and final yield (DJ ml^{-1}) of the X21L3 pool were compared to the parental lines and the commercial line HB4. The highest DJ recovery was recorded in the commercial line HB4 ($74 \pm 2.1\%$), while XX21 had the lowest recovery ($51 \pm 1.99\%$). For final DJ yield, the highest DJ yield was observed for IL3 ($(236 \pm 4.77) \times 10^3$ DJ ml^{-1}), while XX21 had the lowest DJ yield ($193 \pm 2.88 \times 10^3$ DJ ml^{-1}). Recovery ($58 \pm 1.7\%$) and DJ yield ($(203 \pm 71.4) \times 10^3$ DJ ml^{-1}) for the X21L3 pool were higher than for the parental line XX21. It is noteworthy that even though the X21L3 pool had a lower recovery than the commercial line HB4, no significant difference was observed between the two in final DJ yield. Significant differences ($F = 55.4$, df = 3, $P < 0.001$) were observed among the lines for DJ recovery (Fig. 2A) and final DJ yield ($F = 4.39$, df = 3, $P = 0.013$) (Fig. 2B).

Phenotyping X21L3 pool for oxidative stress tolerance

To mitigate the time required for DJ longevity and cold tolerance evaluation, DJ were subjected to oxidative stress conditions at 2 and 25°C. DJ mortality was evaluated every 2-3 days for 30 days and the MTS_{50} calculated. An example of a fitted logistic regression model to predict the MTS_{50} and MTS_{75} of the X21L3 pool under oxidative stress at 2°C storage is shown in Figure 3. The MTS_{50} and MTS_{75} of other lines were also predicted the same way. Under cold storage temperature (2°C), the X21L3 pool had the longest survival with an MTS_{50} of 8.1 ± 0.48 days, whereas the parental line, IL3, had the shortest survival (5.7 ± 0.51 days) with the differences in survival significantly different among the tested lines ($F = 72.0$, df = 3, $P < 0.001$) (Fig. 3B). For oxidative stress at 25°C storage, the parental line XX21, had the longest survival with an MTS_{50} of 6.9 ± 0.52 days while the parental line IL3 had the lowest (4.5 ± 0.42 days). Significant differences were also observed among the lines at 25°C ($F = 101.5$, df = 3, $P < 0.001$) (Fig. 3C).

Phenotyping X21L3 pool for storage potential

DJ from each line were harvested, formulated and stored for 4 weeks and 9 weeks at 2 and 7.5°C, respectively. At 2°C storage, the commercial line HB4 had the highest MTS_{75} (38 ± 3.6 days), while XX21 had the lowest (14 ± 1.8 days). The MTS_{75} (16 ± 1.8 days) of the X21L3 pool was higher than for the parental line XX21 with significant differences among observed lines ($F = 22.2$, df = 3, $P < 0.001$) (Fig. 4A). At 7.5°C storage, the commercial line HB4 survived the longest with an MTS_{75} of 52 ± 2.66 days whereas the parental line

Table 5. SNP allele profiles of RILs found in X21L3 hybrid pool of *Heterorhabditis bacteriophora*.

	SNP ID								
	CT1	CT2	RP1	RP2	L/V1	V1	V2	V3	V4
Associated trait	Cold-tolerance	Cold-tolerance	R. potential	R. potential	Virulence, longevity	Virulence	Virulence	Virulence	Virulence
Desired allele	C	C	T	A	T	T	T	G	G
X21L3-18	C	C	T	A	T	T	T	G	T
X21L3-19	C	C	T	A	T	T	T	G	T
X21L3-2	C	C	T	A	T	T	T	G	T
X21L3-20	C	C	T	A	T	T	T	A	T
X21L3-22	C	C	T	A	T	T	T	G	T
X21L3-23	C	C	T	A	T	T	T	G	T
X21L3-24	C	C	T	A	T	T	T	G	T
X21L3-26	C	C	T	A	T	T	T	G	T
X21L3-28	C	C	T	A	T	T	T	G	T
X21L3-30	C	C	T	A	T	T	T	G	T
X21L3-31	C	C	T	A	T	T	T	G	T
X21L3-32	C	C	T	A	T	T	T	G	T
X21L3-33	C	C	T	A	T	T	T	G	T
X21L3-37	C	C	T	A	T	T	T	G	T
X21L3-39	C	C	T	A	T	T	T	G	T
X21L3-4	C	C	T	A	T	T	T	G	T
X21L3-41	C	C	T	A	T	T	T	-	T
X21L3-42	C	C	T	A	T	T	T	A	T
X21L3-49	C	C	T	A	T	T	T	G	T
X21L3-55	C	C	T	A	T	T	T	G	T
X21L3-60	C	C	T	A	T	T	T	G	T
X21L3-8	C	C	T	A	T	T	T	G	T

All X21L3 RILs have the same profiles except for X21L3-20 and X21L3-42, which have allele A, and X21L3-41 which is heterozygous for the SNP-marker V3.

XX21 survived the shortest time (26 ± 1.54 days). Similarly, the X21L3 pool also had a higher MTS_{75} (31 ± 1.6 days) than the parental line XX21. Significant differences among the materials were also observed ($F = 88.58$, df = 3, $P < 0.001$) (Fig. 4B).

Phenotyping X21L3 pool for virulence during storage time

DJ previously stored at 2 and 7.5°C in formulation were evaluated for virulence assessed as mortality (%) of *T. molitor* larvae after different storage periods. For DJ virulence after storage at 2°C (Fig. 5A) a similar trend of decreasing virulence along storage time was observed for all lines. The initial virulence of the parental line XX21 was the highest (96.9 ± 1.81%), whereas the X21L3 pool had the lowest initial virulence (78.8 ± 2.28%). Similarly, at week 2, XX21 maintained the highest virulence (92.5 ± 1.76%) while X21L3 had the lowest (59.2 ± 2.38%). At week 4, XX21 still had the highest virulence (74 ± 1.93%) but this time the parental line IL3 had the lowest virulence (48 ± 2.7%). The differences in virulence at all the observation time points were significantly different among the lines: initial ($F = 47.16$, df = 3, $P < 0.001$), week 2 ($F = 83.27$, df = 3, $P < 0.001$), and week 4 ($F = 24.01$, df = 3, $P < 0.001$).

The virulence (%) at 7.5°C (Fig. 5B) was evaluated for up to 6 weeks. After 2 weeks, XX21 again had the highest virulence (95 ± 1.95%), while the X21L3 pool had the lowest (76.46 ± 2.18%). At week 4, the commercial line HB4 had the highest virulence (96.46 ± 1.36%) while the X21L3 pool again had the lowest virulence (78.75 ± 2.53%). The commercial line HB4 maintained a high virulence after 6 weeks (97.08 ± 1.52%), whereas the X21L3 pool still had the lowest virulence (76.04 ± 1.82%). The virulence of all the lines was still above 50% after 6 weeks, which is the accepted commercial

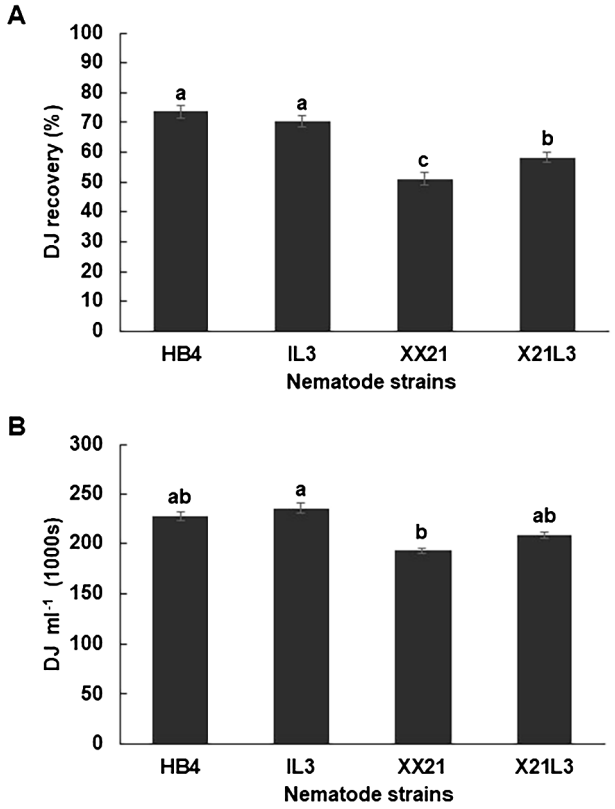

Fig. 2. Reproductive potential of *Heterorhabditis bacteriophora* in liquid culture: Average dauer juvenile (DJ) recovery (%) (A) and average final DJ yield (DJ ml^{-1}) (B) of X21L3 pool in comparison to the parents, XX21 and IL3, and commercial line HB4. DJ recovery and DJ yield were evaluated on day 3 and day 12 after DJ inoculation, respectively. Three independent experiments with each three replicates were carried out. Error bars: Average SD of three experiments. Letters above error bars indicate significant differences (Tukey HSD tests at $P \leqslant 0.05$).

standard after 6 weeks storage. Significant differences were observed in the virulence at all time points among the lines: week 2 ($F = 54.99$, df = 3, $P < 0.001$), week 4 ($F = 45.29$, df = 3, $P < 0.001$), and week 6 ($F = 86.29$, df = 3, $P < 0.001$).

Discussion

Genetic improvement of EPN is predominantly through classical breeding (Glazer, 2015), first involving phenotypic characterisation of a diverse collection of nematode material followed by hybridisation and/or artificial selection for the desirable trait (Strauch *et al*., 2004; Mukuka *et al*., 2010b). Following part of this blueprint, WT inbred

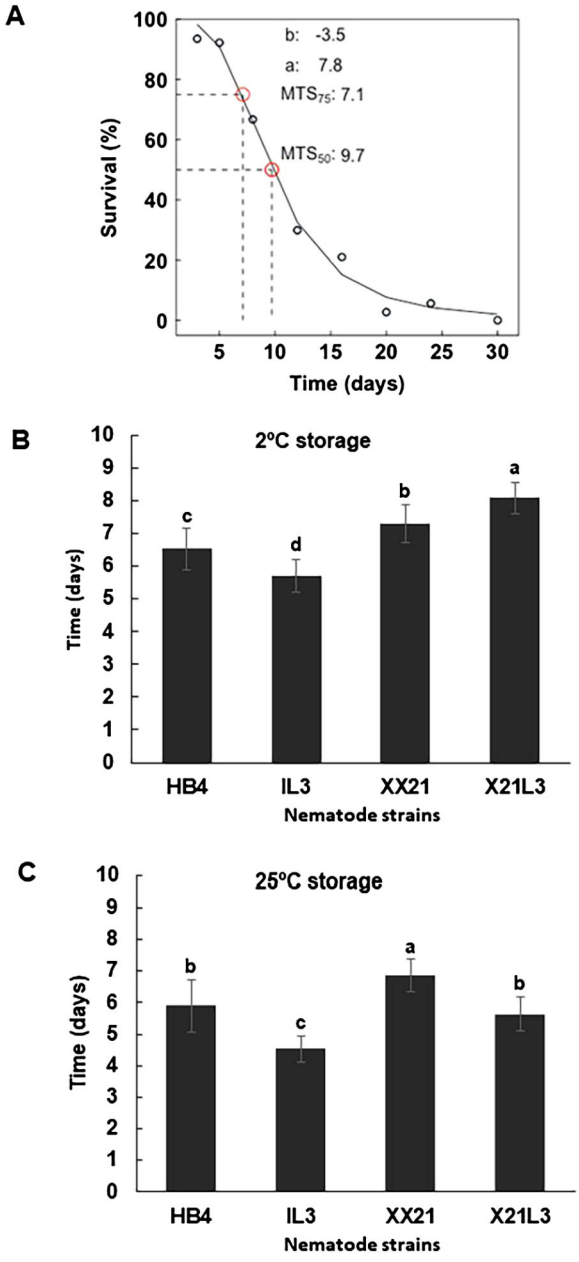

Fig. 3. A logistic model predicting the mean time survived by 50% (MTS_{50}) and 75% (MTS_{75}) of dauer juvenile (DJ) populations of *Heterorhabditis bacteriophora*. The intercept and slope in the logistic regression equation are denoted by 'a' and 'b', respectively (A). Mean time survived by 50% (MTS_{50}) of the X21L3 pool in comparison to the parents, XX2-IL1 and IL3, and commercial line, HB4 under oxidative stress (70 mM H_2O_2) at 2°C (B) and 25°C (C). Three independent experiments with each three replicates were carried out. Error bars: Average SD of three experiments. Letters above error bars denote significant differences (Tukey HSD tests at $P \leqslant 0.05$).

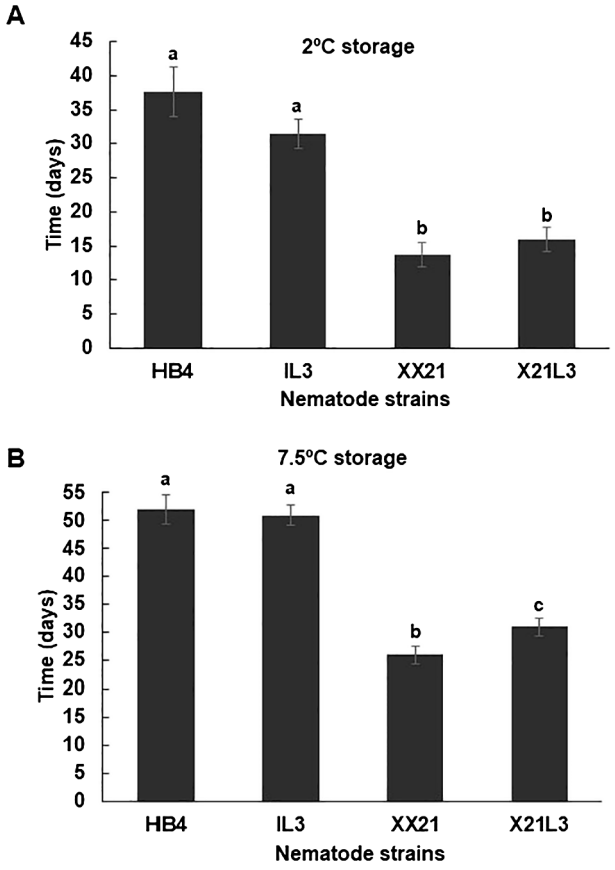

Fig. 4. Mean time of *Heterorhabditis bacteriophora* survived by 75% (MTS_{75}) of the X21L3 pool in comparison to the parents, XX21 and IL3, and commercial line, HB4 after formulation stored at 2°C (A) for 4 weeks and 7.5°C (B) for 9 weeks. Three independent experiments with each three replicates were carried out. Error bars: Average SD of three experiments. Letters above error bars indicate significant differences (Tukey HSD tests at $P \leqslant 0.05$).

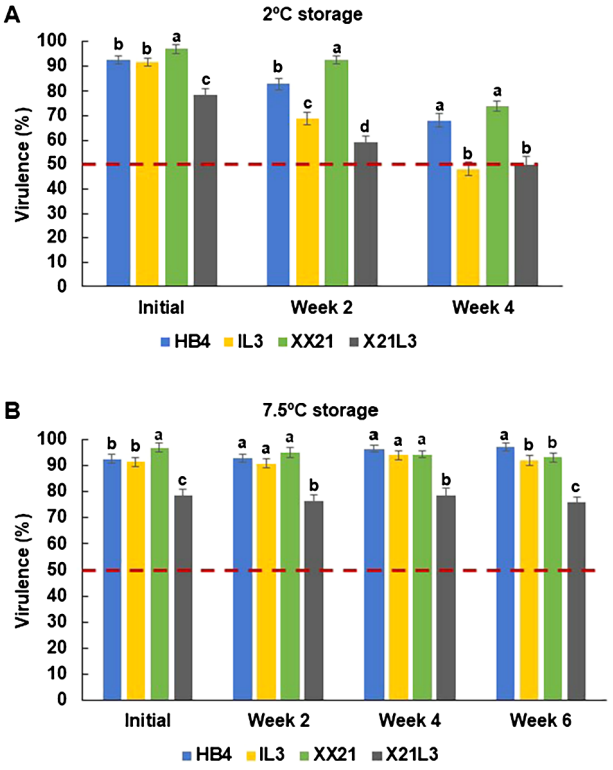

Fig. 5. Mean virulence (% mortality) against meal worm larvae (*Tenebrio mollitor*) of the X21L3 pool of *Heterorhabditis bacteriophora* in comparison to the parents, XX21 and IL3 and commercial line HB4 along storage time at 2°C (A) and 7.5°C (B). Three independent experiments with four technical replicates were carried out with a dose of 20 dauer juveniles (DJ) per insect (40 insects per bioassay). Broken line (red) indicates the standard virulence (%) according to quality control assay at e-nema GmbH. Error bars: Average SD of three experiments. Letters above error bars indicate significant differences (Tukey HSD tests at $P \leqslant 0.05$).

line XX21 with multiple desirable traits was identified as a candidate for genetic crosses with a commercial line IL3. We deviated from the classical genetic blueprint by leveraging SNP genotypic information availed by the works of Godina *et al*. (2022) further identifying additional SNP markers linked to reproductive potential, stress tolerance and virulence in this nematode collection. Surprisingly, none of these SNP markers was identical to the 20 identified by Godina *et al*. (2023) even for similar traits like oxidative stress, even though both SNP marker CT1 (Ogaya *et al*., 2024) and SNP marker OS9 (Godina *et al*., 2023) associated with oxidative stress tolerance were localised on scaffold1330. It is plausible this was due to differences in nematode material as heterozygosity in the Godina *et al*. (2023) study was expected to be more pronounced in the WT strains compared to the homozygous WT ILs phenotyped in this study, thereby decreasing the chances of identical SNP marker discovery. Association analysis was also carried out with a smaller set of SNPs in the Godina *et al*. (2023) study (192), compared to the 720 used in our ILs, increasing the chance of different SNP marker discovery. What was predominant was localisation of SNPs associated with different traits on the same scaffold. SNP markers RP2 and RP3 associated with reproductive potential shared scaffold1351 with SNP marker LH3 associated with longevity from Godina *et al*. (2023). SNP marker RP1 (reproductive potential) and HT1 (Godina *et al*., 2023) associated with heat

tolerance were localised on scaffold1357. Scaffold1080 localised SNP marker RP4 (reproductive potential) and SNP marker LH1 associated with longevity (Godina *et al*., 2023). Finally, virulence SNP marker V1 and oxidative stress tolerance SNP marker OS8 (Godina *et al*., 2023) were both localised on scaffold1351. Without extensive functional gene studies in *H. bacteriophora*, like those done in *Caenorhabditis elegans* (Evans *et al*., 2021), we can only speculate that this localisation of these SNP markers on the same scaffold could be due to pleiotropy. However, they are excellent molecular markers for breeding purposes and the uncharacterised genes on scaffolds of interest can be considered important for further investigation.

We avoided phenotyping the X21L3 RILs for desirable traits and instead genotyped them using the seqSNP platform previously reported by Godina *et al*. (2023). The polymorphic 33 SNP markers discovered in this material verified success of this cross and allele segregation. From this known genotype we could rapidly identify promissory RILs with beneficial SNP markers omitting the need for prior phenotyping of individual RILs. The advantage of using pools of RILs over the use of single RILs is that commercial production is started with a broader genetic variability.

Since all RILs included in the pool possessed two SNP markers for reproductive potential, it was no surprise that DJ recovery and DJ yield were improved in this pool. Owing to the high number of RILs in this pool (22), further increase in reproductive potential through selection is envisioned because subsequent sub-culturing will always favour the high reproducing RILs resulting in further trait stability. Specifically, DJ yield is a highly heritable trait (Johnigk *et al*., 2002). Improvements in oxidative stress tolerance at (2°C) were even more remarkable with X21L3 outperforming all tested lines. This heterosis effect was also observed by Sumaya *et al*. (2018) with the hybrid pool living 2.6 days longer than the parents, further elucidating the heritability of this trait. All RILs in this pool also possessed two SNP markers for oxidative stress tolerance at 2°C. Phenotypic results from reproductive potential and oxidative stress tolerance unrefutably show the precision that SNP marker-assisted breeding added to improvements of these traits.

Robust correlation studies are valuable for rapid EPN characterisation. For example, Sumaya *et al*. (2017) showed that DJ longevity is robustly predictable through oxidative stress tolerance. However, the high reproduction and stress tolerance of X21L3 pool did not translate to a superior shelf-life post formulation in agreement with Ogaya *et al*. (2024). SNP markers for desiccation tolerance, a very important trait in diatomaceous earth formulated nematodes, were absent in our pool thus require consideration in future. It could also be that in formulation the symbiotic bacteria could be playing an outsized role in nematode survival explaining why, even at the optimal storage temperature of 7.5°C, X21L3 and XX21 had a lower survival. IL3 and the commercial line HB4 have been sub-cultured for years in the commercial bacteria and hence might have developed better adaptations with the commercial DE2 symbiotic bacteria in comparison to XX21 and X21L3, so far cultured minimally in this strain of bacteria. Evidence exists showing a link between symbiotic bacteria quality and *H. bacteriophora* stress tolerance (Bilgrami *et al*., 2006). Perennial laboratory subculturing induced selection pressure on quantitative nematode traits in *C. elegans* is well elucidated (McGrath *et al*., 2009; Sterken *et al*., 2015).

Virulence being a complex trait requiring contribution of both nematode and bacteria partners for successful infection (Han & Ehlers, 2000), it is probable that despite low survival in formulation of XX21, the surviving DJ still efficiently deliver *Photorhadus* spp. cells into the insect host, explaining the higher virulence despite presumably lower symbiotic bacteria retention, while IL3 compensates for inferior virulence by retaining a higher symbiotic cell load, thus giving the observed high virulence and survival. We expect improvement in storage stability in the X21L3 pool with subsequent sub-culturing, as the same phenomena was observed for the commercial line HB4. Quantifying the symbiotic bacteria load in the respective materials would shed light on this through qPCR assays.

In conclusion, this study avails novel SNP molecular markers associated with reproductive potential, oxidative stress tolerance and virulence. We successfully thereafter deployed these SNP markers to fast-track the *H. bacteriophora* breeding process realising significant improvements in reproductive potential, stress tolerance and virulence in a new pool of RILs. This is the first study to use molecular genetics to breed improved material of EPN, in particular *H. bacteriophora*.

Acknowledgements

The scholarship by the German Academic Exchange Service (http://www.daad.de) to the first author and the scholarship by the Flemish University Cooperation for

Development (VLIR-UOS, http://www.vliruos.be) to the second and third authors are much appreciated.

References

Addis, T., Teshome, A., Strauch, O. & Ehlers, R.-U. (2014). Life history trait analysis of the entomopathogenic nematode *Steinernema riobrave*. *Nematology* 16, 929-936. DOI: 10.1163/15685411-00002819

Bai, C., Shapiro-Ilan, D.I., Gaugler, R. & Hopper, K.R. (2005). Stabilization of beneficial traits in *Heterorhabditis bacteriophora* through creation of inbred lines. *Biological Control* 32, 220-227. DOI: 10.1016/j.biocontrol.2004.09.011

Bilgrami, A.L., Gaugler, R., Shapiro-Ilan, D.I. & Adams, B.J. (2006). Source of trait deterioration in entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* during *in vivo* culture. *Nematology* 8, 397-409. DOI: 10.1163/156854106778493394

Dhakal, M., Nguyen, K.B., Hunt, D.J., Ehlers, R.-U., Spiridonov, S.E. & Subbotin, S.A. (2020). Molecular identification, phylogeny and phylogeography of the entomopathogenic nematodes of the genus *Heterorhabditis* Poinar, 1976: a multigene approach. *Nematology* 23, 451-466. DOI: 10.1163/15685411-bja10052

Eleftherianos, I., Joyce, S., ffrench-Constant, R.H., Clarke, D.J. & Reynolds, S.E. (2010). Probing the tri-trophic interaction between insects, nematodes and *Photorhabdus*. *Annual Review of Immunology* 137, 1695-1706. DOI: 10.1017/S0031182010000508

Evans, K.S., van Wijk, M.H., McGrath, P.T., Andersen, E.C. & Sterken, M.G. (2021). From QTL to gene: *C. elegans* facilitates discoveries of the genetic mechanisms underlying natural variation. *Trends in Genetics* 37, 933-947. DOI: 10.1016/j.tig.2021.06.005

Fu, Z., Crossley, M.S., Epstein, B., Bates, C., Crowder, D.W., Elling, A.A., Hohenlohe, P.A., Jabbour, R., Ramirez, R.A. & Snyder, W.E. (2021). Using fine-scale relatedness to infer natural enemy movement. *Biological Control* 160, 104662. DOI: 10.1016/j.biocontrol.2021.104662

Gaugler, R. & Han, R. (2002). Production technology. In: Gaugler, R. (Ed.). *Entomopathogenic nematology*. Wallingford, UK, CABI International, pp. 289-310.

Glazer, I. (2015). Improvement of entomopathogenic nematodes: a genetic approach. In: Campos-Herrera, R. (Ed.). *Nematode pathogenesis of insects and other pests. Sustainability in plant and crop protection*. Cham, Switzerland, Springer International Publishing, pp. 29-55.

Godina, G., Kirsch, C., Dörfler, V., Barg, M., Singh, P.R., Vandenbossche, B., Strauch, O., Ehlers, R.-U. & Molina, C. (2022). Single nucleotide polymorphism markers in *Heterorhabditis bacteriophora* associated with virulence at low temperature. *Nematology* 24, 925-938. DOI: 10.1163/15685411-bja10181

Godina, G., Vandenbossche, B., Centurión, A., Dörfler, V., Barg, M., Ehlers, R.-U. & Molina, C. (2023). New genotyping rescues old phenotypes: beneficial traits in *Heterorhabditis bacteriophora* wild type material and association to single nucleotide polymorphisms. *Nematology* 25, 761-773. DOI: 10.1163/15685411-bja10255

Grewal, P.S., Koppenhöfer, A.M. & Choo, H.Y. (2005). Lawn, turfgrass and pasture applications. In: Grewal, P.S., Ehlers, R.-U. & Shapiro-Ilan, D.I. (Eds). *Nematodes as biocontrol agents*. Wallingford, UK, CABI International, pp. 115-146. DOI: 10.1079/9780851990170.0115

Griffin, C.T. (2012). Perspectives on the behavior of entomopathogenic nematodes from dispersal to reproduction: traits contributing to nematode fitness and biocontrol efficacy. *Journal of Nematology* 44, 177-184.

Han, R. & Ehlers, R.-U. (2000). Pathogenicity, development and reproduction of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* under axenic *in vivo* conditions. *Journal of Invertebrate Pathology* 75, 55-58. DOI: 10.1006/jipa.1999.4900

Hirao, A. & Ehlers, R.-U. (2009). Influence of cell density and phase variants of bacterial symbionts (*Xenorhabdus* spp.) on dauer juvenile recovery and development of biocontrol nematodes *Steinernema carpocapsae* and *S. feltiae* (Nematoda: Rhabditida). *Applied Microbiology and Biotechnology* 84, 77-85. DOI: 10.1007/s00253-009-1961-4

Iraki, N., Salah, N., Sansour, M.A., Segal, D., Glazer, I., Johnigk, S.-A., Hussein, M.A. & Ehlers, R.-U. (2000). Isolation and characterization of two entomopathogenic nematode strains, *Heterorhabditis indica* (Nematoda, Rhabditida), from the West Bank, Palestinian Territories. *Journal of Applied Entomology* 124, 375-380. DOI: 10.1046/j.1439-0418.2000.00450.x

Johnigk, S.-A. & Ehlers, R.-U. (1999). *Endotokia matricida* in hermaphrodites of *Heterorhabditis* spp. and the effect of the food supply. *Nematology* 1, 717-726. DOI: 10.1163/156854199508748

Johnigk, S.-A., Hollmer, S., Strauch, O., Wyss, U. & Ehlers, R.-U. (2002). Heritability of the liquid culture mass production potential of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Biocontrol Science and Technology* 12, 267-276. DOI: 10.1080/09583150120124504

Levy, N., Faigenboim, A., Salame, L., Molina, C., Ehlers, R.-U., Glazer, I. & Ment, D. (2020). Characterization of the phenotypic and genotypic tolerance to abiotic stresses of natural populations of *Heterorhabditis bacteriophora*. *Scientific Reports* 10, 1-16. DOI: 10.1038/s41598-020-67097-0

Lunau, S., Ehlers, R.-U., Stoessel, S. & Schmidt-Peisker, A.J. (1993). Establishment of monoxenic inocula for scaling up *in vitro* cultures of the entomopathogenic nematodes *Steinernema* spp. and *Heterorhabditis* spp. *Nematologica* 39, 385-399. DOI: 10.1163/187529293X00330

McGrath, P.T., Rockman, M.V., Zimmer, M., Jang, H., Macosko, E.Z., Kruglyak, L. & Bargmann, C.I. (2009). Quantitative

mapping of a digenic behavioral trait implicates globin variation in *C. elegans* sensory behaviors. *Neuron* 61, 692-699. DOI: 10.1016/j.neuron.2009.02.012

Mukuka, J., Strauch, O. & Ehlers, R.-U. (2010a). Variability in desiccation tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology* 12, 711-720. DOI: 10.1163/138855409X12607871174454

Mukuka, J., Strauch, O., Hoppe, C. & Ehlers, R.-U. (2010b). Fitness of heat and desiccation tolerant hybrid strains of *Heterorhabditis bacteriophora* (Rhabditidomorpha: Heterorhabditidae). *Journal of Pest Science* 83, 281-287. DOI: 10.1007/s10340-010-0296-3

Ogaya, C., Moges, Y., Dörfler, V., Barg, M., Ehlers, R.-U. & Molina, C. (2024). Genotypic markers associated with cold storage survival of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology* 26, 107-118. DOI: 10.1163/15685411-bja10295

Sterken, M.G., Snoek, L.B., Kammenga, J.E. & Andersen, E.C. (2015). The laboratory domestication of *Caenorhabditis elegans*. *Trends in Genetics* 31, 224-231. DOI: 10.1016/j.tig.2015.02.009

Strauch, O. & Ehlers, R.-U. (1998). Food signal production of *Photorhabdus luminescens* inducing the recovery of entomopathogenic nematodes *Heterorhabditis* spp. in liquid culture. *Applied Microbiology and Biotechnology* 50, 369-374. DOI: 10.1007/s002530051306

Strauch, O., Stoessel, S. & Ehlers, R.-U. (1994). Culture conditions define automictic or amphimictic reproduction in entomopathogenic rhabditid nematodes of the genus *Heterorhabditis*. *Fundamental and Applied Nematology* 17, 575-582.

Strauch, O., Oestergaard, J., Hollmer, S. & Ehlers, R.-U. (2004). Genetic improvement of the desiccation tolerance of the entomopathogenic nematode *Heterorhabditis bacteriophora* through selective breeding. *Biological Control* 31, 218-226. DOI: 10.1016/j.biocontrol.2004.03.009

Stuart, R.J., Barbercheck, M.E. & Grewal, P.S. (2015). Entomopathogenic nematodes in the soil environment: distributions, interactions and the influence of biotic and abiotic factors. In: Campos-Herrera, R. (Ed.). *Nematode pathogenesis of insects and other pests. Sustainability in plant and crop protection*. Cham, Switzerland, Springer International Publishing, pp. 97-137. DOI: 10.1007/978-3-319-18266-7_4

Sumaya, N.H., Aryal, S., Vandenbossche, B., Barg, M., Dörfler, V., Strauch, O., Molina, C. & Ehlers, R.-U. (2017). Phenotyping dauer juvenile oxidative stress tolerance, longevity and persistence within wild type and inbred lines of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Nematology* 19, 971-986. DOI: 10.1163/15685411-00003100

Sumaya, N.H., Gohil, R., Okolo, C., Addis, T., Dörfler, V., Ehlers, R.-U. & Molina, C. (2018). Applying inbreeding, hybridization and mutagenesis to improve oxidative stress tolerance and longevity of the entomopathogenic nematode *Heterorhabditis bacteriophora*. *Journal of Invertebrate Pathology* 151, 50-58. DOI: 10.1016/j.jip.2017.11.001

Wang, Z., Ogaya, C., Dörfler, V., Barg, M., Ehlers, R.-U. & Molina, C. (2023). Pheno- and genotyping *in vitro* dauer juvenile recovery in the nematode *Heterorhabditis bacteriophora*. *Applied Microbiology and Biotechnology* 107, 7181-7196. DOI: 10.1007/s00253-023-12775-y

Acknowledgements

Special appreciation to Prof. Dr. Ralf-Udo Ehlers for granting me the opportunity to pursue this Ph.D even if it meant you had to supervise me past your official retirement. Talking to you during periods of extreme pressure was always calming and reassuring. Working with you has provided me with a priceless template on how to be a good scientist. To Dr. Carlos Molina, I know I was a very stubborn student but your supervision style pre-dominated by kindness and humor made it exceptionally joyful to work with you. Your valuable input made this work “sexy”, to borrow your term. I would not have asked for a better supervisor. Yohannes Moges Ferede, Nontarak Huong, Anjeline Wamalu and Michelle Ann Diano, what a joy it was to work with all of you. You taught me valuable lessons in teamwork and this dissertation is a testament to that. To Innocent Hategekimana, special thanks for carrying out the genetic crosses in this work. Verena Dörfer and Mike Barg, the fruition of this work is a result of you always happily sharing your technical expertise and I thank you for that. Dr. Maria Touceda-González, I was a novice in technical molecular aspects. You patiently guided me through it all and for that, you have my gratitude. To Dr. Giulia Godina, I followed your template while writing this thesis and I appreciate your advice along this process. To Dr. Bart Vandenbosch and Dr. Olaf Strauch, thank you for always finding the time to advise on different ways to analyze data and set up experiments. Blanca von Stülpnagel, thank you very much for helping with all the German translations in this document. Big thank you to the lab colleagues and all e-nema staff for creating such a wonderful working environment. Working and interacting with you all has been a joy. This work would not have been possible without funding from the German Academic Exchange Service (DAAD) and my life has taken a different trajectory thanks to you. Finally, I would love to thank my dear family for being my biggest cheerleader along this journey and all friends that I cannot all mention here but have walked this journey with me. I hope the culmination of this work makes you proud.