Introduction

The insect pathogenic group of nematodes known as entomopathogenic nematodes (EPN) have been explored in temperate, sub-tropical and tropical regions for their effective use in the control of insect pests of over 250 insect species. The infective juveniles (IJs) of EPN of the genera *Heterorhabditis* and *Steinernema* have been exploited for the control of insect pests due to their interaction with the enteric bacteria symbionts from the *Photorhabdus* and *Xenorhabdus* species, respectively. Thus, this nematode-bacteria association presents a benign alternative to the use of chemical insecticides mainly because of their ability to locate insects in cryptic habitats, high mortality rate on insect pests, their high reproductive ability and the simplicity of mass producing them and moreover, there are no reported hazards to human or other non-target organisms (Lacey et al., 2015). More than 90% of insects have at least one stage of their lifecycle in the soil especially the larval and pupal stage and EPNs have the potentials to exploit a range of hosts that spans nearly all insect orders (Půža, 2015). Therefore, EPNs offer an opportunity for an effective management programme and are excellent candidates in the integrated pest management strategies of insect pests especially targeting specific pest species when the ability of the target species to relocate is much reduced.

A plethora of work has already been reported on the substantial improvement of stress tolerance traits in EPNs via selection approaches (John Mukuka et al., 2010; Sumaya et al., 2018; Uhlmann et al., 2013). To complement this approach, the use of molecular genetics for further improvement should...
The cross between H. bact IL-1 and H. bact IL-2 inbred lines was done using males and virgin females at the fourth juvenile stage (J4) according to (Iraki et al., 2000; J. Mukuka et al., 2010). Unfertilized J4 females were identified and separated by absence of eggs in the uterus and copulation plug in the vulva with the aid of a stereomicroscope (Dix, 1994).

Two methods were compared for the production of males and virgin females; in vitro and in vivo, however, the production of males was only possible from the last instar of G. mellonella larvae after infection with 50 IJs/larva. Six days after infection, clearly differentiable J4 males and unfertilized females were recovered from the dissected cadaver and selected for crossing. All crosses were performed in a 12-cell well plate containing 500 µl of NGG pre-coated with 100 µl of P. luminescens diluted in semi-solid NGG. In individual cell wells, 10 male and 5 female nematodes from each strain were placed to mate at 25°C in a dark incubation room. Each cross was repeated 4 times. Reciprocal crosses were done in the same manner. For virginity tests, five females from the same batch as the crosses were placed in separate cell wells without males. As a control cross, males and females from the same inbred line were incubated in separate cell wells (as described above). Successful mating was confirmed by the presence of a mating plug in the vulva of the female observed under a stereomicroscope. The products of the crosses between the two strains were validated as true progenies only when the cell wells containing females alone had no progenies while cell wells containing males and females of the same strain produced progenies.

For this study, two strains with contrasting performance to stress factors were selected as parents for the design of a genetic cross. IJs of H. bact -1 was superior in tolerance to stress factors compared to IJs of H. bact -2. Both inbred lines were cultured and maintained in vitro on nematode growth gelrite (NGG) media (1.5 g gelrite, 1.25 g peptone, 1.5 NaCl, 500 µl CaCl₂×2H₂O, 500 µl MgSO₄×7H₂O, 12.5 ml KH₂PO₄, and 500 µl Cholesterol dissolved in 486 ml of mineral water) pre-inoculated with a lawn of the symbiotic bacteria Photorhabdus luminescens and used for physiological tests. In vivo culture of the lines was done in the last instar larval stage of the Great Wax Moth, Galleria mellonella as described by (Dutky et al., 1964) and used for genetic crosses. Both in vitro and in vivo propagation of the inbred lines were done in 4 different batches.

Development of H. bact-1 x H. bact-2 Recombinant Inbred Line Population

After successful mating, the F₁ progenies was allowed to self-fertilize on NGG media to produce the F₂ generations. Thereafter, single hermaphrodites at endotokia matricida stage were picked from the F₂ population into single NGG cell wells inoculated with P. luminescens. This way of propagation by selfing was maintained for 4 further generations to produce the population of recombinant inbred lines (RILs). Each RIL was transferred to the next generation from a single hermaphrodite of the previous cycle (Fig. 1). The harvested IJs were washed in Ringer's solution using a vacuum pump, stored in sterile culture flasks at 15°C and used within 7 days for molecular tests. Each culture flask of RILs contained between 3-5 ml of IJs in Ringer's solution.
Genomic DNA was extracted from freshly harvested IJs of each RIL and the parental lines using the KAPA Express Extract kit (Kapa Biosystem, Wilmington, USA). Thereafter, 10 µl of Kapa extraction buffer and 2 µl of Kapa thermostable protease enzyme were added. The DNA extraction was done with the aid of a GeneTouch™ Thermocycler (Bioer, Hangzhou, China) using the following temperature profile: 75°C for 10 min, and 95°C for 5 min. The extraction products were vortexed and centrifuged at high speed for 1 min. The DNA-containing supernatant was transferred to sterile tubes and stored at -20°C.

The amplification of the DNA by polymerase chain reaction (PCR) was performed using PCR thermocycler program for the amplification of genomic DNA of *Heterorhabditis bacteriophora* using SSR primers. Taq DNA Polymerase, 0.2 mM dNTPs, 10 mM Tris-HCl with pH 8.8 at 25 °C, 50 mM KCl, 0.01% Tween 20 and 1.5 mM MgCl2), 1.5 µl of 5 µM primer pair, 1 µl of 30 ng/µl DNA template and remaining part was filled with PCR-graded water. Amplifications were carried out in a Gene Touch™ Thermocycler (Bioer, Hangzhou, China) according to the procedures described in Fig. 2. PCR amplicons were separated in 1.75% agarose and stained with GelRed™ (Biotuem, California, USA), following the manufacturer’s instructions. As size standard, the GeneRuler marker (Thermo Fisher Scientific, Schwerte, Germany) was used. PCR amplicons were visualized using UV light in a GenDoc system (Vilbar Loumart, Germany).

All primer sequences were synthesized by Eurofins Genomics (München, Germany). After the PCR amplification and evaluation of the RILs genomic DNA, banding patterns were scored for presence or absence of genetic markers across the mapping population relative to the parental lines.

DNA Extraction and Polymorphic Screening

Genomic DNA was extracted from freshly harvested IJs of each RIL and the parental lines using the KAPA Express Extract kit (Kapa Biosystem, Wilmington, USA). Thereafter, 10 µl of Kapa extraction buffer and 2 µl of Kapa thermostable protease enzyme were added. The DNA extraction was done with the aid of a GeneTouch™ Thermocycler (Bioer, Hangzhou, China) using the following temperature profile: 75°C for 10 min, and 95°C for 5 min. The extraction products were vortexed and centrifuged at high speed for 1 min. The DNA-containing supernatant was transferred to sterile tubes and stored at -20°C.

The amplification of the DNA by polymerase chain reaction (PCR) was performed under a ClearView Hood (Biozym Scientific, Oldendorf, Germany) using peqGOLD™ HotStart Mix (VWR International, Erlangen, Germany). Amplifications were performed in 20-µl final volume containing 10 µl HotStart Mix (12.5 µl Taq DNA Polymerase, 0.2 mM dNTPs, 10 mM Tris-HCl with pH 8.8 at 25 °C, 50 mM KCl, 0.01% Tween 20 and 1.5 mM MgCl2), 1.5 µl of 5 µM primer pair, 1 µl of 30 ng/µl DNA template and remaining part was filled with PCR-graded water. Amplifications were carried out in a Gene Touch™ Thermocycler (Bioer, Hangzhou, China) according to the procedures described in Fig. 2. PCR amplicons were separated in 1.75% agarose and stained with GelRed™ (Biotuem, California, USA), following the manufacturer’s instructions. As size standard, the GeneRuler marker (Thermo Fisher Scientific, Schwerte, Germany) was used. PCR amplicons were visualized using UV light in a GenDoc system (Vilbar Loumart, Germany).

All primer sequences were synthesized by Eurofins Genomics (München, Germany). After the PCR amplification and evaluation of the RILs genomic DNA, banding patterns were scored for presence or absence of genetic markers across the mapping population relative to the parental lines.

---

**Fig. 1.** Design of genetic cross between *H. bact* -1 ♀ (*H.b-1*) and *H. bact* -2 ♂ (*H.b-2*) and subsequent development of recombinant inbred lines. = self-fertilization; = crossed with.

**Fig. 2.** PCR thermocycler program for the amplification of genomic DNA of *Heterorhabditis bacteriophora* using SSR primers.
Results

Genetic Crosses and Development of Recombinant Inbred Lines

The crossing scheme depicted in Figure 1 was designed for H. bact-1 ♀ and H. bact-2 ♂ and H. bact-1 ♂ x H. bact-2 ♀. However, only the first crossing set up successfully produced viable F₁ progenies. Fertilized gravid females were observed 3-5 days after incubation (Fig. 3). Each H. bact-1 ♀ was combined with at least 10 H. bact-2 ♂ and produced an average of 12 ± 2.85 progenies, meanwhile, no progeny was observed in the cross between H. bact-1 ♂ x H. bact-2 ♀ after 4 crossing attempts.

In the test crosses between males and females from the same strains, H. bact-1 had an average of 21 ± 5.31 progenies per female and was significantly different from H. bact-2, which produced an average of 32 ± 1.65 progenies per female (t₁ = 2.73, P = 0.037). No progenies were recorded in cell wells containing only females (Table 1), confirming that the individuals used for the crosses contained virgin females, validating the progenies from the crosses as hybrids.

| Female | Male | Adults | Progenies |
|--------|------|--------|-----------|
| H. bact-1 | H. bact-2 | 5♀, 10♂ | 12 ± 2.85 |
| H. bact-2 | H. bact-1 | 5♀, 10♂ | 0 |
| H. bact-1 | H. bact-1 | 5♀, 5♂ | 21 ± 5.31a |
| H. bact-2 | H. bact-2 | 5♀, 5♂ | 32 ± 1.65a |
| H. bact-1 | --- | 5♀ | 0 |
| H. bact-2 | --- | 5♀ | 0 |

NGG media for subsequent development to individual lines. These were advanced by self-fertilization for 6 generations each starting from a single hermaphrodite. As outcome, 60 separate lines were developed from the cross of H. bact-1 ♀ x H. bact-2 ♂ (Table 2). The infective juveniles from each RIL were harvested, washed in Ringer’s solution and stored at 15°C in sterile culture flasks.

Table 1: Number of starting individuals and resulting progenies from the cross between H. bact-1 ♀ x H. bact-2 ♂ inbred lines. Each cross-replicate contained an equal number of males and females. Cell wells containing only females served as check for virginity. Values are depicted as means ± SD. Different letters indicate significant differences (P = 0.037).
Table 2: Development of recombinant inbred lines from *H. bact-1 ♀ x H. bact-2 ♂* through advancement of *F₂* to *F₆* IJs by single hermaphrodite selection. Production of *F₂* was initiated from a pool of 20 *F₁* hybrids. *F₂* to *F₅* were propagated on *NGG* while *F₆* was propagated in *Galleria mellonella* larvae. Starting with 108 *F₁* hermaphrodites, the number of hermaphrodites, which produced viable infective juveniles, reduced in each generation for either *in vitro* or *in vivo* propagation.

| Generations | Number of IJ lines derived after every generation |
|-------------|--------------------------------------------------|
| *F₂*        | 108                                              |
| *F₃*        | 95                                               |
| *F₄*        | 80                                               |
| *F₅*        | 68                                               |
| *F₆*        | 60                                               |

*Molecular Screening for Polymorphism*  
*H. bact -1 ♀ x H. bact -2 ♂* inbred lines were genotyped for polymorphism using intron-directed primers, HSPs, SSRs and RAPD markers. Of the 227 primers used, 18 primers (7.9%) detected polymorphisms between the two parental inbred lines (Table 3). The polymorphisms found between the two inbred lines were based on differences in DNA fragments of the amplified regions, depicted by the presence or absence of bands (Fig. 4 a).

The polymorphic markers detected in the parental inbred lines were screened across the genomic DNA of all the RILs for segregating allelic loci. Presence or absence of bands was scored as alleles due to the homogeneity attained by the RILs after 6 generations of self-fertilization (Fig. 4 b).

From the polymorphic markers detected, 19 were scored throughout the RIL population. The sizes of the scored genetic markers ranged from 150 bp (SSRm150) to 1800 bp.

Table 3: Molecular maker types and number of primers used for screening *H. bact -1* and *H. bact -1* inbred lines for polymorphism. *SSR-mixes* were produced by a combination of a forward sequence 5'-3' of one primer and paired with the reverse sequence 3'-5' of another primer.

| Marker Types | Total Number of Primers tested | Number of primers showing polymorphism | Number of polymorphic bands |
|--------------|--------------------------------|----------------------------------------|-----------------------------|
| SSR          | 40                             | 1                                      | 1                           |
| Intron-directed Primers | 20 | 0                                      | 0                           |
| Random Primers | 14 | 3                                      | 8                           |
| HSP          | 3                              | 0                                      | 0                           |
| SSR-mixes    | 150                            | 14                                     | 16                          |
| **Total**    | **227**                        | **18**                                 | **25**                      |
Fig. 4. a. PCR amplification of six SSR primer mixes (1-6) used for polymorphism screening in the genomic DNA of H. bact-1 and H. bact-2 infective juveniles shown in paired lanes (A-L). Amplicons were separated by 1.75% (w/v) Agarose gel and visualized by UV light after staining with GelRed™. Polymorphic regions were detected by the presence or absence of band patterns shown in the boxes. Molecular weight (M) 1Kb was used as size standard.

Fig. 4. b. Segregation of polymorphic loci across

Discussion

The use of Heterorhabditis bacteriophora for biological control leverages on the genetic improvement aimed at enhancing IJs tolerance to environmental stress mainly through series of selection processes following adaptation phases (Anbesse et al., 2013; Sumaya et al., 2018; Susurluk et al., 2007). This strategy is often not sustainable due to environmental influences and instability of the improved traits after removal of selection pressure (Bilgrami et al., 2006). Therefore, it is imperative to target at the genetic components controlling traits of interest by marker-assisted selection.

The cross, carried out on NGG-

Photorhabdus luminescens media, yielded only 20 F₁ hybrids from a combination of 10 ♂ and 5 ♀ of each strain in 12 replicates. The low number F₁ was due to the loss of young hybrids within the media. Subsequent advancement and development of the RILs were done on NGG media and this posed significant challenges as a considerable number of the prospective inbred lines were lost (45%) during the process. The loss of the inbred lines could be as a result of the lack of development in lines with an accumulation of deleterious alleles. Another possible reason for the low output of RIL population could be the medium of propagation. Given the relatively high salt concentration in the media, the use of NGG for developing inbred lines could perhaps be replaced by a more suitable option such as liquid culture as reported by (Anbesse et al., 2013), after successfully maintaining inbred lines in liquid culture.

The cross progenies from Brecon IL-1 ♂ X EN01.sel15 ♀ produced viable progenies whereas the reciprocal cross, Brecon IL-1 ♂ X EN01.sel15 ♀ did not produce any progeny. Similar failure of inter-strain cross-hybridisation was reported in C. elegans by (Ayyadevara et al., 2001) for the cross Bergerac-BO X RC301. They combined 1 Bergerac-BO ♂ to 3 RC301 ♂ and produced viable progenies, whereas, the reciprocal cross failed to produce any progeny. They suggested male sterility as possible cause of the recombinant inbred lines (RILs) developed from the cross between H. bact-1 (P1) and H. bact-2 (P2) inbred lines of H. bacteriophora after genotyping with random primer. Segregating RILs (lanes 1-23) for each polymorphic marker are determined by the presence or absence of a band relative to the parental line, shown in the black boxes.

Fig 4. c. Sample of initial amplification of parental lines with SSR primers. Codes written within the gel are IDs for the SSR of the failed cross, however, care must be taken to draw conclusions based on findings from C. elegans given the differences in crossing methods. The progeny of the successful single reciprocal cross was still suitable for molecular or genotypic analyses. This supports the use of the RILs generated in this study for mapping genetic markers.

The presence of polymorphic markers from the parental lines in the RILs clearly depicts the heritability of the characters encoded by the portion of the genome amplified. This shows that new strains can be screened by these markers in a marker assisted selection. The RILs from this work can as well be used as a mapping population to develop a genetic linkage map depicting region in that controls the expressions of traits of interest. Therefore, contributing to the improvement of EPN as biocontrol agents to insect pests of crops.

Acknowledgement

Special thanks for the scholarship to the first author by the Flemish University Cooperation for Development – VLIR-UOS (https://www.vliruos.be). I appreciate the support by the staff of E-nema Biotechnology Company, Germany (https://e-nema.de).

References

Anbesse, S., Sumaya, N.H., Dorfler, A.V, Strauch, O., Ehlers, R.-U.R-U.U., 2013. Selective breeding for desiccation tolerance in liquid culture provides genetically stable inbred lines of the entomopathogenic nematode Heterorhabditis bacteriophora. Appl. Microbiol. Biotechnol. 97, 731–739. https://doi.org/10.1007/s00253-012-4227-5

Ayyadevara, S., Ayyadevara, R., Hou, S., Thaden, J.J., Shmookler Reis, R.J., 2001. Genetic Mapping of Quantitative Trait Loci Governing Longevity of Caenorhabditis elegans in Recombinant-Inbred Progeny of a Bergerac-BO 3 RC301 Inter-strain Cross. Genetics 157, Pp. 655–666.
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. https://doi.org/10.1163/15685410678493394

Dix, I., 1994. Use of second generation *Heterorhabditis* females for genetic crosses, in: Burnell, A.M., Ehlers, R.U., Masson, J.P. (Eds.), Genetics of Entomopathogenic Nematode-Bacterium Complexes. COST 812 Biotechnology. European Commission, Directorate General XII, Luxembourg, Pp. 190–193.

Dutky, S. R., Thompson, J. V, Cantwell, G. E., 1964. A technique for the mass production of DD-136 nematode. J. Insect Pathol. 6, 417–422.

Iraki, N., Salah, N., Sansour, M. A., Segal, D., Glazer, I., Johnik, 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. J. Appl. Entomol. 124, 375–380. https://doi.org/10.1046/j.1439-0418.2000.00450.x

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., Goettel, M. S. 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132, 1–41. https://doi.org/10.1016/j.jip.2015.07.009

Mukuka, J., Strauch, O., Ehlers, R. U., Strauch, O., 2010. Variability in desiccation tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*. Nematology 12, 711–720. https://doi.org/10.1163/138855409x1260787174454

Mukuka, J., Strauch, O., Hoppe, C., Ehlers, R. U., 2010. Improvement of heat and desiccation tolerance in *Heterorhabditis bacteriophora* through cross-breeding of tolerant strains and successive genetic selection. BioControl. https://doi.org/10.1007/s10526-010-9271-4

Půža, V., 2015. Control of Insect Pests by Entomopathogenic Nematodes, in: Lugtenberg, B. (Ed.), Principles of Plant-Microbe Interactions. Springer International Publishing, Pp. 175–183. https://doi.org/10.1007/978-3-319-08575-3_19

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*. J. Invertebr. Pathol. 151, 50–58. https://doi.org/10.1016/j.jip.2017.11.001

Susurluk, A., Tarasco, E., Ehlers, R., Triggiani, O., 2007. Molecular Characterisation of Entomopathogenic Nematodes Isolated in Italy By Pcr-Rflp Analysis of the Its Region of the Ribosomal Dna Repeat Unit. Nematol. Mediterr. 35, 23–28.

Uhlmann, F., Strauch, O., Nimkingrat, P., Ehlers, R. U., 2013. Desiccation tolerance of dauers of entomopathogenic nematodes of the genus *Steinernema*. Nematology. https://doi.org/10.1163/15685411-00002692