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Molecular screening of *Heterorhabditis bacteriophora* inbred lines for polymorphism and genetic crosses for the development of recombinant inbred lines

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Abstract

Males and females of two entomopathogenic nematode (EPN) inbred lines, H. bact -1 and H. bact -2, whose infective juveniles expressed contrasting performance for stress factors, were used as candidates in genetic crosses. The crosses produced an initial pool of 108 F_2 progenies and these were advanced by self-fertilization for 6 generations, each starting from a single hermaphrodite. After 6 generations and about 96% of homozygosity, 60 separate recombinant inbred lines (RILs) were recovered. The parental lines were previously subjected to molecular screening for polymorphism. 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 gel bands were scored as alleles due to the homogeneity attained by the inbred lines after 6 generations of self-fertilization. From the polymorphic markers detected, 19 were scored throughout the RIL population. This indicates the existence of a genetic heritable component for traits observed in the parental line of *H. bacteriophora* and therefore can be used to screen new population for desirable traits through marker assisted selection and could be useful for developing a genomic linkage map.

Keywords: Genetic-crosses, RILs, Polymorphisms, Genetic-Markers, EPN

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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 pest 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

correlate the environmental factors with the underlying genes linked to these traits.

Many genes control desirable traits and the phenotypic expression of these traits are additive effects, each contributing gene interacting with the environment. To further gain insight on the interaction between the phenotypic expression and the influencing genes, there is the need to develop a mapping population to help identify the regions on the genome containing markers that are linked to the variations in the important traits such longevity, persistence and virulence in *H. bacteriophora* IJs.

Therefore, the main objectives of this study were;

i. to develop population of recombinant inbred lines from the crosses between two *Heterorhabditis bacteriophora* strains with contrasting in performance to stress factors and

ii. to screen for polymorphic genetic markers between the parental lines to identify genetic link in the RILs to traits of interest.

Materials and Methods

Nematode Strains and Culture

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 $CaCl_2 \times 2H_2O$, 500 µl $MgSO_4 \times 7H_2O$, 12.5 ml KH_2PO_4 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.

Genetic Crosses

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.

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

After successful mating, the F_1 progenies was allowed to self-fertilize on NGG media to produce the F_2 generations. Thereafter, single hermaphrodites at *endotokia matricida* stage were picked from the F_2 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.

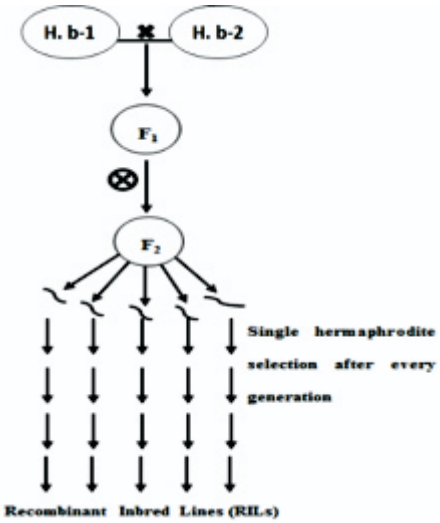

Fig. 1. Design of genetic cross between H. bact -1 ♀ (**H.b-1**) and H. bact-2 ♂ (**H.b-2**) and subsequent development of recombinant inbred lines. = self-fertilization; = crossed with.

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 Gene Touch™ 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™ (Biotuim, 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 makers across the mapping population relative to the parental lines.

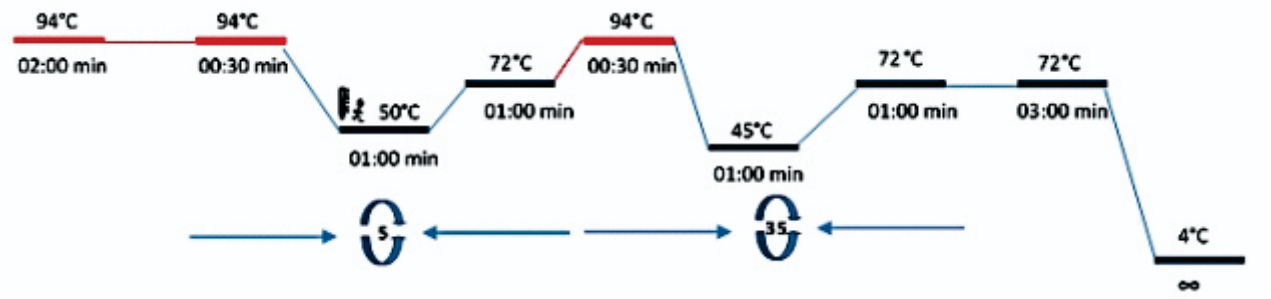

Fig. 2. PCR thermocycler program for the amplification of genomic DNA of *Heterorhabditis bacteriophora* using SSR primers.

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 F_1 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 ($t_{df = 5}$ = 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.

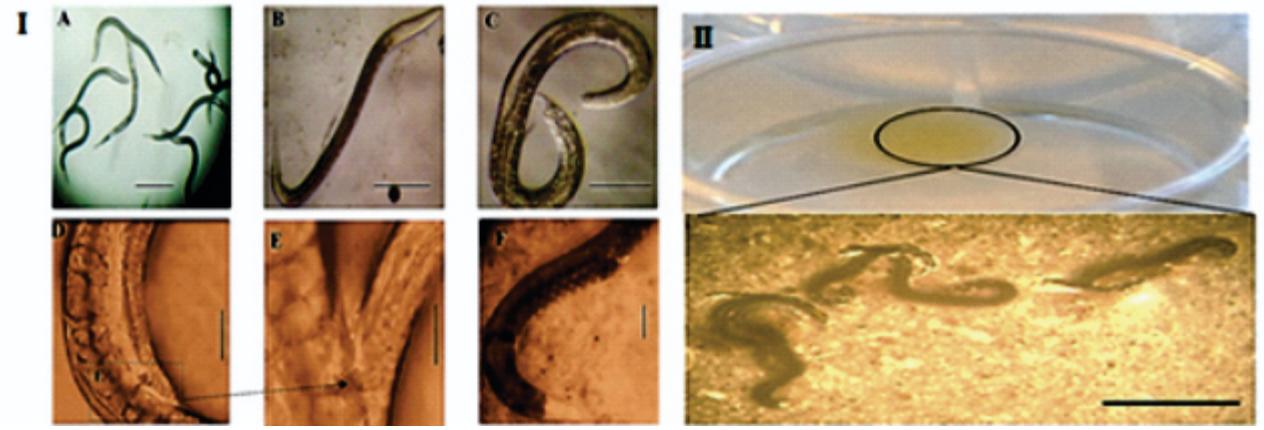

Fig. 3. I. Different developmental stages of individuals from *H. bact -1* and *H. bact -2* inbred lines.

- Freshly harvested infective juveniles from Galleria mellonella.
- Unfertilized J4 female *H. bact -1*
- J4 male from *H. bact -2*
- Coupled male *H. bact -1* and female *H. bact -2* to form a genetic cross
- View of the spicule insertion into the vagina of a J4 female.
- Fertilized gravid *H. bact -2* female.

(Scale bar: A, B, C = 300 µm, D, E, F = 100 µm.)

Fig. 3. II. Initiation of the genetic cross between H. bact -1 ♀ **X** H. bact -2 ♂ inbred lines. Each cell contained NGG pre-inoculated with *Photorhabdus luminescens* before introducing the male and female nematodes. Magnified view of the nematodes within a cell well (Scale bar = 300 µm.).

From the outcome, 108 F_2 hermaphrodites in E*ndotokia matricida* stage (H_{em}) were recovered and each H_{em} was placed in a single cell well of 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).

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± 5.31[b]
H. bact -2	H. bact -2	5♀, 5♀	32± 1.65[a]
H. bact -1	---	5♀	0
H. bact -2	---	5♀	0

Table 2: Development of recombinant inbred lines from *H. bact-1* ♀ *x H. bact-2* ♂ through advancement of F_2 to F_6 IJs by single hermaphrodite selection. Production of F_2 was initiated from a pool of 20 F_1 hybrids. F_2 to F_5 were propagated on NGG while F_6 was propagated in *Galleria mellonella* larvae. Starting with 108 F_2 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
F_2	108
F_3	95
F_4	80
F_5	68
F_6	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

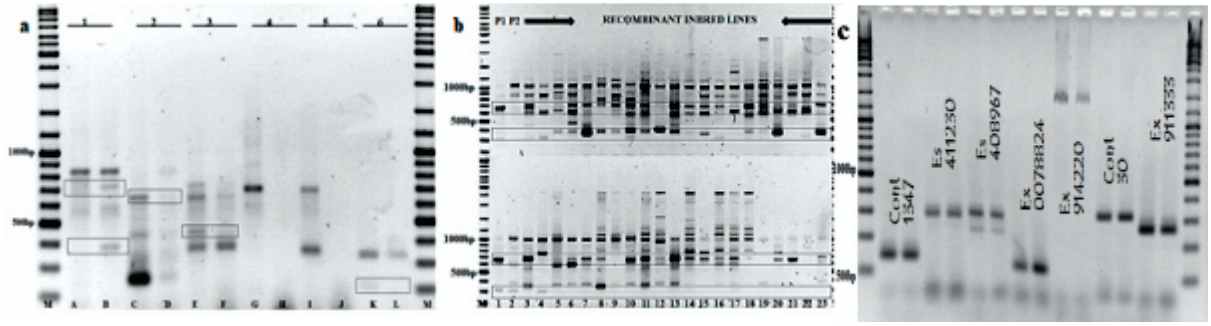

Fig. 4. a. PCR amplification of six SSR primer mixes (1-6) used for polymorphism screening in the genomic DNA of *H. bact -1* and *H. bact -2* infective juveniles shown in paired lanes (A-L). Amplicons were separated by 1.75% (w/v) Agarose gel and visualized by UV light after staining with GelRed™. Polymorphic 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 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

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 F_1 hybrids from a combination of 10 ♂ and 5♀ of each strain in 12 replicates. The low number F_1 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 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.

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