Heat tolerance among different strains of the entomopathogenic nematode *Heterorhabditis bacteriophora*

John Mukuka · Olaf Strauch ·
Lieven Waeyenberge · Nicole Viaene ·
Maurice Moens · Ralf-Udo Ehlers

Abstract Quality of biological control products based on entomopathogenic nematodes can be severely damaged due to exposure to high temperature surpassing 40°C. The study screened 36 natural populations and 18 hybrid or inbred strains of *Heterorhabditis bacteriophora* for their response to high temperature. Nematodes were tested with or without prior adaptation to heat at 35°C for 3 h. Five strains of *H. indica* and one of *H. megidis* were also included. Molecular identification using nuclear ribosomal DNA sequences confirmed the designation to the three *Heterorhabditis* spp. The mean tolerated temperature ranged from 33.3°C to 40.1°C for non-adapted and from 34.8°C to 39.2°C for adapted strain populations. *H. indica* was the most tolerant, followed by *H. bacteriophora* and *H. megidis*. No correlation was recorded between tolerance assessed with and without adaptation to heat, implying that different genes are involved. Correlation between heat tolerance and mean annual temperature at place of origin of the strains was weak. A high variability in tolerance among strains and the relatively high heritability ($h^2 = 0.68$) for the adapted heat tolerance recorded for *H. bacteriophora* provide an excellent foundation for future selective breeding with the objective to enhance heat tolerance of *H. bacteriophora*.

Keywords Biological control · Adaptation · *H. indica* · *H. megidis* · Selective breeding · Enhanced heat tolerance

Introduction

Of all nematodes studied for biocontrol of insects, members of the genera *Steinernema* (Panagrolaimomorpha: Steinernematidae) and *Heterorhabditis* (Rhabditida: Heterorhabditidae) have received the most attention. They live in a symbiotic relation with bacteria of the genera *Xenorhabdus* and *Photorhabdus* (Enterobacteriacae), respectively (Ciche et al. 2006). The bacto-helminthic complexes possess many attributes of effective (e.g., Grewal et al. 2005) and safe (Ehlers 2003) biocontrol agents and they can be commercially mass-produced in large scale liquid culture (Ehlers 2001). These nematodes produce dauer juveniles (DJs), third stage non-feeding and infective juveniles, which are able to persist in the soil

J. Mukuka · O. Strauch · R.-U. Ehlers (✉)
Department for Biotechnology and Biological Control,
Institute for Phytopathology,
Christian-Albrechts-University Kiel,
Hermann-Rodewald Str. 9, 24118 Kiel, Germany
e-mail: ehlers@biotec.uni-kiel.de

L. Waeyenberge · N. Viaene · M. Moens
Institute for Agricultural and Fisheries Research,
Burg. Van Gansberghelaan 96,
9820 Merelbeke, Belgium

environment outside an insect host (Susurluk and Ehlers 2008). The DJs are resistant to shear stress and can therefore be applied with conventional spraying technology (Wright et al. 2005). Compared to the developmental EPN stages inside a host insect, the DJs are more resistant to environmental stress, like high temperature and desiccation (Glazer 2002). Upon entry into the host's haemocoel, the DJs release cells of their symbiotic bacteria, which proliferate and serve as food source for the nematodes (Han and Ehlers 2000). The insect is killed by septicaemia (Dowds and Peters 2002).

The effect of temperature on infectivity, survival and persistence of steinernematids and heterorhabditis is well documented (Molyneux 1986; Griffin and Downes 1991; Kung et al. 1991; Wright 1992; Grewal et al. 1993, 2002). Somasekhar et al. (2002) reported survival between 37% and 82% among 14 strains of *S. carpocapsae* exposed to 40°C for 2 h. Hybrid strains of *H. bacteriophora* had a mean survival temperature (temperature at which 50% survive) of 39.2°C for 2 h (Ehlers et al. 2005). Extended exposure to temperatures below 0°C and above 40°C is lethal to most EPN species but the effect depends on exposure time (Koppenhöfer 2000). The synthesis of heat shock proteins (HSP) as a response to increasing temperature can enhance tolerance of *Heterorhabditis* spp. to higher temperature and a correlation between geographical origin and polymorphism of the heat shock protein gene *hsp 70A* was reported (Hashmi et al. 1997). Both, heat-shock and cold-shock result in an accumulation of trehalose in *H. bacteriophora* (Selvan et al. 1996; Jagdale et al. 2005).

For commercial use in biological control of insect pest, nematodes are preferably produced in liquid culture (Ehlers 2001) and stored in different formulations. The survival in these formulations is limited to only a few weeks and is much reduced at room temperature (Strauch et al. 2000). Short-term exposure of DJs to temperatures above 35°C, for instance during transportation, can hamper reproduction, activity and viability and thus spoil nematode products (Grewal et al. 1994). Heat tolerance is a heritable trait (Glazer et al. 1991), therefore increase of heat tolerance through selective breeding is possible. During four selection steps, using a hybrid strain pooling seven strains of *H. bacteriophora*, the mean tolerated temperature increased from 38.5°C to 39.2°C

(Ehlers et al. 2005). This limited improvement of the heat tolerance motivated screening for higher heat tolerance among natural populations isolated from warmer regions. The aim of the current study was to evaluate the heat tolerance of different *H. bacteriophora* strains of diverse geographical origin. The heat tolerance assessment was conducted for non-adapted and adapted nematode populations. In addition, to confirm species designation for each strain, the internal transcribed spacer regions ITS1 and ITS2 together with the 5.8S rRNA gene of the ribosomal DNA tandem repeat was sequenced and compared with available data (Nguyen 2007).

Materials and methods

Nematode strains

Thirty-six *H. bacteriophora* strains of different geographical origin (Table 1) and 18 hybrid strains or inbred lines (Table 2) were used. For comparison, five *H. indica* strains and one *H. megidis* strain were included in the study. The strain collection was maintained in liquid nitrogen as described by Curran et al. (1992), except strains HY-AA7 and HY-AA8, which had been stored at 4°C only. All nematode strains cryo-preserved in liquid nitrogen were thawed in Ringer's solution for 24 h and then cultured in vivo using last instar of *Galleria mellonella* (Lepidoptera, Pyralidae) as described by Dutky et al. (1962). Subculturing in *G. mellonella* was repeated twice and dead cadavers were incubated on damp paper and transferred to modified White traps for collection of DJs (White 1927). The nematodes were stored in Ringer's solution at 10°C and used within one week after emergence.

Molecular identification

The identification of the non-hybrid strains was done by sequence comparison of the ITS rDNA region (Internal Transcribed Spacer) with sequences available in Genbank (Nguyen 2007). DNA was extracted from three individuals, which were hand-picked, placed in a 40 μl drop of double-distilled water on a glass slide and cut into two or more pieces under a dissecting microscope. Nematode fragments were transferred in 10 μl water to a sterile Eppendorf tube

Table 1 Strains of *Heterorhabditis* spp., geographical origin, mean annual temperatures at place of origin and source of each strain

Strain	Species	Place of isolation	°C[a]	Source
AU 1	*H. bacteriophora*	Brecon, Australia	16.9	R.J. Akhurst
AU 2	*H. bacteriophora*	Brecon, Australia	16.9	R.J. Akhurst
CN 1	*H. indica*	Guangzhou, China	21.9	R.C Han
CN 2	*H. bacteriophora*	Guangzhou, China	21.9	R.C.Han
CN 3	*H. bacteriophora*	Guangzhou, China	21.9	R.C.Han
CN 4	*H. bacteriophora*	Shandong, China	14.0	R.C.Han
CZ 1	*H. bacteriophora*	Ceske B., Czech Republic	8.8	P. Hyrsl
CZ 2	*H. bacteriophora*	Ceske B., Czech Republic	8.8	P. Hyrsl
DE 1	*H. megidis*	Selent, Germany	7.7	R.-U. Ehlers
DE 2	*H. bacteriophora*	Darmstadt, Germany	11.0	H. Bathon
DE 3	*H. bacteriophora*	B. Gladbach, Germany	7.7	R.-U.Ehlers
DE 4	*H. bacteriophora*	Darmstadt, Germany	9.8	H. Bathon
DE 5	*H. bacteriophora*	Darmstadt, Germany	9.8	H. Bathon
DE 6	*H. bacteriophora*	Berlin, Germany	8.9	H. Sermann
EG 1	*H. indica*	Cairo, Egypt	21.0	M. Saleh
EG 2	*H. bacteriophora*	Cairo, Egypt	21.0	M. Saleh
HU 1	*H. bacteriophora*	Budapest, Hungary	10.4	A. Fodor
HU 2	*H. bacteriophora*	Budapest, Hungary	10.4	A. Fodor
IL 1	*H. indica*	Béer Sheva, Israel	17.3	I. Glazer
IN 1	*H. indica*	Tamil Nadu, India	27.0	S.K.Easwaramoorthy
IN 2	*H. indica*	Tamil Nadu, India	27.0	S.K.Easwaramoorthy
IN 3	*H. bacteriophora*	Tamil Nadu, India	27.0	S.K.Easwaramoorthy
IR 1	*H. bacteriophora*	Karaj, Iran	16.6	J. Karimi
IR 2	*H. bacteriophora*	Karaj, Iran	16.6	J. Karimi
IR 3	*H. bacteriophora*	Karaj, Iran	16.6	J. Karimi
IR 4	*H. bacteriophora*	Karaj, Iran	16.6	J. Karimi
IR 5	*H. bacteriophora*	Karaj, Iran	16.6	J. Karimi
IR 6	*H. bacteriophora*	Karaj, Iran	16.6	J. Karimi
IT 1	*H. bacteriophora*	Lucera, Italy	15.4	E. Tarasco
IT 2	*H. bacteriophora*	Bari, Italy	15.7	E. Tarasco
IT 3	*H. bacteriophora*	Margherira de Savoia, Italy	13.8	E. Tarasco
IT 4	*H. bacteriophora*	St. Pietro Vernotico, Italy	15.9	E. Tarasco
IT 5	*H. bacteriophora*	Lavello, Italy	16.0	E. Tarasco
IT 6	*H. bacteriophora*	Bologna, Italy	13.5	K.V.Deseö
NZ 1	*H. bacteriophora*	Christ Church, New Zealand	12.1	W. Wouts
PAL 1	*H. bacteriophora*	Bethlehem, Palestine	20.0	N. Iraki
TR 1	*H. bacteriophora*	Aydin, Turkey	18.0	S. Hazir
TR 2	*H. bacteriophora*	Aydin, Turkey	18.0	S. Hazir
TR 3	*H. bacteriophora*	Aydin, Turkey	18.0	S. Hazir
TR 4	*H. bacteriophora*	Aydin, Turkey	18.0	S. Hazir
TT 1	*H. bacteriophora*	Trinidad & Tobago	26.0	T. Ciche
US 1	*H. bacteriophora*	California, USA	25.0	H. Kaya
US 2	*H. bacteriophora*	New Jersey, USA	11.5	R. Gaugler

[a] Mean annual temperature at place of origin obtained from http://www.worldclimate.com/

Table 2 Hybrid and inbred lines of *Heterorhabditis bacteriophora* used for heat tolerance assays

Strain	Comment	Reference
HY-EN 01	Commercial hybrid strain	Johnigk et al. (2002)
HY-PS7	Hybrid strain of EN01 and HO6	Ehlers et al. (2005)
HY- IL B4	Inbred line of PS7	Strauch et al. (2004)
HY- IL C4a	Inbred line of PS7	Strauch et al. (2004)
HY- IL C7a	Inbred line of PS7	Strauch et al. (2004)
HY- IL D4	Inbred line of PS7	Strauch et al. (2004)
HY- IL E2	Inbred line of PS7	Strauch et al. (2004)
HY- IL B3	Inbred line of PS7	Strauch et al. (2004)
HY- IL B6a	Inbred line of PS7	Strauch et al. (2004)
HY- IL B8	Inbred line of PS7	Strauch et al. (2004)
HY- MM4	PS7 after 4 selection steps for virulence against *M. melolontha*	Berner et al. (2001)
HY- MM6	PS7 after 6 selection steps for virulence against *M. melolontha*	Berner et al. (2001)
HY- MM8	PS7 after 8 selection steps for virulence against *M. melolontha*	Berner et al. (2001)
HY- MM14	PS7 after 14 selection steps for virulence against *M. melolontha*	Berner et al. (2001)
HY-AA7	PS 7 after 7 selection steps for desiccation tolerance[a]	Strauch et al. (2004)
HY-AB7	PS 7 after 7 selection steps for desiccation tolerance	Strauch et al. (2004)
HY-AA8	PS 7 after 8 selection steps for desiccation tolerance[a]	Strauch et al. (2004)
HY-AB8	PS 7 after 8 selection steps for desiccation tolerance	Strauch et al. (2004)

[a] Without prior storage in liquid nitrogen

containing 8 μl lysis buffer (final concentration: 50 mM KCl, 10 mM Tris–Cl pH 8.3, 1.5 mM MgCl$_2$, 1 mM DTT, 0.45% Tween 20). Two μl of proteinase K (600 μg ml^{-1}) were added and nematode lysates were frozen at -70°C for a minimum 15 min. The samples were then removed from the freezer and immediately incubated at 65°C for 1 h, followed by 10 min at 95°C. After centrifugation (1 min at 16,000g), 5 μl of the crude DNA extract was used for PCR. The remainder of the crude DNA-extract was stored at -20°C for future use.

An rDNA fragment containing the internal transcribed spacer regions ITS 1 and ITS 2, and the 5.8S rRNA gene was amplified by PCR using the forward primer TW81 5′-GTTTCCGTAGGTGAACCTGC-3′ and the reverse primer AB28 5′-ATATGCTTAAGTT CAGCGGGT-3′ (Joyce et al. 1994). The PCR reaction mixture contained 5 μl 10× PCR reaction buffer, 2 mM MgCl$_2$, 200 μM of each dNTP, 1 μM forward and reverse primer, 2 U Taq polymerase (Invitrogen, Merelbeke, Belgium), 5 μl crude DNA-extract and sterile water up to a volume of 50 μl. The PCR-programme settings were as described by Joyce et al. (1994). After electrophoresis of 5 μl PCR product in a 1.5% TAE buffered agarose gel (1 h,

100 V), the gel was stained in an ethidium bromide bath (1 mg l^{-1}) for 10 min and photographed under UV-light. In case of a positive result, the remainder of the PCR product was purified after electrophoresis in a 1% TAE buffered agarose gel (1 h, 100 V) following the instructions included in the Wizard SV Gel and PCR Clean-Up System Kit (Promega Benelux, Leiden, The Netherlands). Subsequently, the concentrations of the purified PCR products were measured using a UV spectrophotometer (Nanodrop ND-1000, Isogen Life Sciences, Sint-Pieters-Leeuw, Belgium). Purified PCR products were sequenced (Macrogen Inc, Seoul, Korea) in both directions to obtain overlapping sequences of the forward and reverse DNA strand. Finally, the sequences were visualised, edited and analysed using software packages Chromas 2.00 (Technelysium Pty, Helensvale, Australia) and BioEdit 7.0.4.1 (Hall 1999). The obtained sequences were aligned together with sequences from the same region of *Heterorhabditis* species available in Genbank (NCBI).

To obtain the alignments, ClustalX 1.64 (Thompson et al. 1997) was used (pairwise alignment parameters: gap opening set on 10, gap extension set on 0.1; multiple alignment parameters: gap opening set on 10,
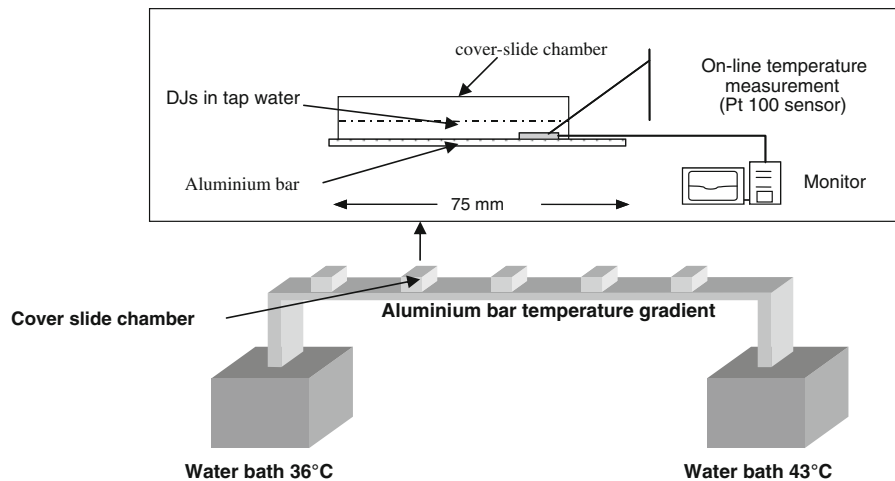
Fig. 1 Experimental design for the evaluation of the heat tolerance. A temperature gradient is produced by placing the ends of the aluminium bar in low and high temperature. Nematodes were placed into the chambers, which were positioned at different temperatures on the bar. The temperature in the chamber was recorded with a Pt 100 sensor connected to a PC (according to Ehlers et al. 2005)

gap extension set on 0.2). The alignments were subsequently used in PAUP 4.0b10 (Swofford 1998) to construct a Maximum Parsimony tree (MP). A *Steinernema* population (*S. affine* AY171298) was used as outgroup, default parameters were maintained with exception of the calculation by a heuristic search with 100 replicates. Subsequently, bootstrapping was done with 100 replicates to calculate the robustness of the trees.

Determination of heat tolerance

The assessment of the heat tolerance was done as described by Ehlers et al. (2005). Five cover-slide chambers (Lab-Tek® Chamber SLide 1, Nunc, Naperville, IL) containing 5 ml tap water were filled with 200 DJs each of one strain. The chambers were then distributed on a temperature gradient generated on an aluminium bar at temperatures between 32°C and 41°C for 2 h (Fig. 1). The temperature at the bottom of the chambers was recorded by a platinum Pt 100 thin layer sensors (M-FK 422, Heraeus Sensor-Nite GmbH, Kleinostheim, Germany).

For adaptation to high temperature, DJs were exposed to 35°C for 3 h. Afterwards they were left to recover for 1 h at 25°C and then exposed to different temperatures on the gradient as described above.

After exposure to the heat treatment, active and inactive nematodes were separated using water traps (Strauch et al. 2004) and counted. All treatments were performed three times, each time with another production batch of each nematode strain.

Statistical analysis

In order to determine the mean heat tolerance, a cumulative normal distribution was fitted to the original data (percentage of active DJs and temperature). This was performed by reducing the χ^2 through comparing the original data and the theoretical normal distribution. The mean and standard deviation of the fitted normal distribution was used as an estimation for the mean and standard deviation of the heat tolerance in a given nematode population (Example HYB-IL C4a in Fig. 2). The means of three replicates were used to calculate the mean temperature tolerated by 50% of the population (MT_{50}). This parameter was used to compare strains and identify the strain with the most tolerant individuals. The mean temperature tolerated by only 10% of the population (MT_{10}) was also determined (10% quantile of the normal distribution). Differences in MT_{50} or MT_{10} of the different strains were assessed through Tukey's HSD test ($P \leq 0.05$). To check whether heat tolerance of heat adapted and non-adapted are related, data were correlated using the Pearson's correlation coefficient at a 5% confidence level. Correlations were also calculated between the mean annual
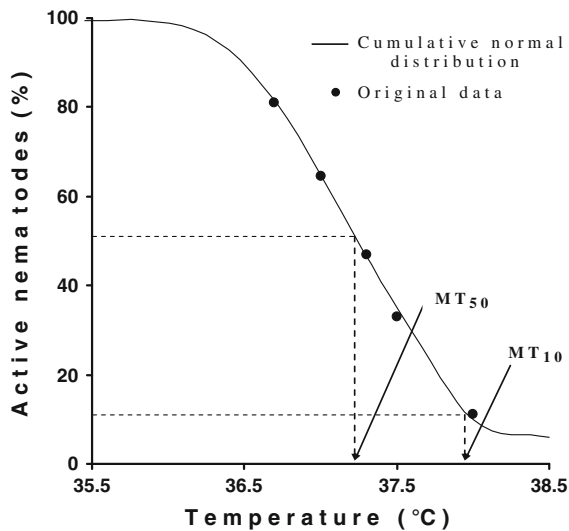
Fig. 2 Percentage active nematodes after exposure to different temperatures and cumulative normal distribution used to calculate the median of the tolerated temperature (MT_{50}) and mean temperature tolerated by only 10% of the population (MT_{10}). Example with data obtained for heat tolerance of *H. bacteriophora* strain HY IL C4a

temperature of the place of origin of each strain and their MT_{50} or MT_{10} for adapted and non-adapted populations (Table 1).

Results

Identification and phylogenetic analysis

PCR yielded a fragment of approximately 850 bp. Based on the phylogenetic analysis of the obtained sequences together with existing sequences available in Genbank, the strain DE 1 from Germany was identified as *Heterorhabditis megidis*, five strains CN 1, EG 1, IN 1, IN 2, IL 1 as *H. indica* and the remaining 36 strains as *H. bacteriophora* (Fig. 3). These findings are supported by corresponding bootstrap values of 100%.

The cluster comprising all populations identified as *H. bacteriophora*, shows a subgroup supported by a bootstrap value of 63%. This subgroup contains the sequence of Genbank (EF469774), US 2, DE 3, PAL 1, IR 4, IR 1, DE 5 and IR 3. The same can be noticed for the *H. indica* cluster but the subgroup (IN 2, IN 3 and the sequence of Genbank EF043445) is supported by a bootstrap value of 56% only.
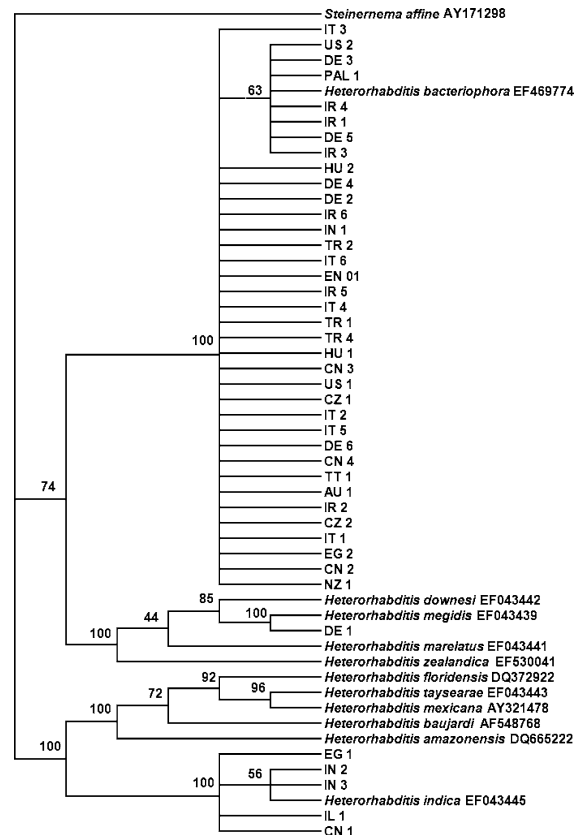

Fig. 3 Phylogenetic relationship of *Heterorhabditis* spp. based on the analysis of the internal transcribed spacer 1 and 2 sequences of the ribosomal DNA using *Steinernema affine* (AY171298) as an out-group. The 60% majority rule consensus tree of 39 equally most parsimonious trees was obtained after phylogenetic analysis with Paup v 4.0 beta 10 based on 236 parsimony-informative characters of totally 792 characters. Bootstrap values are indicated and were obtained after heuristic search with 100 replicates

Heat tolerance

When nematodes had not been adapted to higher temperature, the MT_{50} ranged from 33.3°C to 40.1°C, representing a temperature difference of 6.8°C. There were significant differences ($F = 1.65$; $df = 59, 179$; $P < 0.01$) between some of the strains. Nematode strain CN 1 (*H. indica*) from China tolerated the highest temperature ($MT_{50} = 40.1$°C), followed by *H. bacteriophora* IR 4 from Iran ($MT_{50} = 38.5$°C) and the inbred line HY IL B6a ($MT_{50} = 38.3$°C). The least tolerant strain was *H. megidis* DE 1 ($MT_{50} = 33.3$°C) from Germany followed by two *H. bacteriophora* strains from Iran, IR 6 ($MT_{50} = 34.7$°C) and IR 3 ($MT_{50} = 34.9$°C; Fig. 4).
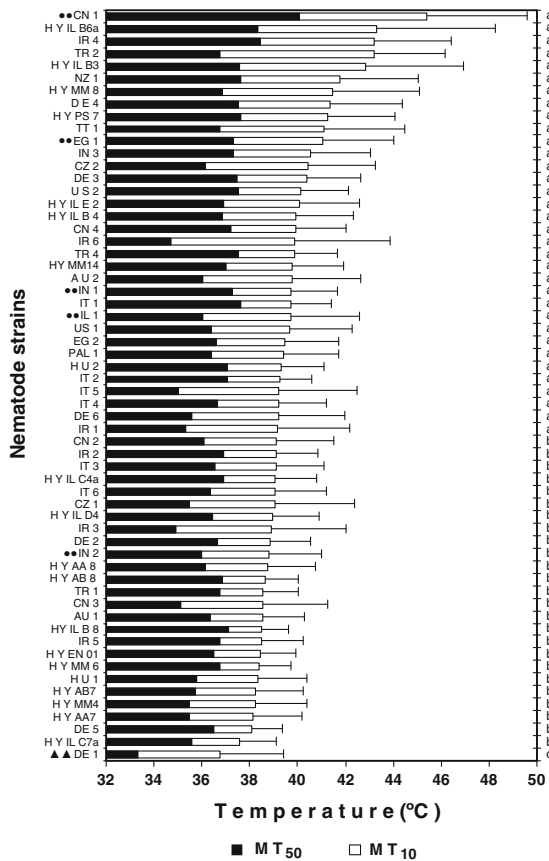
Fig. 4 Mean temperature tolerated by 50% of the population of each strain (MT_{50}) (black bars) and mean temperature tolerated by only 10% of the population (MT_{10}) (white bars) for non-adapted nematode populations of *H. bacteriophora*, *H. indica* (double filled circle) and *H. megidis* (double filled triangle). Error bars at the columns indicate standard deviation of MT_{50} and letters on the right axis indicate significant differences between MT_{10}. Different letters indicate significant differences at $P < 0.05$

The MT_{10}, representing the temperature, which only 10% of the population survived, ranged from 36.8°C to 45.4°C, representing a difference of 8.6°C and with significant differences ($F = 2.001$; $df = 59, 179$; $P < 0.001$) between some strains (Fig. 4). Again, *H. indica* CN 1 tolerated the highest temperature ($MT_{10} = 45.4$°C), followed by *H. bacteriophora* inbred line HY IL B6a ($MT_{10} = 43.3$°C) and then IR 4 from Iran ($MT_{10} = 43.2$°C). The least tolerant three strains were *H. megidis* DE 1 ($MT_{10} = 36.8$°C), followed by *H. bacteriophora* inbred line HY IL C7a ($MT_{10} = 37.6$°C) and DE 5 from Germany ($MT_{10} = 38.1$°C; Fig. 4).

Results for the tolerance recorded after adaptation are presented in Fig. 5. The MT_{50} ranged from 34.8°C to 39.2°C, representing a temperature difference of 4.4°C, with significant differences ($F = 1.797$; $df = 59, 179$; $P < 0.004$) between some of the strains. *H. indica* EG 2 from Egypt was the most tolerant, followed by *H. bacteriophora* inbred line HY IL B6a and NZ 1 from New Zealand with a $MT_{50} = 39.2, 39.1$ and 38.5°C, respectively. The least tolerant were *H. megidis* DE 1 and *H. bacteriophora* HU 1 from Hungary (both $MT_{50} = 34.8$°C), followed by the inbred line HY IL MM8 ($MT_{50} = 35.8$°C).

The MT_{10} ranged from 36.7°C to 41.8°C, representing a temperature difference of 5.1°C and with significant differences ($F = 2.54$; $df = 59$, 179; $P < 0.001$) between some strains. The most tolerant after temperature adaptation was *H.bacteriophora* IR 3 from Iran, IT 1 from Italy and NZ 1 from New Zealand with $MT_{10} = 41.8, 41.7$ and 41.3°C, respectively. The least tolerant was again *H. megidis* DE 1 ($MT_{10} = 36.7$°C), followed by *H. bacteriophora* HU 1 from Hungary and DE 2 from Germany (both $MT_{10} = 38.6$°C) (Fig. 5).

The least tolerant species is *H. megidis* (Fig. 6). When DJs had not been adapted to high temperature, a significant difference was recorded only between *H. megidis* and *H. indica* ($F = 5.33$, $df = 2$, 122; $P = 0.006$), whereas the differences was also significant to *H. bacteriophora* for the adapted tolerance ($F = 8.12$; $df = 2, 122$; $P = 0.0005$; Fig. 6).

Influence of adaptation on heat tolerance

Adaptation to higher temperature before measuring the heat tolerance significantly increased the tolerance of all species. The increase in MT_{50} (average of all strains) for *H. bacteriophora*, *H. indica* and *H. megidis* was 1.37, 1.1 and 0.39°C and in MT_{10} 1.59, 1.92 and 1.5°C, respectively. The increase in heat tolerance was significantly higher in the MT_{10} than in the MT_{50} ($F = 8.91$; $df = 5, 66$; $P < 0.0001$). The tolerance measured as MT_{50} increased for all strains, except for *H. indica* strain CN1, whereas the tolerance of eight strains decreased after adaptation when comparing data recorded for MT_{10}. Significant differences between species were noted for MT_{50} ($F = 5.42$; $df = 2$, 24; $P < 0.012$) and MT_{10} ($F = 3.736$; $df = 2, 41$; $P < 0.03$).
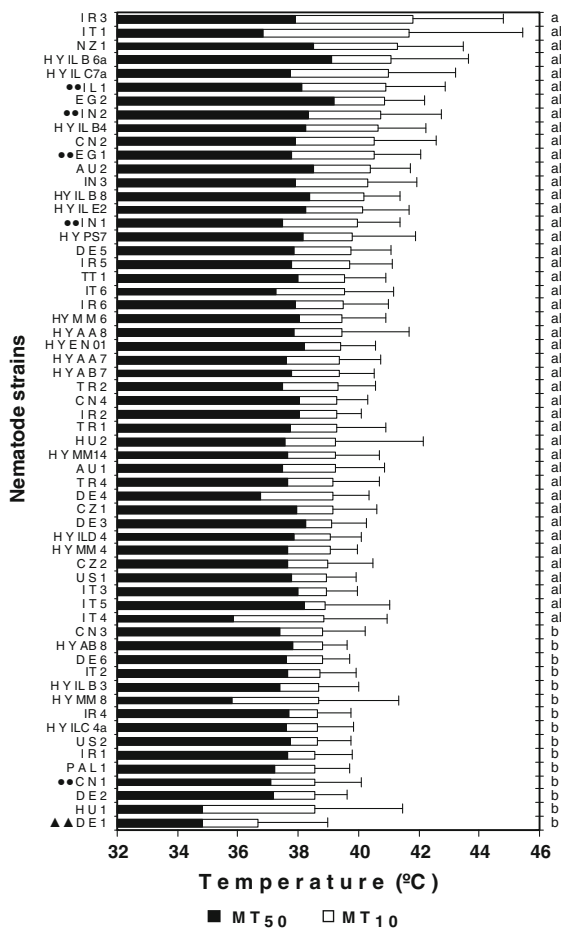
Fig. 5 Mean temperature tolerated by 50% of the population of each strain (MT$_{50}$) (black bars) and mean temperature tolerated by only 10% of the population (MT$_{10}$) (white bars) after adaptation to high temperature of *H. bacteriophora*, *H. indica* (double filled circle) and *H. megidis* (double filled triangle). Error bars at the columns indicate standard deviation of MT$_{50}$ and letters on the right axis indicate significant differences between MT$_{10}$. Different letters indicate significant differences at $P < 0.05$

A low correlation was recorded between the tolerance before and after adaptation, which was not significant, neither for the MT$_{50}$ ($y = 0.15x + 32.1$; $r = 0.22$; $P = 0.1$) nor for the MT$_{10}$ ($y = 0.07x + 36.9$; $P = 0.36$).

Correlation between heat tolerance and mean annual temperature at place of origin

The MT$_{50}$, as well as the MT$_{10}$ for heat adapted populations was faintly correlated with the mean temperature at the place of origin (Fig. 7C, D). The

same was true for the MT$_{10}$ of non-adapted populations (Fig. 7B), but no significant correlation was recorded for MT$_{50}$ without adaptation (Fig. 7A). Moreover, when only considering data obtained for *H. bacteriophora* strains (elimination of the data points for *H. megidis* and *H. indica*), a significant correlation was recorded only for the MT$_{10}$ for the adapted tolerance (data not shown).

Discussion

This comprehensive investigation assessed the heat tolerance of several strains of the biological control agent *H. bacteriophora*. To compare the results with other species, some strains of *H. indica* and one of *H. megidis* were included. Prior to the investigation of the heat tolerance, sequencing the ITS regions of the strains supported earlier identifications based on morphometric data. Although *H. bacteriophora* is recorded from many different regions of the world, it could not be excluded that other *Heterorhabditis* spp. might have been among the material, as many new species of the genus have recently been described, which often overlap in morphometric measurements (Nguyen and Hunt 2007).

The obtained subgroups within *H. bacteriophora* and *H. indica,* which are not very well supported because of rather low bootstrap values (Fig. 3), do not correlate in any way with the biological data available in Table 1 (place of isolation and annual temperature) nor to the obtained results from the heat tolerance assays. This is to be expected since the resolution of the ITS-region is compared to other DNA-regions less useful for population studies based on genetic variation (which was not a goal of this paper).

The results show variations in heat tolerance among the three *Heterorhabditis* spp. with the highest heat tolerance recorded for *H. indica*, followed by *H. bacteriophora* and then *H. megidis*. Conclusions for *H. megidis* need confirmation as only one strain was investigated.

The heat tolerance of the different strains is defined by their MT$_{50}$ and MT$_{10}$. The normal distribution fitted to the temperature-effect-response is a good measure to assess the variation among strains of one species. The temperatures tolerated by only 10% of the population is also presented apart from the MT$_{50}$ to provide an indication about the

Fig. 6 Mean tolerated temperature (MT_{50}) (black bars) and mean temperature tolerated by only 10% of the population (MT_{10}) (white bars) of *Heterorhabditis indica, H. bacteriophora and H. megidis* of non-adapted (**A**) and adapted populations (**B**). Error bars on the columns indicate standard deviation of the MT_{50}. Different letters on columns indicate significant differences at $P < 0.05$
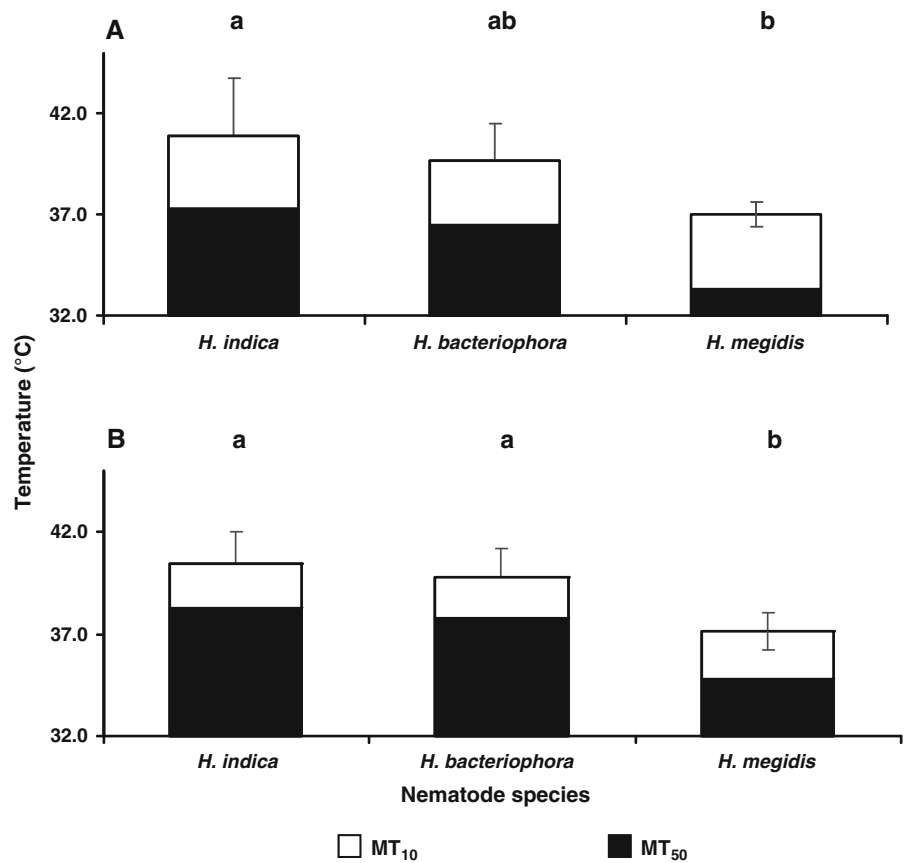

potential of one strain for inclusion into a programme for selective breeding for improved heat tolerance. Such an approach would choose those strains with the highest tolerance and high variability. For rapid progress only the best 10% of a population would be included into a selection programme and the MT_{10} would be a good indicator for the temperature to be chosen as selection pressure.

This investigation was motivated by the results obtained by Ehlers et al. (2005), who improved the heat tolerance after adaptation for a hybrid strain from 38.5°C to 39.2°C. They proposed to use heat tolerant wild type strains from relatively warm regions to identify strains with the heat tolerance superior to what they obtained by selective breeding. However, this goal was not achieved. The maximum MT_{50} recorded after adaptation was 39.2°C, thus no higher tolerance was found among the natural populations used in this study. When analysing the results obtained for the non-adapted tolerance we have to conclude that no significant differences were

detected among the *H. bacteriophora* strains. The most tolerant strain CN1 is *H. indica* and the least tolerant *H. megidis* (Fig. 4). When these two strains were excluded from the analysis, data on the heat tolerance without adaptation for the strains were not statistically different. However, significant differences were recorded for the tolerance obtained after adaptation (Fig. 5). These results would not justify substitution of the commercial strain (HY EN01) with any of the other strains characterized during this study as none of the *H. bacteriophora* strains was significantly better in tolerance compared to the commercial strain.

When analysing the relation between tolerance before and after adaption to heat, the correlation was low and not significant. This is surprising as one would expect that the tolerance to heat, whether before or after adaptation, is influenced by the same set of genes. Extraordinary is the *H. indica* strain CN1, which was the best performing for non-adapted tolerance (Fig. 4) but was among the least tolerant

Fig. 7 Correlation of MT_{50} (**A** and **C**) and MT_{10} (**B** and **D**) for non-adapted (**A** and **B**) and adapted (**C** and **D**) populations with the mean annual temperature recorded at place of isolation of the strains for *H. bacteriophora*, *H. indica* and *H. megidis*. (Pearson's correlation)

● *H.bacteriophora*   □ *H.megidis*   △ *H.indica*
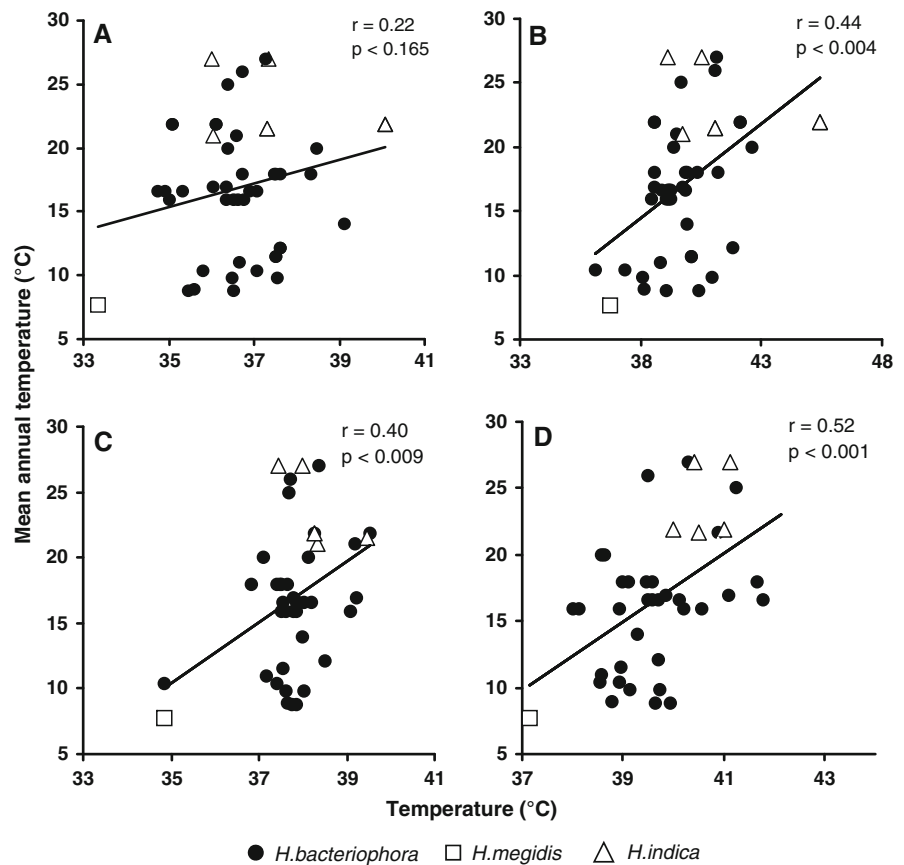
after adaptation (Fig. 5). It even was the only strain, which had a lower MT_{50} after adaptation to heat. Thus different genes might be responsible for the heat tolerance before and after adaptation, a fact that has to be considered for future breeding programmes. The mechanisms of heat resistance and possible genes involved have been described also for EPN (e.g., Grewal et al. 2006). It would be interesting to relate our results to the molecular background responsible for increase heat tolerance. Sequence data resulting from the *H. bacteriophora* genome project (Ciche 2007) would be useful for such approach. However, these data are not publically available. Strains with enhanced tolerance without prior adaptation should be crossed with those with enhanced tolerance after adaptation in order to pool the different genes responsible for the tolerance in one strain.

The relation of the enhanced tolerance to higher temperature with the mean annual temperature at the place of origin is not well pronounced, particularly when the data analysis was limited to *H.*

bacteriophora. The correlation might be better when one would perform the analysis with the maximum temperature recorded in those regions. However, apart from the top soil horizons, soil temperatures have much lower amplitudes than air temperatures and the influence of the origin of the strains on their tolerance might be less important. Consequently we can expect higher differences between the species rather than between strains of one species. Grewal et al. (1994) suggested that each nematode species has a relatively well-defined thermal niche breadth, which is not necessarily affected by the climatic conditions at their site of isolation. However, just switching to another species for heat tolerance might involve tradeoffs. Strauch et al. (2000) reported that the more heat tolerant species *H. indica* is not surviving well at lower storage temperatures. As the major approach to prolong shelf-life of EPN products still is cold storage, switching to *H. indica* would have more disadvantages than advantages obtained by a higher tolerance to heat.

Some of the screened strains can tolerate temperatures of over 40°C for 2 h or more. This is a starting point in obtaining hybrids with improved heat tolerance. An enhanced ability of nematodes to withstand higher temperature of over 40°C could improve quality and shelf-life of nematodes and ease the commercial handling of nematode-based products, providing the trait is stable in the offspring generations. Whether the increased tolerance has consequences also on mobility and infectivity of the more tolerant strains under field conditions was not investigated and needs additional investigations.

At first glance the results of this study might appear disappointing. However, the opposite is true. The results indicate a high variability among strains and a high tolerance when only considering the 10% survivors, which will be the source for crosses and genetic selection. The heritability for adapted heat tolerance recorded for *H. bacteriophora* by Ehlers et al. (2005) was $h^2 = 0.68$, which implies a high probability for success of selective breeding. The task for future studies is to enlarge the genetic pool by cross-breeding the best performing strains with each other and then to select for improved heat tolerance. These results obtained during this study lay the foundation for a future breeding programme.

References

Berner M, Ehlers R-U, Schnetter W (2001) Genetic variability and discrimination of isolates, inbred lines and hybrids of *Heterorhabditis bacteriophora* via RAPD-PCR. In: 34th annual meeting of the society of invertebrate pathology. Noordwijkerhout, p 7

Ciche T (2007) The biology and genome of *Heterorhabditis bacteriophora*. In: WormBook (ed) The *C. elegans* research community. http://www.wormbook.org/chapters/www_genomesHbacteriophora/genomesHbacteriophora.html

Ciche TA, Darby C, Ehlers R-U, Forst S, Goodrich-Blair H (2006) Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. Biol Control 38:22–46

Curran J, Gilbert C, Butler K (1992) Routine cryopreservation of *Steinernema* and *Heterorhabditis* spp. J Nematol 24:1–3

Dowds BCA, Peters A (2002) Virulence mechanisms. In: Gaugler R (ed) Entomopathogenic nematology. CABI Publishing, UK, pp 79–98

Dutky SR, Thompson JV, Cantwell GE (1962) A technique for mass rearing the greater wax moth (Lepidopera: Galleriidae). Proc Ent Soc Wash 64:56–58

Ehlers R-U (2001) Mass production of entomopathogenic nematodes for plant protection. Appl Microbiol Biotechnol 56:623–633

Ehlers R-U (2003) Biocontrol nematodes. In: Hokkanen HMT, Hajek AE (eds) Environmental impacts of microbial insecticides. Kluwer Academic Publishers, The Netherlands, pp 177–220

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

Glazer I (2002) Survival biology. In: Gaugler R (ed) Entomopathogenic nematology. CABI Publishing, UK, pp 169–187

Glazer I, Gaugler R, Segal D (1991) Genetics of the entomopathogenic nematode *Heterorhabditis bacteriophora* (strain HP88): the diversity of beneficial traits. J Nematol 23:324–333

Grewal PS, Gaugler R, Kaya HK, Wusaty M (1993) Infectivity of the entomopathogenic nematode *Steinernema scapterisci* (Nematoda: Steinernamatidae). J Invertebr Pathol 62:22–28

Grewal PS, Selvan S, Gaugler R (1994) Thermal adaptation of entomopathogenic nematodes: Niche breadth for infection, establishment and reproduction. J Thermal Biol 19:245–253

Grewal PS, Wang X, Taylor RAJ (2002) Dauer juvenile longevity and stress tolerance in natural populations of entomopathogenic nematodes: is there a relationship? Int J Parasitol 32:717–725

Grewal PS, Ehlers R-U, Shapiro-Ilan DI (2005) Nematodes as biocontrol agents. CABI International, UK

Grewal PS, Bornstein-Forst S, Burnell AM, Glazer I, Jagdale GB (2006) Physiological, genetic, and molecular mechanisms of chemoreception, thermobiosis, and anhydrobiosis in entomopathogenic nematodes. Biol Contr 38:54–65

Griffin CR, Downes MJ (1991) Low temperature activity in *Heterorhabditis* sp. (Nematoda: Heterorhabditidae). Nematologica 37:83–91

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows95/98/NT. Nucl Acids Symp Ser 41:95–98

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

Hashmi GS, Hashmi S, Selvan S, Grewal PS, Gaugler R (1997) Polymorphism in heat shock protein gene (hsp70) in entomopathogenic nematodes (Rhabditida). J Therm Biol 22:143–149

Jagdale GB, Grewal PS, Salminen SO (2005) Both heat-shock and cold-shock influence trehalose metabolism in an entomopathogenic nematode. J Parasitol 91:988–994

Johnigk SA, 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 Sci Technol 12:267–276

Joyce SA, Burnell AM, Powers TO (1994) Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA genes. J Nematol 26:260–270

Koppenhöfer AM (2000) Nematodes. In: Lacey LA, Kaya HK (eds) Field manual of techniques in invertebrate pathology. Kluwer Academic Press, The Netherlands, pp 283–301

Kung SP, Gaugler R, Kaya HK (1991) Effects of soil temperature, moisture and relative humidity on entomopathogenic nematode persistence. J Invertebr Pathol 57:242–249

Molyneux AS (1986) *Heterorhabditis* spp. and *Steinernema* (=*Neoaplectana*) spp.: temperature and aspects of behaviour and infectivity. Exp Parasit 62:169–180

Nguyen KB (2007) Methodology, morphology and identification. In: Nguyen KB, Hunt DJ (eds) Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. Hunt DJ, Perry RN (Series eds) Nematology monographs and perspectives, Brill NV, The Netherlands, pp 59–119

Nguyen KB, Hunt DJ (2007) Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. Hunt DJ, Perry RN (Series eds) Nematology monographs and perspectives, Brill NV, The Netherlands

Selvan S, Grewal PS, Leustek T, Gaugler R (1996) Heat shock enhances thermotolerance of infective juvenile insect-parasitic nematodes *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae). Experientia 52:727–730

Somasekhar N, Grewal PS, Klein MG (2002) Genetic variability in stress tolerance and fitness among natural populations of *Steinernema carpocapsae*. Biol Control 23:303–310

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

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. Biol Control 31:218–226

Susurluk A, Ehlers R-U (2008) Field persistence of the entomopathogenic nematode *Heterorhabditis bacteriophora* in different crops. BioControl 53:627–641

Swofford DL (1998) PAUP, phylogenetic analysis using parsimony and other methods. Version 4. Sinauer Associates, Sunderland, MA, p 128

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows inference: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acid Res 24:4876–4882

White F (1927) A method for obtaining infective juveniles from cultures. Science 66:1666–1670

Wright PJ (1992) Cool temperature reproduction of steinernematid and heterorhabditid nematodes. J Invertebr Pathol 60:148–151

Wright DJ, Peters A, Schroer S, Fife JP (2005) Application technology. In: Grewal PS, Ehlers R-U, Shapiro-Ilan DI (eds) Nematodes as biocontrol agents. CABI Publishing, UK, pp 91–106