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Title: Heat tolerance among different strains of the entomopathogenic nematode Heterorhabditis bacteriophora

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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 thirty six natural populations and 18 hybrid or inbred strains of Heterorhabditis bacteriophora for their response to high temperature. Nematodes were tested without or with prior adaptation to heat at 35°C for 3 hours. 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 to 40.1°C for non-adapted and from 34.8 to 39.2°C for adapted strain populations. Among the Heterorhabditis spp., H. indica was the most tolerant, followed by H. bacteriophora and H. megidis. No correlation was recorded between tolerance assessed with or 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.

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

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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 thirty six natural populations and 18 hybrid or inbred strains of *Heterorhabditis bacteriophora* for their response to high temperature. Nematodes were tested without or with prior adaptation to heat at 35°C for 3 hours. 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 to 40.1°C for non-adapted and from 34.8 to 39.2°C for adapted strain populations. Among the *Heterorhabditis* spp., *H. indica* was the most tolerant, followed by *H. bacteriophora* and *H. megidis.* No correlation was recorded between tolerance assessed with or 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

Running title: Heat tolerance of *H. bacteriophora* strains

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 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). Somasekhar et al. (2002) reported survival between 37% and 82% among fourteen strains of *S. carpocapsae* exposed to 40°C for 2 h. Hybrid strains of *H. bacteriophora* had a mean survival temperature of 39.2ºC 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).

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 4 selection steps, using a hybrid strain pooling 7 strains of *H. bacteriophora*, the mean tolerated temperature increased from 38.5 to 39.2°C. This limited improvement of the heat tolerance motivated Ehlers et al. (2005) to propose a screening for higher heat tolerance among natural populations isolated from warmer regions. The aim of this 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 order 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 AA7 and 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 10ºC 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 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) 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 16,000 *g*), five 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'-ATATGCTTAAGTTCAGCGGGT-3' (Joyce et al. 1994). The PCR reaction mixture contained 5 µl 10x 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 (1mg 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, Clustal X 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, 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). A batch of 200 DJs of one strain was transferred into a cover-slide chamber (Lab-Tek® Chamber SLide 1, Nunc, Naperville, IL) containing 5 ml tap water and exposed to five different temperatures between 32°C and 41°C on a temperature gradient generated on an aluminium bar for 2 h (Fig. 1). The temperature on 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 done 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 median and standard deviation of the heat tolerance in a given nematode population (Example

HYB-IL C4a in Fig. 2). The mean temperature tolerated by 50% of the population (MT_{50}) 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 evaluated (10% quantile of the normal distribution). Treatment differences were detected through ANOVA ($P \leq 0.05$) and differences between treatments were compared using Tukey's HSD test. 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 for the MT_{50} and MT_{10} with the mean annual temperature of the place of origin of each strain (see Tab. 1)

Results

Identification and phylogenetic analysis

PCR yielded a fragment of approximately 850bp. 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.

183 Heat tolerance

184

185 When nematodes had not been adapted to higher temperature, the MT_{50} ranged from 33.3 to
186 40.1°C, representing a temperature difference of 6.9 ºC and with significant differences (F =
187 1.65; df = 59, 179; p < 0.01) between some of the strains. Nematode strain CN 1 (*H. indica*)
188 from China tolerated the highest temperature (MT_{50} = 40.1ºC) , followed by *H. bacteriophora*
189 IR 4 from Iran (MT_{50} = 38.5ºC) and the inbred line HY IL B6a (MT_{50} = 38.3 ºC). The least
190 tolerant strain was *H. megidis* DE 1 (MT_{50} = 33.3 ºC) from Germany followed by two *H.*
191 *bacteriophora* strains from Iran, IR 6 (MT_{50} = 34.7 ºC) and IR 3 (MT_{50} = 34.9 ºC) (Fig. 4).

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

199 Results for the tolerance recorded after adaptation are presented in Figure 5. The MT_{50}
200 ranged from 34.8 to 39.2ºC, representing a temperature difference of 4.4ºC, with significant
201 differences (F = 1.797; df = 59, 179; p <0.004) between some of the strains. *H. indica* EG 2
202 from Egypt was the most tolerant, followed by *H. bacteriophora* inbred line HY IL B6a and
203 NZ 1 from New Zealand with a MT_{50} = 39.2ºC, 39.1ºC and 38.5ºC, respectively. The least
204 tolerant were *H. megidis* DE 1 and *H. bacteriophora* HU 1 from Hungary (both MT_{50} =
205 34.8ºC), followed by the inbred line HY IL MM8 (MT_{50} =35.8ºC).

206 The MT_{10} ranged from 36.7 to 41.8°C, representing a temperature difference of 5.1°C
207 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 41.3ºC, respectively. The least tolerant was again *H. megidis* DE 1 (MT_{10} = 36.7ºC), followed by *H. bacteriophora* HU 1 from Hungary and DE 2 from Germany (both MT_{10} = 38.6ºC) (Fig. 5).

The mean tolerance recorded for the different species is presented in Figure 6. The least tolerant species is *H. megidis*. 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} 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 *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} ($F = 3.736$; $df = 2, 41$; $p < 0.03$).

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

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

The correlation between the heat tolerance and mean annual temperature at place of origin is presented in Figure 7. Only in the MT_{50} for non-adapted tolerance the correlation was not significant. However, the elimination of the data points for *H. megidis* and *H. indica* results in a lower correlation for *H. bacteriophora*, which is only significant 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 on *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} in order to provide an indication about the 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 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). If these two strains are excluded from the analysis, data on the heat tolerance without adaptation for the strains are not statistically different. But significant differences are recorded for the tolerance obtained after adaptation (Fig. 5). However, 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.*

283 *bacteriophora* strains was significantly better in tolerance compared to the commercial strain
284 (Fig. 4 and 5).

285 When analysing the relation between tolerance before and after adaption to heat, the
286 correlation is low and not significant. This is surprising as one would expect that the tolerance
287 to heat, whether before or after adaptation, is influenced by the same set of genes.
288 Extraordinary is the *H. indica* strain CN1, which is best performing for non-adapted tolerance
289 (Fig. 4) but is among the least tolerant after adaptation (Fig. 5) and is even the only strain,
290 which was less well performing regarding the MT_{50} after adaptation. Thus different genes
291 might be responsible for the heat tolerance before and after adaptation, a fact that has to be
292 considered for future breeding programmes. Strains with enhanced tolerance without prior
293 adaptation should be crossed with those with enhanced tolerance after adaptation in order to
294 pool the different genes responsible for the tolerance in one strain.

295 The relation of the enhanced tolerance to higher temperature with the mean annual
296 temperature at the place of origin is not well pronounced; particularly when the data analysis
297 was limited to *H. bacteriophora*. Possibly the correlation might be better when one would do
298 the analysis with maximum temperatures recorded in those regions. However, apart from the
299 top soil horizons, soil temperatures have much lower amplitudes than air temperatures and the
300 influence of the origin of the strains on their tolerance might be less important. Consequently
301 we can expect higher differences between the species rather than between strains of one
302 species. Grewal et al (1994) suggested that each nematode species has a relatively well-
303 defined thermal niche breadth, which is not necessarily affected by the climate conditions at
304 their site of isolation. However, just switching to another species for heat tolerance might
305 involve tradeoff effects. Strauch et al. (2000) reported that the more heat tolerant species *H.*
306 *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.

Our selected strains can tolerate temperature of over 40^0C for 2 hours 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^oC 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.

At first glance the results of this study might appear disappointing. But the opposite is true. The results indicate a high variability among strains and a high tolerance when only looking at 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 now to enlarge the genetic pool by cross-breeding the best performing strains with each other and then start selection for improved heat tolerance. These results lay the foundation for a future breeding programme.

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Figures texts:

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).

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.

Fig. 3 Phylogenetic relationship *Heterorhabditi*s spp. based on the analysis of the internal transcribed spacer 1 and 2 sequences of the ribosomal DNA gene using *Steinernema affine* (AY171298) as an out-group. 60% majority rule consensus tree of 39 equally most parsimonious trees 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 heuristic search after 100 replicates.

Fig. 4 Mean temperature tolerated by 50% of the population of each strain (MT_{50}) in black bars and mean temperature tolerated by only 10% of the population (MT_{10}) in white bars for non-adapted nematode populations of *H. bacteriophora*, *H. indica* (●●) and *H. megidis* (▲▲). Error bars at the columns indicate standard deviation of MT_{50} and letters above the bars indicate significant differences between MT_{10}. Different letters at the columns indicate significant differences at $P < 0.05$.

Fig. 5 Mean temperature tolerated by 50% of the population of each strain (MT_{50}) in black bars and mean temperature tolerated by only 10% of the population (MT_{10}) in white bars after adaptation to high temperature of *H. bacteriophora*, *H. indica* (●●) and *H. megidis* (▲▲). Error bars at the columns indicate standard deviation of MT_{50} and letters above the bars indicate significant differences between MT_{10}. Different letters at the columns indicate significant differences at $P < 0.05$.

Fig. 6 Mean tolerated temperature (MT_{50}) in black bars and mean temperature tolerated by only 10% of the population (MT_{10}) in 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} and letters above the bars indicate significant differences between MT_{10} of the nematode population. Different letters on columns indicate significant differences at $P < 0.05$.

Fig. 7 Correlation between MT_{50} (A and C) and MT_{10} (B and D) and mean annual temperature recorded at place of isolation of the strains for *H. bacteriophora*, *H. indica* and *H. megidis.* (Pearson ’s correlation at a 5% confidence level).

Table 1: Strains of *Heterorhabditis* spp., geographical origin, mean annual temperatures at by of isolation and source.

Strain	Species	Place of isolation	°C [1]	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*	Ber 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

[1] Mean annual temperature at place of origin obtained from 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	Strauch et al. 2004
HY-AB7	PS 7 after 7 selection steps for desiccation tolerance[1]	Strauch et al. 2004
HY-AA8	PS 7 after 8 selection steps for desiccation tolerance[1]	Strauch et al. 2004
HY-AB8	PS 7 after 8 selection steps for desiccation tolerance	Strauch et al. 2004

[1] Without prior storage in liquid nitrogen

Fig. 1

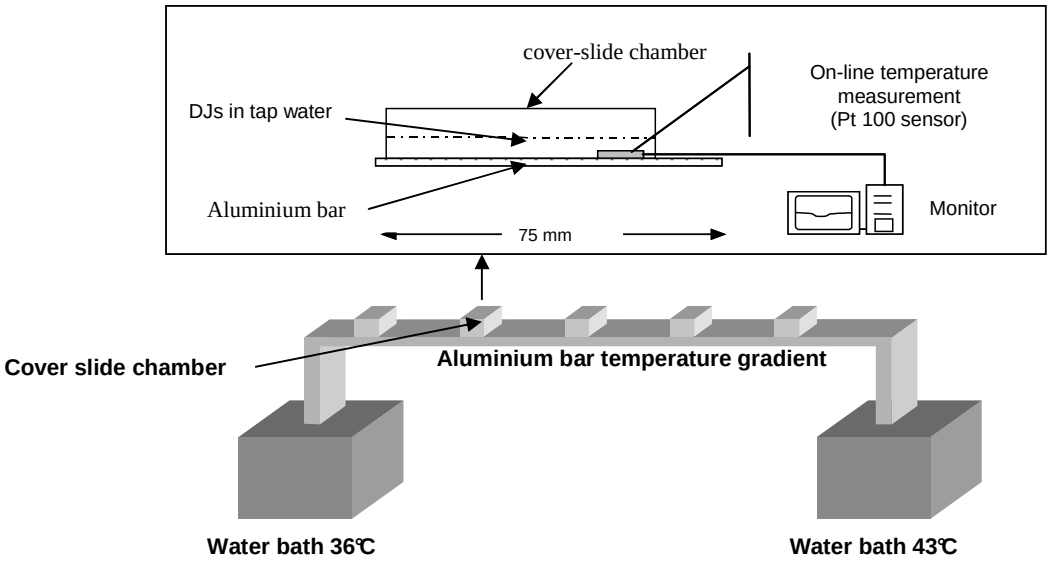
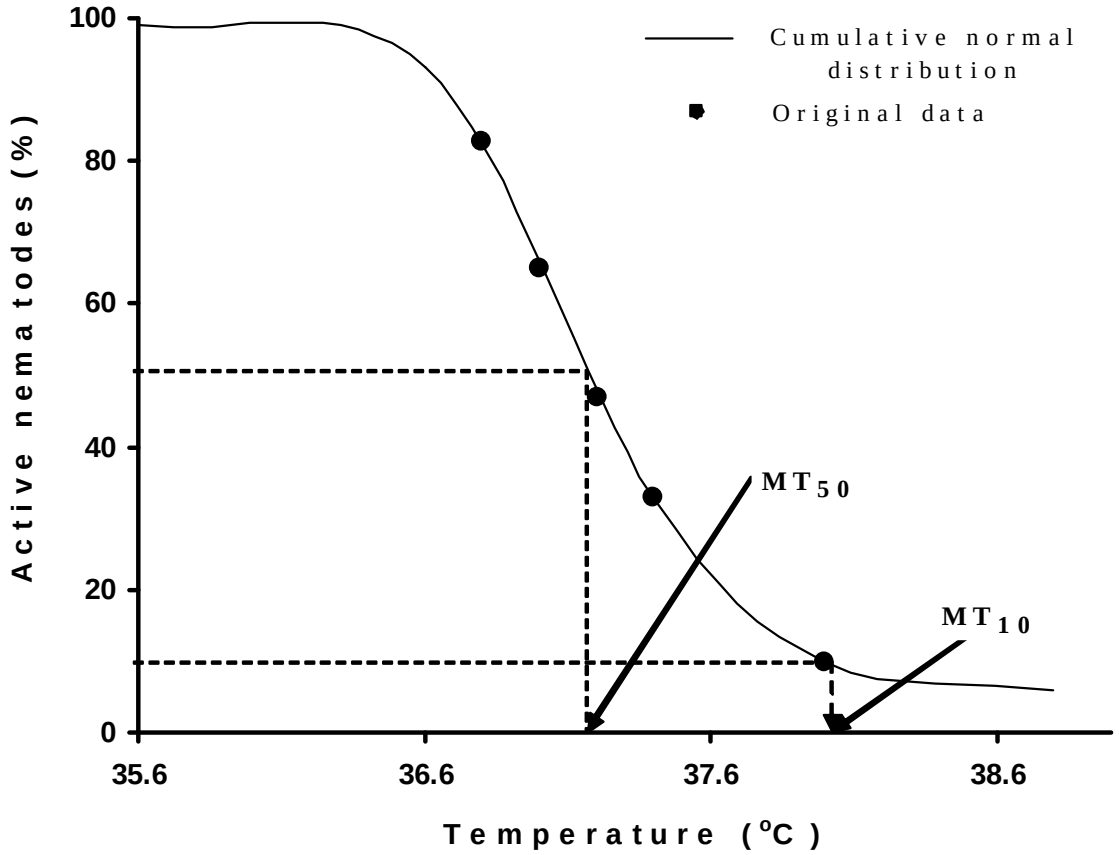
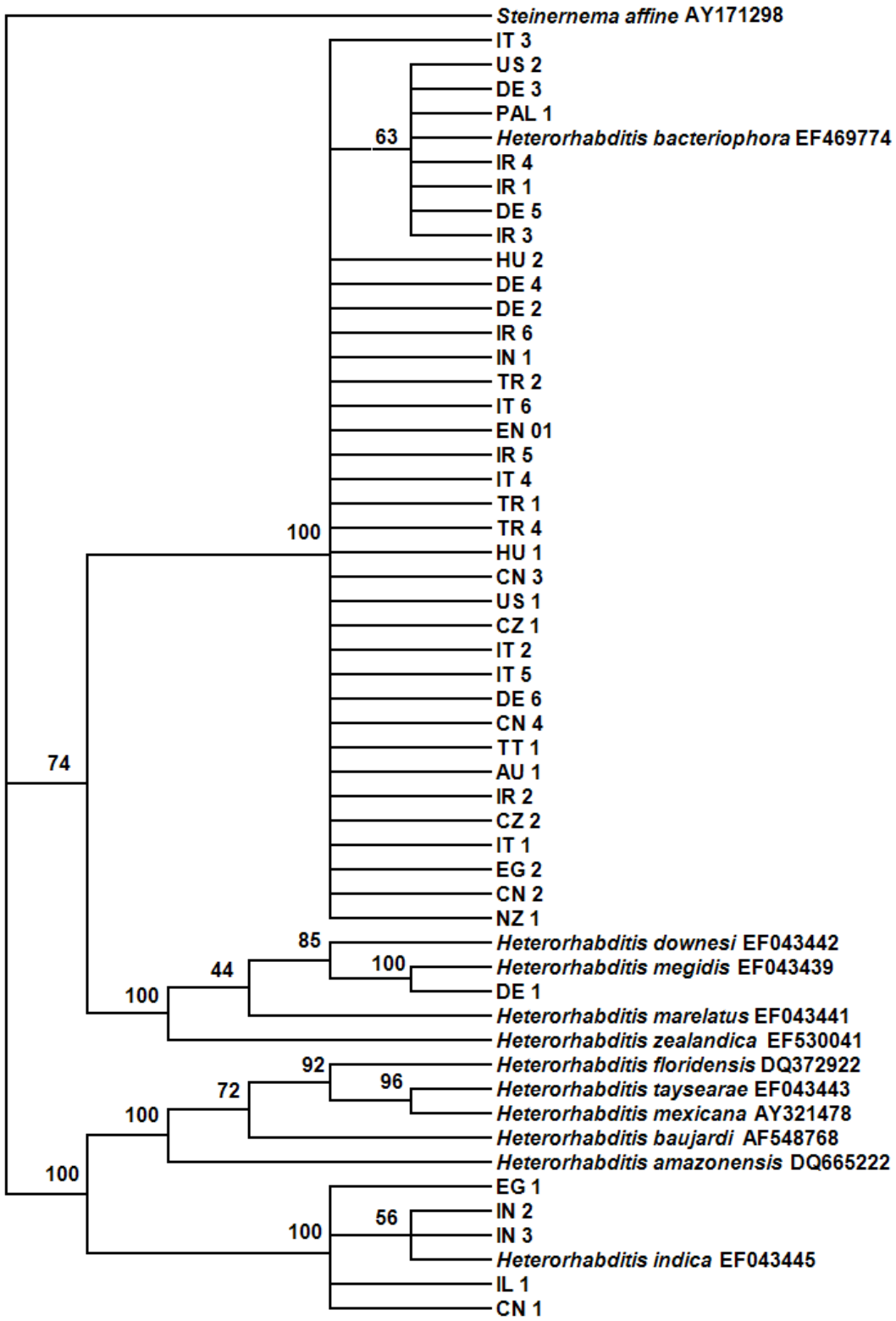

cover-slide chamber
On-line temperature measurement (Pt 100 sensor)
DJs in tap water
Aluminium bar
Monitor
75 mm
Cover slide chamber
Aluminium bar temperature gradient
Water bath 36℃
Water bath 43℃

Fig. 2

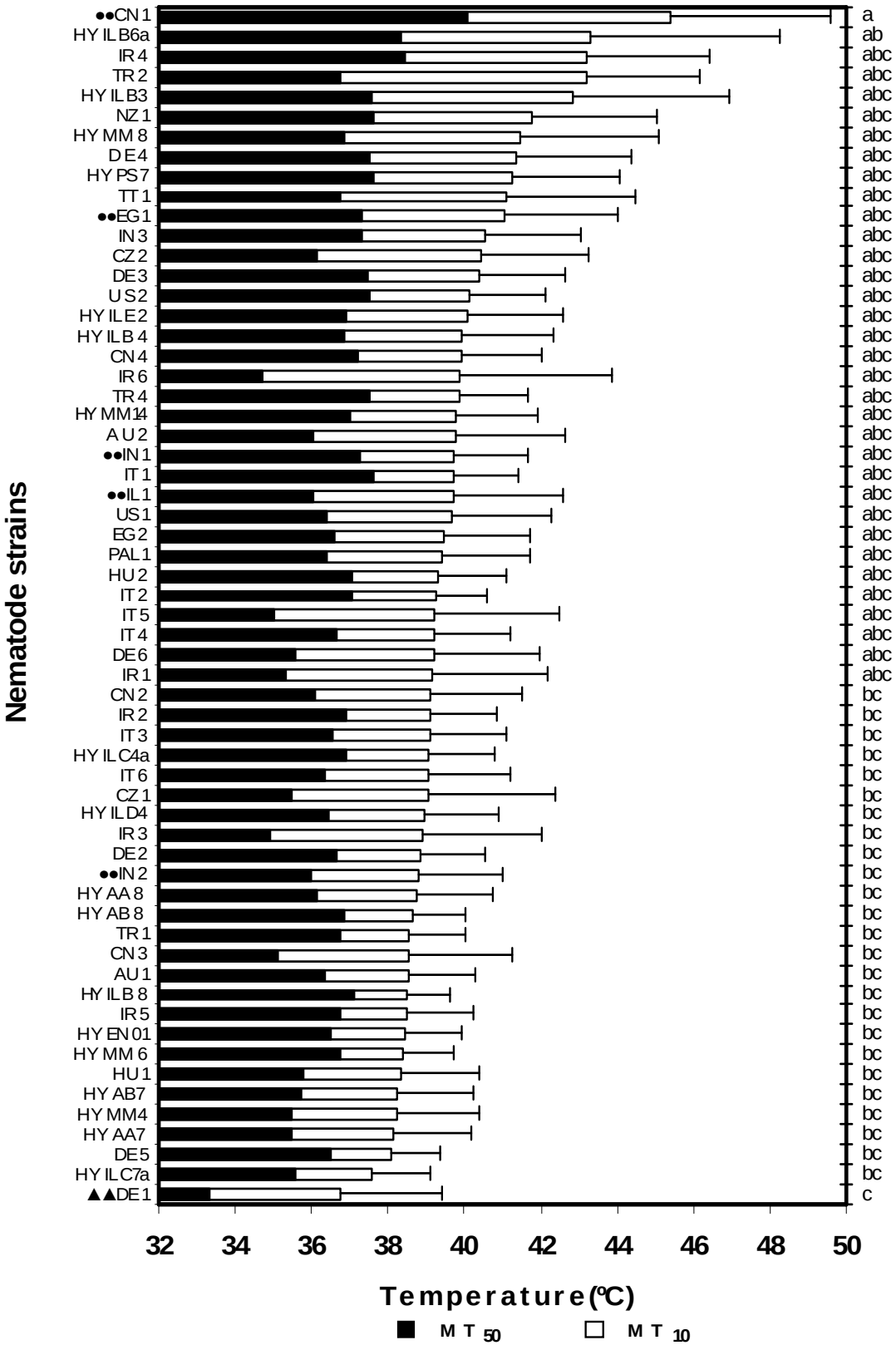

Cumulative normal distribution
Original data
Active nematodes (%)
100
80
60
40
20
0
35.6
36.6
37.6
38.6
Temperature (°C)
MT50
MT10

550 **Fig.3**



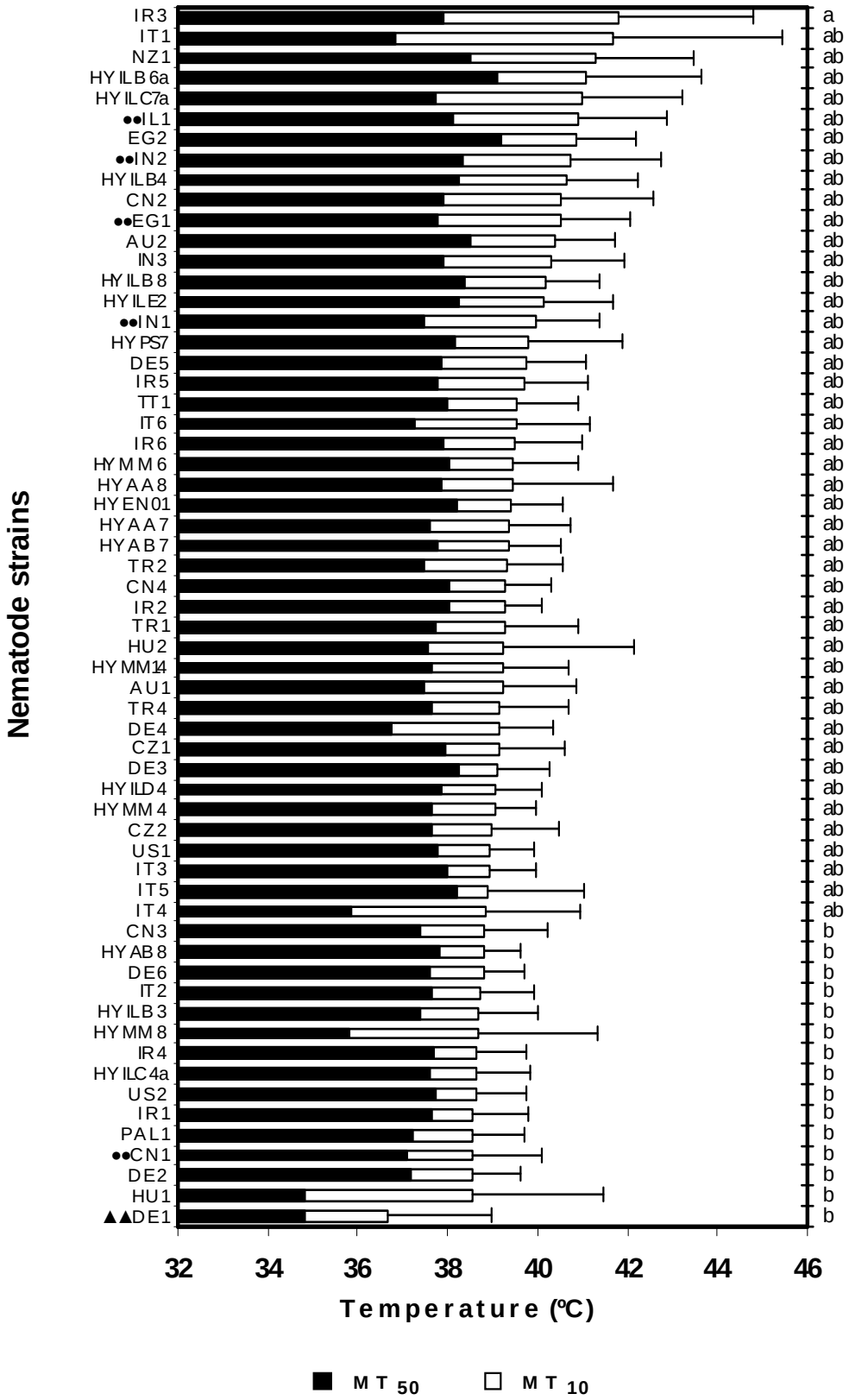
551
552
553

554 **Fig. 4**



555
556

557 **Fig. 5**


IR3 a
IT1 ab
NZ1 ab
HYILB6a ab
HYILC7a ab
●●IL1 ab
EG2 ab
●●IN2 ab
HYILB4 ab
CN2 ab
●●EG1 ab
AU2 ab
IN3 ab
HYILB8 ab
HYILE2 ab
●●IN1 ab
HYPS7 ab
DE5 ab
IR5 ab
TT1 ab
IT6 ab
IR6 ab
HYMM6 ab
HYAA8 ab
HYEN01 ab
HYAA7 ab
HYAB7 ab
TR2 ab
CN4 ab
IR2 ab
TR1 ab
HU2 ab
HYMM14 ab
AU1 ab
TR4 ab
DE4 ab
CZ1 ab
DE3 ab
HYILD4 ab
HYMM4 ab
CZ2 ab
US1 ab
IT3 ab
IT5 ab
IT4 ab
CN3 b
HYAB8 b
DE6 b
IT2 b
HYILB3 b
HYMM8 b
IR4 b
HYILC4a b
US2 b
IR1 b
PAL1 b
●●CN1 b
DE2 b
HU1 b
▲▲DE1 b
Nematode strains
32 34 36 38 40 42 44 46
Temperature (ºC)
MT50 MT10

558

Fig. 6

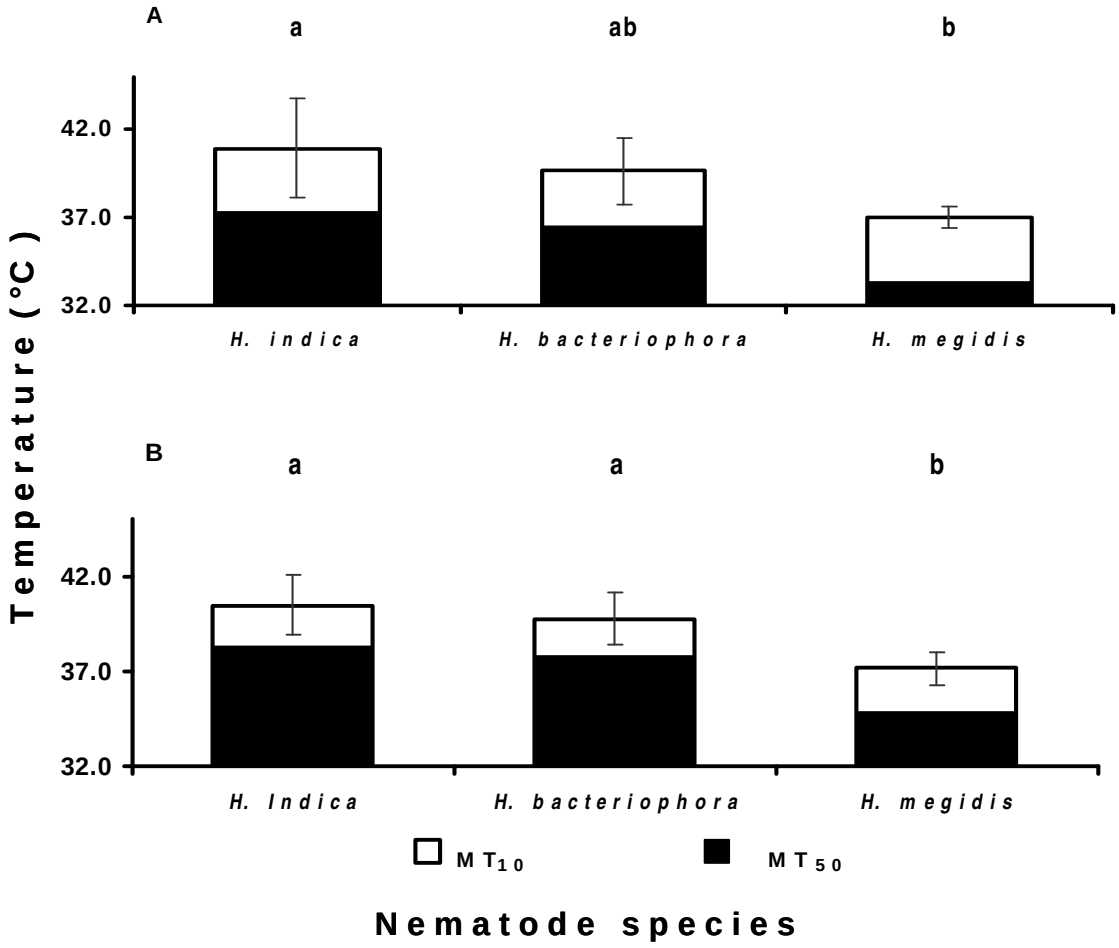
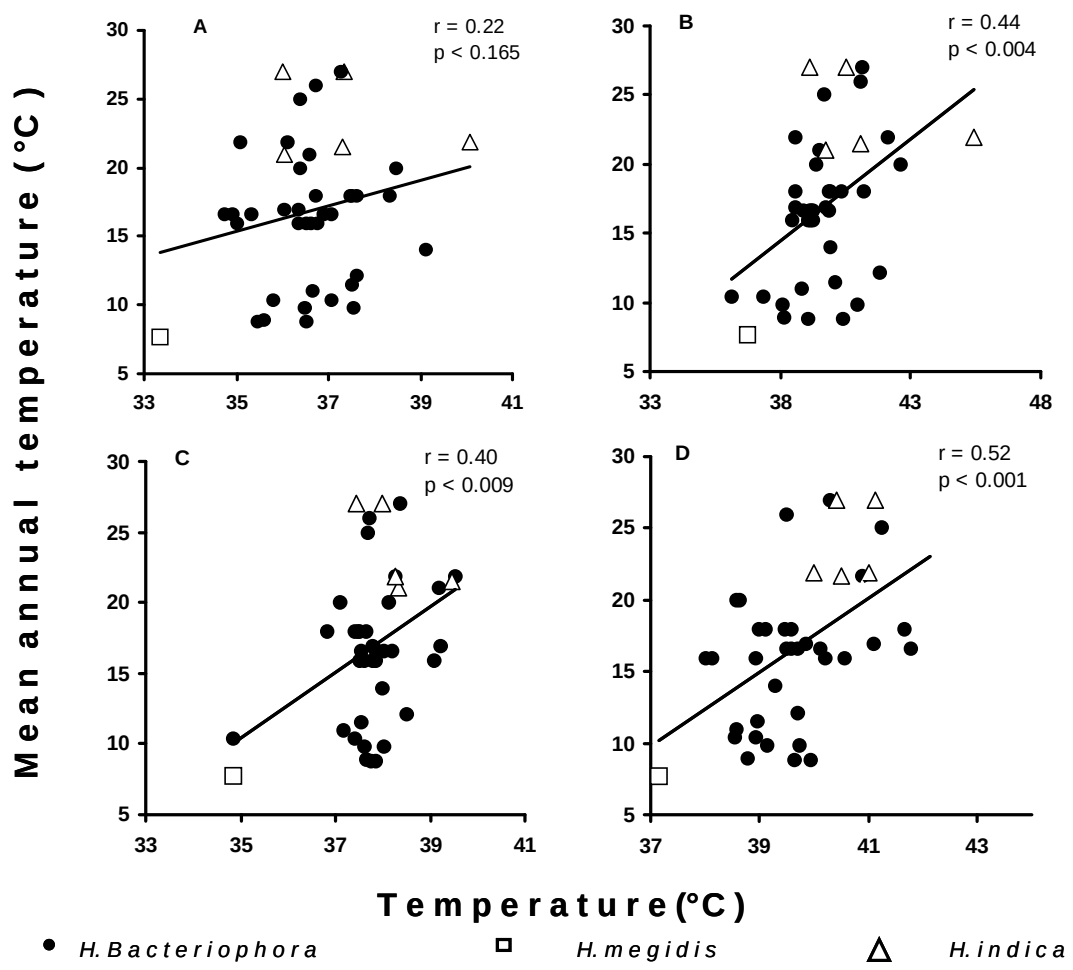

A
a
ab
b
42.0
37.0
32.0
H. indica
H. bacteriophora
H. megidis
B
a
a
b
42.0
37.0
32.0
H. Indica
H. bacteriophora
H. megidis
MT10
MT50
Temperature (°C)
Nematode species

Fig. 7


A
r = 0.22
p < 0.165
B
r = 0.44
p < 0.004
C
r = 0.40
p < 0.009
D
r = 0.52
p < 0.001
Mean annual temperature (°C)
Temperature (°C)
H. Bacteriophora
H. megidis
H. indica

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