

Orijinal araştırma (Original article)

Comparison of virulence of hybridized entomopathogenic nematode *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) strains and their parents¹

Hibrit entomopatojen nematod *Heterorhabditis bacteriophora* ırklarının ebeveynlerine göre etkinliklerinin karşılaştırılması

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Summary

Efficacy of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) species can be changed on targeted insect pests in different climatic zone. In this study, six different *H. bacteriophora* strains, which isolated in different cities [Antalya (H.b. 6), Çanakkale (H.b. 876), Kırklareli (H.b. 17), İzmir (H.b. HIZ) and Adana (H.b.10)] from Turkey, were hybridized and their virulence were determined on the last instar of *Galleria mellonella* (Lepidoptera: Pyralidae) at six different dosages. Male and female individuals of having lowest virulence were crossbred with other strain females and males *in vitro* conditions. Ten different strains were obtained by hybridization and their virulence was compared with their parent and each other on the last instar larvae of *G. mellonella*. The results showed that 70% of hybridized strains were more effective than their parents. The results indicated that male and female individuals have different effect on the virulence of the hybridized strains. The hybridized strains, which were more effective than their parents, were influenced from female with 42.9% and from male with 57.1%.

Key words: *Heterorhabditis bacteriophora*, entomopathogenic nematodes, virulence, hybridization, *in vitro*

Özet

Yüksek biyolojik mücadele potansiyeline sahip olan *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) türünün hedef alınan zararlı böcek üzerindeki etkinliği farklı iklim özelliklerine sahip olan bölgelerde değişiklik göstermektedir. Bu çalışmada; Antalya (H.b. 6), Çanakkale (H.b. 876), Kırklareli (H.b. 17), İzmir (H.b. HIZ), Şanlıurfa (H.b. HSU) ve Adana (H.b. 10) illerinden izole edilmiş olan 6 farklı *H. bacteriophora* izolatu kullanılmıştır. Bu izolatların etkinlikleri *Galleria mellonella* (Lepidoptera: Pyralidae)'nin son dönem larvaları üzerinde 6 farklı uygulama dozunda belirlenmiştir. En düşük etkinlik değerine sahip olan izolatın erkek ve dişi bireyleri ile denemelerde kullanılan diğer tüm ırkların erkek ve dişi bireyleri *in vitro* ortam koşullarında hibritlenerek 10 farklı ırk elde edilmiştir. Hibridizasyon sonucunda elde edilen ırkların *G. mellonella* larvaları üzerindeki etkinlikleri ebeveyn ırklar ile aynı şekilde belirlenmiştir. Çalışma sonunda ebeveyn ve hibrit ırklara ait olan etkinlik verileri karşılaştırılmış ve elde edilen 10 yeni hibrit ırkın %70'inin ebeveynlerine göre yüksek, %30'unun ise ebeveynlerine göre daha düşük değerinde etkinlik gösterdiği tespit edilmiştir. Ayrıca hibrit ırkların böcek üzerindeki etkinlik değeri üzerine erkek ve dişi bireylerin farklı etkiler gösterdiği de tespit edilmiştir. Ebeveynlerine göre yüksek etkinlik değerine sahip olan hibrit ırkların etkinlik değerlerinin %57.1'inin erkek, %42.9'unun ise dişi bireylerin etkisi altında kaldığı bulunmuştur.

Anahtar sözcükler: *Heterorhabditis bacteriophora*, entomopatojen nematod, etkinlik, hibridizasyon, *in vitro*

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Introduction

Heterorhabditis bacteriophora (Rhabditida: Heterorhabditidae) Poinar 1976, is an entomopathogenic nematode (EPNs) species used successfully in biological control against insect pests (Ehlers, 1996). This species is well known all over the world and were isolated from Turkey as well (Özer et. al., 1995; Susurluk et. al., 2003; Kepenekçi, 2002; Hazır et. al., 2003; Kepenekçi & Susurluk, 2003; Susurluk, 2006; Susurluk et. al., 2009, 2011). The 3rd juvenile stage of this nematode, which is named infective juvenile (IJ) also, is non-feeding and free living in soil. Furthermore it is symbiotically associated with bacterium, *Photorhabdus luminescens*, which is carried in the intestine of IJs at varying number ranging 200 from 2000 (Bird & Akhurst, 1983). IJs can survive until finding new hosts up to 8 months in soil (Ehlers, 1996). When they find a suitable host, they enter their hosts via natural opening such as mouth, anus, spiracles, or directly the cuticle (Bedding & Molyneux, 1982; Poinar, 1990; Wang & Gaugler, 1998). Then, IJs release their symbiotic bacterium cells into the haemocoel (Poinar et. al., 1977). IJs feed on bacterium cells and host tissue while the bacterium cells grow in the host. After penetration the host is killed by means of septicemia within 24-48 h (Akhurst, 1983; Forst & Neelson, 1996). Developing IJs of *H. bacteriophora* reach self-fertile hermaphrodites that are female phenotype in first generation. Then, they generate amphimictic males and females and IJs in second generation (Poinar, 1975; Strauch et. al., 1994). New adult generations develop if enough nutrients exist in haemocoel of insects. Otherwise they create IJs, which emerge into the soil where they seek out a new host (Kaya & Gaugler, 1993; Grewal et. al., 1994; Ehlers, 1996).

EPNs can be produced *in vivo* and *in vitro* (Friedman, 1990; Lunau et. al., 1993). White Trap method is a basic method for *in vivo* production (White, 1927; Flanders et. al., 1996; Kaya & Stock, 1997). Generally, greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) last instars larvae, which has high susceptibility to EPNs, are used in this method (Shapiro-Ilan & Gaugler, 2002). Another technique for production of EPNs is *in vitro* on solid or liquid cultures (Fridman et. al., 1990; Lunau et. al., 1993; Ehlers, 1996, 2001; Gaugler & Han, 2002). Isolations of symbiotic bacteria and nematode eggs are the most important points at EPN production for both the solid culture and liquid culture techniques.

Previous study showed that there were individuals, having different reactions in the population of the same EPN species (Gaugler et. al., 1989; Glazer et. al., 1991). *H. bacteriophora* is hermaphrodite in the first generation, which enables to save any character of *H. bacteriophora* structure. Female and male individuals, which are seen in the second generation, are fertile and their characters are pass down other generations. Thus, a feature can be transferred to *H. bacteriophora* structure by hybridization of females and males (Zioni et. al., 1992; Johnigk & Ehlers, 1999 a,b). Moreover, the feature can be protected through hermaphrodites. As a result, tolerance of *H. bacteriophora* is improved against extreme environmental conditions via a selective breeding, which helps obtaining the resistant strains (Strauch et. al. 2004; Ehlers et. al., 2005; Mukuka et. al., 2010 a,b,c,d Mukuka et. al., (2010 a, b) had some findings about hybrid vigor. Hybrid vigor and breakdown are usually ascribed to the average fitness of the hybrid offspring, although there will be individual genotypes within the recombinant hybrid offspring that do better or worse than the average (Edmands 1999).

The aim of this study was to compare the virulence of ten different *H. bacteriophora* strains obtained by hybridization with their parent and each other on the last instar larvae of *G. mellonella*.

Material and Methods

Isolations of *Heterorhabditis bacteriophora* and rearing of *Galleria mellonella* larvae

Six different *H. bacteriophora* strains, which were isolated from different cities of Turkey, were used in the experiments. *H. bacteriophora* H.b. 6, H.b. 17, H.b. 10, H.b. HIZ, H.b. HSU and H.b. 876 strains were isolated from Antalya, Kırklareli, Adana, İzmir, Şanlıurfa and Çanakkale, respectively. All of them were identified by molecular techniques (PCR-RFLP) (unpublished data).

The last instar larvae of greater wax moth, *G. mellonella*, were used in the all experiments because of being sensitive to EPNs. The larvae of the insect were reared in 1000 ml glass jars at $30\pm 2^\circ\text{C}$ on an artificial media according to Wiesner (1993).

Virulence tests of parent strains

G. mellonella last instars larvae were used in the laboratory tests to determine the virulence of nematode strains,. Virulence of all strains were tested on the last instars larvae at the six different dosages 5, 10, 20, 50, 75 and 100 IJs/Larva. The IJs were 2-week-old. The experiments were carried out in the 24 well plates (each well 1.6 cm diameter, 1.8 cm high). Each larva was placed at the bottom of each well and covered with 3 cm^3 of sterile sand (particle size: 300-400 μm), which had a water content of 3%. Each larvae was inoculated with different dose, which was applied in 300 μl of Ringer's solution (Susurluk et. al., 2001). The well plates were closed with parafilm and incubated at $25\pm 1.5^\circ\text{C}$ for 4 days. twenty larvae were used in an experiment to assess the virulence of a strain,.. After incubation, the larvae were removed from the sand and the larvae killed by IJs, were counted to evaluate of efficacy. The results were recorded as Lethal Dose [LD_{50} , LD_{90} and $\text{LD}_{\text{mean}}(\text{LD}_{50} + \text{LD}_{90})/2$] at the end of experiments. The experiment was repeated three times for each dose

Hybridization of *H. bacteriophora* strains on *in vitro* conditions

Hybridization processes contained the following steps:

Bacteria isolation

Five *G. mellonella* last instars larvae were infected with *H. bacteriophora* at the range of 100 IJs per larva as mentioned above (Susurluk et al., 2001, 2003). After infection, larvae were incubated $25\pm 1.5^\circ\text{C}$ for 24 h. At the end of the time, treated larvae were washed with Ringer's solutions and then sterilized with 70% ethanol for 5 minutes. Each sterilized larva was drilled with a sterile pin for the purpose of obtaining haemolymph where grow bacteria. A drop of haemolymph was taken using a loop and transferred on NBTA agar. Inoculated NBTA agar with bacteria was incubated at $25\pm 1.5^\circ\text{C}$ for 3 days. Then, a colony was taken by loop from colonized bacteria and transferred in the YS liquid medium. The YS medium was incubated at $25\pm 1.5^\circ\text{C}$ for 24h in orbital shaker at 200 rpm in the dark (Akhurst 1980). sterile glycerol was added to previously produced bacteria in YS medium at the range of 15% to storage of the bacteria, It was stocked at -20°C for using the next experiments.

Egg isolation:

Ten *G. mellonella* last instar larvae were inoculated with strains of *H. bacteriophora* as the range of 50 IJs each larva according to Susurluk et. al. (2001, 2003). Treated larvae were incubated at $25\pm 1.5^\circ\text{C}$ for 3 days. Then, infected larvae were dissected and hermaphrodite individuals were collected. Approximately 150 or 200 individuals were enough for successful egg isolation. Fertilized hermaphrodite individuals were transferred into Ringer's solutions a glass tube containing pieces of razor blade.. To separate eggs from hermaphrodites, glass tube was treated with vortex for a minute. Then, suspension of egg-Ringer's was centrifuged for 2 minutes at 3000 rpm. The eggs were got a sediment deep of centrifuge tube. Then, supernatant was removed and filled up with fresh Ringer's solution and centrifuged. This treatment was repeated until supernatant was clear. After this process, supernatant was removed and the sterilization solution (12% NaOCl, 0.5 ml; 4 M NaOH, 1.5 ml; distilled water, 10 ml) was added on the eggs. Tubes containing eggs were shaken for 4 minutes and centrifuged at 2000 rpm for 2 minutes. After centrifugation, the sterilization solutions were removed from the eggs and added YS medium. Centrifugation was repeated as described above. Eggs were transferred into the YS medium in the well plates after last centrifugation and then incubated $25\pm 1.5^\circ\text{C}$ for 2 days (Lunau et al., 1993).

Prepare of monoxenic nematode culture:

The YS medium was inoculated with *P. luminescens* that previously isolated and then incubated in the orbital shaker at 200 rpm and 25±1.5 °C in dark conditions for 24h. One or two drops were taken from grown bacteria in the YS and transferred to the WOUTS agar. The first stage juveniles, which had occurred hatching of eggs, were transferred to the WOUTS agar, being inoculated with the bacteria. Petri dishes were covered with parafilm and incubated at 25±1.5 °C in the dark. The experiment was repeated five times for each strains of *H. bacteriophora*. The petri dishes were checked daily for observation of IJs growing (Lunau et al., 1993).

Production of H. bacteriophora strains on in vitro liquid culture:

Symbiotic bacteria were prepared according to Akhurst (1980) for *in vitro* liquid culture production. A drop of bacteria from the YS medium was transferred to the BSA medium and this medium was incubated at 25±1.5 °C in orbital shaker at 200 rpm in the dark for 24 h. This treatment was applied for each strain and the control. On the other hand, new generation IJs were climbed up the lid of petri dishes two week after the monoxenic nematode culture process. The IJs were placed into BSA, being inoculated with bacteria, and incubated at 25±1.5 °C in orbital shaker at 200 rpm in the dark, as well (Lunau et. al., 1993; Ehlers et. al., 1998; Ehlers, 2001). Fertile male and female individuals were appeared 10 days after incubation. New generation IJs were obtained approximately 3 weeks after inoculation.

Hybridization of strains:

At the start of this study, virulence of parent strains was determined. H.b. 6 (Antalya) strain with lowest virulence was used as standard negative control. Male and female individuals of H.b. 6 (Antalya) were crossbred with females or males of other strains on *in vitro* conditions.

Egg isolation, preparation of monoxenic nematode culture, production in BSA of H.b. 6, H.b. 17, H.b. 10, H.b. HIZ, H.b. HSU and H.b. 876 strains were carried out for hybridization as described above. 10th day of EPN production in BSA liquid medium, fertile female and male individuals were arisen. As from 10th day, BSA medium containing strains were opened and removed approximately 2 ml and transferred into Petri dishes in the sterile conditions. This application was performed for each strain. All strains were examined under the inverted microscope individually. After microscopic examination, male or female individuals of H.b. 6 were selected and transferred the WOUTS agar, which had been inoculated with *P. luminescens* and incubated at 25±1.5 °C for 2 days. Female or males of any strain was transferred to same conditions with H.b. 6 for crossbreeding (Table 1). Petri dishes (6 cm diameter) were covered with parafilm and incubated at 25±1.5 °C for 2 weeks. Petri dishes were controlled daily whether there was any mating between females and males.. Ten males and females, total twenty individuals were used for each strain in the experiment. Additionally, five unfertilized females were transferred to the WOUTS agar for each strain for the control. The treatment was repeated three times for all strains (Lunau et. al., 1993; Iraki et. al., 2000; Susurluk et. al., 2001; Ehlers et. al., 2001; Mukuka et. al., 2010 a,b,c,d).

Table1. Parent and Hybrid Strains

Parent Strains	Hybrid Strains
H.b. 6 (Antalya) ♀ X H.b. 876 (Çanakkale) ♂	H.b. A
H.b. 6 (Antalya) ♂ X H.b. 876 (Çanakkale) ♀	H.b. B
H.b. 6 (Antalya) ♀ X H.b. 17 (Kırklareli) ♂	H.b. C
H.b. 6 (Antalya) ♂ X H.b. 17 (Kırklareli) ♀	H.b. D
H.b. 6 (Antalya) ♀ X H.b. HIZ (İzmir) ♂	H.b. E
H.b. 6 (Antalya) ♂ X H.b. HIZ (İzmir) ♀	H.b. F
H.b. 6 (Antalya) ♀ X H.b. HSU (Şanlıurfa) ♂	H.b. G
H.b. 6 (Antalya) ♂ X H.b. HSU (Şanlıurfa) ♀	H.b. H
H.b. 6 (Antalya) ♀ X H.b. 10 (Adana) ♂	H.b. K
H.b. 6 (Antalya) ♂ X H.b. 10 (Adana) ♀	H.b. L

The new generation IJs, which composed of hybridization, were climbed up the lid of petri dishes. They were harvested from Petri dishes and stored in the Ringer's solution at 4-8°C (Kaya & Stock, 1997).

Virulence tests of hybrid strains

Virulence of hybrid strains was determined with the same method as parent strains. The results were recorded as Lethal Dose (LD₅₀, LD₉₀ and LD_{mean}) at the end of experiments.

Statistical analysis

Data belong to virulence value of parents and hybridized strains were analyzed by one-way ANOVA and followed by Least Significant Deviance (LSD) test for post-hoc comparisons using of JMP® 7.0. The minimum level of significance was taken as P<0.05. LD₅₀ and LD₉₀ values of parents and hybridized strains were calculated with probit analysis using program of BioStat ©2010.

Results

Virulence of parent strains

H.b. 876 was the most effective strain against the last instars larvae of *G. mellonella* (LD₅₀=0.58, LD₉₀=21.43, LD_{mean}=11.01). The least effective strain was H.b. 6 (LD₅₀=5.20, LD₉₀=28.89, LD_{mean}=17.05), which was used as a standard for obtaining negative control. For this reason, H.b. 6 was crossbred with the other strains. The results are shown on Table 2 for other strains.

Table 2. LD₅₀, LD₉₀ and LD_{mean} values of parent strains on *Galleria mellonella* larvae

Parent Strains	LD ₅₀	LD ₉₀	LD _{mean}
H.b. 6 (Antalya)	5.20	28.89	17.05
H.b. 876 (Çanakkale)	0.58	21.43	11.01
H.b. 17 (Kırklareli)	4.98	27.56	16.27
H.b. HIZ (İzmir)	5.06	24.26	14.66
H.b. HSU (Şanlıurfa)	5.27	26.53	15.90
H.b. 10 (Adana)	5.42	26.56	15.99

Virulence of strains was increased with increasing application dose. Parent strains caused mortality at the range of 13.33% and 60% against the larvae with dose of 5 IJs/Larva. At the dose of 75 IJs / larva, all strains caused 100% mortality. All results are presented in Table 3.

Table 3. Percent mortalities that parent strains caused on *Galleria mellonella* larvae at different doses.

Strains	Insect Mortality (%)				
	5 IJs/Larva (F=11.62; df=5, 12; P=0.0003)	10 IJs/Larva (F=14.04; df=5, 12; P=0.0001)	20 IJs/Larva (F=1.62; df=5, 12; P=0.2285)	50 IJs/Larva (F=3.39; df=5, 12; P=0.0386)	75 IJs/Larva (F=0; df=5, 12; P=1)
H.b. 6	43.33 bc	78.33 a	91.67 ab	91.67 ab	100.00 a
H.b. 876	60.00 a	83.33 a	100.00 a	100.00 a	100.00 a
H.b. 17	13.33 d	30.00 c	88.33 ab	100.00 a	100.00 a
H.b. HIZ	50.00 ab	85.00 a	83.33 b	100.00 a	100.00 a
H.b. HSU	40.00 bc	83.33 a	91.67 ab	91.67 ab	100.00 a
H.b. 10	31.67 c	50.00 b	88.33 ab	88.33 b	100.00 a

Virulence of hybrid strains

The most and the least effective hybrid strains were H.b. A (LD₅₀=0.0096, LD₉₀=20.6637) and H.b. B (LD₅₀=11.2499, LD₉₀=39.0115). The LD₅₀ values of the all hybrid strains were found below 1 except H.b. B and H.b. L, those of which were detected between ca. 20 and 41. Results are summarized in Table 4.

Table 4. LD₅₀, LD₉₀ and LD_{mean} values of hybrid strains

Hybrid Strains	LD ₅₀	LD ₉₀	LD _{mean}
H.b. A (H.b. 6 ♀ x H.b. 876 ♂)	0.00960	20.6637	10.33665
H.b. B (H.b. 6 ♂ x H.b. 876 ♀)	10.2499	37.0115	24.13070
H.b. C (H.b. 6 ♀ x H.b. 17 ♂)	0.54550	26.1796	13.36255
H.b. D (H.b. 6 ♂ x H.b. 17 ♀)	0.47680	33.4722	16.97450
H.b. E (H.b. 6 ♀ x H.b. HIZ ♂)	0.07880	20.7189	10.39885
H.b. F (H.b. 6 ♂ x H.b. HIZ ♀)	0.23870	21.9252	11.08195
H.b. G (H.b. 6 ♀ x H.b. HSU ♂)	0.15470	29.0246	14.58965
H.b. H (H.b. 6 ♂ x H.b. HSU ♀)	0.40400	29.3109	14.85745
H.b. K (H.b. 6 ♀ x H.b. 10 ♂)	0.38020	24.9347	12.65745
H.b. L (H.b. 6 ♂ x H.b. 10 ♀)	7.32090	41.6751	24.49800

Virulence of hybrid strains varied from 26.67% to 83.3% at the application dose of 5 IJs/Larva. The most effective strain was H.b. E. Besides that, strains of H.b. H and H.b. K and H.b. A were not significantly different from H.b. E at the dose of 5 IJs/Larva. The least effective strain was H.b. B, and followed by H.b. L and H.b. G which were statistically at the same group as H.b. B., at the same application dose. The most virulent dose was 75 IJs/larva and all insect larvae were killed by all strains (Table 5).

Table 5. Virulence values of hybrid strains at all applied doses

Strains	Insect Mortality (%)				
	5 IJ/Larva (F=9.6129; df=9, 20; P=0.0001)	10 IJ/Larva (F=11.9352; df=9, 20; P=0.0001)	20 IJ/Larva (F=11.2540; df=9, 20; P=0.0001)	50 IJ/Larva (F=3.3472; df=9, 20; P=0.0117)	75 IJ/Larva (F=0; df=9, 20; P=1)
H.b. A	70.00 abc	98.33 a	98.33 a	100.00 a	100.00 a
H.b. B	26.67 e	60.00 e	65.00 d	98.33 ab	100.00 a
H.b. C	56.67 bcd	73.33 cd	90.00 abc	98.33 ab	100.00 a
H.b. D	55.00 cd	68.33 de	88.33 bc	95.00 bc	100.00 a
H.b. E	83.33 a	90.00 ab	96.67 ab	100.00 a	100.00 a
H.b. F	65.00 bc	81.67 bc	91.67 abc	100.00 a	100.00 a
H.b. G	40.00 de	88.33 ab	91.67 abc	100.00 a	100.00 a
H.b. H	73.33 ab	85.00 b	86.67 c	96.67 ab	100.00 a
H.b. K	71.67 abc	80.00 bc	93.33 abc	98.33 ab	100.00 a

Comparison of virulence values of parent and hybrid strains

The mean mortality of *G. mellonella* was between 13.33 and 83.33% at the dose of 5 IJs/Larva. The results showed that the most effective hybrid strain was H.b. E (83.33%) followed by H.b. H (73.33%). All hybrid strains were more effective than their parents were except H.b. B (LD₅₀=11.2499, LD₉₀=37.0115) and H.b. L (LD₅₀=7.3209, LD₉₀=41.6751) (F=14.3843; df=15, 32; P=0.0001) (Figure 1).

The results indicated that seven of hybridized strains of *H. bacteriophora* were more effective than their parents (Figure 1). Female and male individuals of parents had different effects on virulence of hybrid strains. Male parents were more effective than females; 57.1 % of more effective hybrid strains and 42.9 % of those were originated from males and females, respectively (Table 3 and 4).

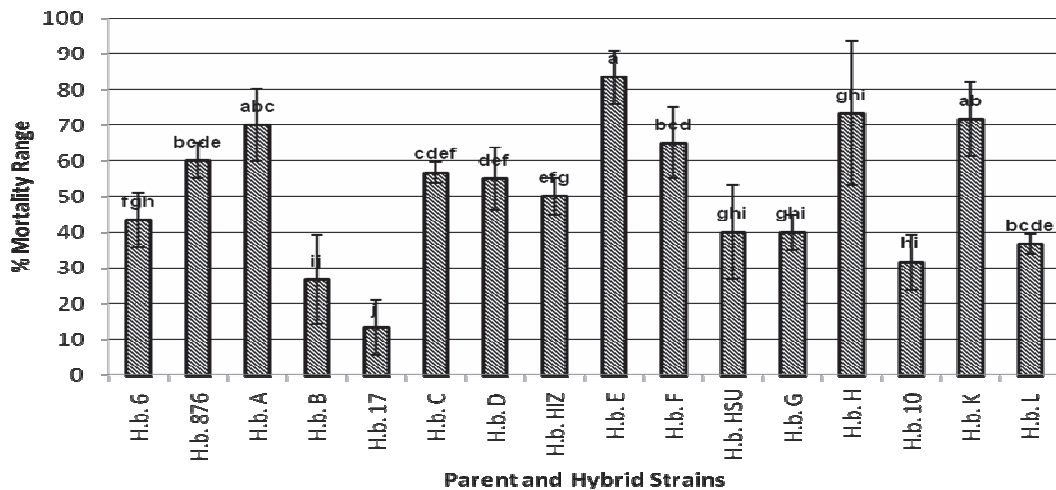


Figure 1. Virulence of parents and hybrids of *H. bacteriophora* at the applied dose of 5 IJ/Larva.

Discussion

Hybridization has been used for improvement of target organisms to get better individuals of the organism. By this means, more productive products or more capable living material can be obtained to serve several areas. Biological control agents are successfully used in some agricultural areas. However, they need to be improved for being more effective. One of the most effective tools is hybridization of them. Entomopathogenic nematodes (EPNs), which are used against various insect pests, are recently hybridized to get more tolerant strains to some environmental conditions that can have major effects on establishment and persistence of EPNs (Susurluk & Ehlers, 2008). Generally, heat and desiccation tolerant strains of EPNs were improved by hybridization (Strauch et. al., 2004; Mukuka et. al., 2010 c, d; Susurluk et. al., 2013 a, b; Ulu & Susurluk, 2014). Very limited fitness/hybrid vigor studies were carried out (Mukuka et. al., 2010 b). In the present study, six strains of *H. bacteriophora*, which were isolated from different regions of Turkey, were hybridized with each other and their virulence were compared with their parents. The aim of the study was to obtain more effective hybrid strains of *H. bacteriophora*, which are used several countries against several insects. This study is also important for detecting if effective features of the species can be transferred by heritability or not. Turkish strains were firstly hybridized to obtain more effective strains. Hybridization of EPNs was firstly advised by Gaugler (1986), he indicated that the stress factors on *H. bacteriophora*, such as heat and desiccation, were reduced via hybridization of strains, which adapted different climatic zone. He showed that producing of tolerant strains against desiccation and heat were possible via crossbreeding,. Crossbreeding method was used for hybridization; moreover this method is generally used for identification of unknown species (Susurluk et al., 2001; Strauch et. al., 2004; Mukuka et. al., 2010 c, d; Susurluk et. al., 2013 a, b).

In the present study, this method was successfully applied in *in vitro* conditions. Mukuka et al. (2010 b) stated that some heat and desiccation tolerant hybrid strains had more virulence than hybrid commercial strain EN01 of *H. bacteriophora*, but some of them had lower effect. However, they were not compared the hybrid strains with their parents for the virulence. They expressed that the virulence depends on the symbiotic bacterium (Han and Ehlers, 2000), so that the bacterium that originated from different females can change virulence capabilities. In the present study, the same bacterium was used in all cross breeding experiment. Therefore, the differences of virulence were not related with the symbiotic bacterium.

As an important finding of the study, female and males of parents play different roles on heritability to virulence of hybrid strains. Males are more effective than females and this is the first report. However, it is now unknown the reason of the difference that depends on the sexuality of parent. After this result, it is needed to detect whether there is also different virulence between female and male IJs or not.

Increasing of doses triggers positively efficacy of EPNs, the present results confirmed this judgment. There was a correlation between increasing of doses and mortality. Several studies were accordance with the present conclusion (Susurluk et al., 2001; Kepenekçi, 2004).

Some studies on hybrid vigor of some animal and plants indicated that if hybrid vigor results in higher densities, larger body sizes, or more rapid growth of organisms in hybrid populations, the impacts of hybridization will almost certainly extend to other members of the ecological community (Benjamin et al. 2007). Similarly, the whole study indicated that hybrid *H. bacteriophora* with high effective capacity can be obtained by the using hybridization. By this means, success and sustainability of EPNs in biological control can be increased. This study is a milestone in contributing to the improvement of more effective *H. bacteriophora*. Thus, new hybrid Turkish strains of *H. bacteriophora* might be successfully used against especially soil dwelling insect pests in Turkey.

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