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## Genomics of Entomopathogenic Nematodes and Implications for Pest Control

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### Abstract

Entomopathogenic nematodes (EPNs) have been used in biological control but improvement is needed to realize their full potential for broader application in agriculture. Some improvements have been gained through selective breeding and the isolation of additional species and populations. Having genomic sequences for at least six EPNs opens the possibility of genetic improvement, either by facilitating the selection of candidate genes for hypothesis-driven studies of gene-trait relationships or by genomics-assisted breeding for desirable traits. However, the genomic data will be of limited use without a more mechanistic understanding of the genes underlying traits important for biological control. Additionally, molecular tools are required to fully translate the genomic resources into further functional studies and better biological control.

### Keywords

Biological control; entomopathogenic nematodes; genomics

### Entomopathogenic nematodes in Biological Control

Global annual crop loss due to herbivory by pests is 32.1% [1]. Farmers and researchers have applied many methods to reduce this crop loss, one of which is the application of specialized insect-parasitic nematodes called entomopathogenic nematodes (EPNs). EPNs differ from other insect-parasitic nematodes in two meaningful ways: 1) EPNs associate with symbiotic bacteria to facilitate pathogenesis and 2) they rapidly kill their hosts, usually within seventy-two hours after infection [2–4] (Figure 1). Entomopathogenic species within the genera *Heterorhabditis* and *Steinernema* are the most extensively studied and most often used in biological control [4–7]. EPNs are highly pathogenic and are used as biological control agents of numerous insect pests. They have been commercialized on several continents and are used in large-scale agriculture and in individual home gardens.

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Despite their promise as biological control agents, lack of consistent efficacy in the field has prevented these nematodes from being more widely used. Researchers have worked on improving efficacy against arthropod pests under field conditions for decades, employing two main strategies: 1) artificial selection and 2) genetic improvement via mutagenesis or other molecular methods (Figure 2). Artificial selection is enhanced by continued collection of new EPN species and/or populations that are adapted to certain environmental conditions and pests (Figure 2). Occasionally, locally adapted EPNs provide superior control when compared to non-native species or populations [8–10]. Many new EPN isolates have been identified, which may lead to increased genetic variation and to the development of new nematode strains [11]. Isolation and/or breeding of EPNs for improved insect pest suppression relies on the identification and manipulation of certain traits [12–14]. These traits include but are not limited to increased tolerance to temperature, desiccation, and ultraviolet light, as well as increased or modified host-seeking ability, virulence, and resistance to nematicides (Figure 3). Improving these traits in EPNs has been done primarily by classical genetic techniques such as breeding and selection. However, traits improved this way are not always stable and individual trait gains can sometime be lost once the selective pressure is removed [13]. Moreover, selection of some traits can lead to inadvertent reduction of others or of overall fitness [12, 15, 16]. Inbreeding depression or other means of fitness loss during EPN-mass production or as a result of continuous laboratory culture are also concerns [17, 18]. The second major strategy to improve EPN field efficacy is to use modern genetic and molecular tools. These tools have not yet been fully employed to improve EPN field efficacy in biological control [12–14]. Progress has been made toward tool development and technology transfer from the *Caenorhabditis elegans* research community, but the application of modern techniques to improve EPN efficacy is still in its infancy. EPNs are model nematode parasites for studies of ecology [19, 20], behaviour [21–23], neurobiology [24], and host-parasite interactions [25, 26]. Manipulation of the bacterial partner is a strategy that may yield improvements in field efficacy related traits, but here we focus on the nematodes and the recently sequenced genomes [15, 16]. The availability of multiple EPN genomes should facilitate new and powerful studies of EPN biology and will be used to decipher the function of individual genes in parasitism [27, 28]. Here we discuss the implications that the recently available EPN genomes will have on their efficacy as biological control agents.

## Traits Important for Biological Control and Their Improvement

Traits important for biological control can be grouped into three main categories: infectivity, persistence, and storage stability [29] (Figure 3). Infectivity refers to the characteristics involved with finding, infecting and killing a target host. Persistence refers to traits that increase survival after application in the field such as temperature, desiccation, and UV tolerance (Figure 3). Storage stability of EPNs involves traits that increase the shelf life necessary for distribution of EPNs. Improving these traits and many others have the potential for ultimately increasing field efficacy.

In order for EPNs to be effective in biological control they must be able to find and kill insect hosts. Thus attempts to increase and modify host-seeking behavior have been popular. Host-seeking has been shown to be a highly heritable trait, which can be enhanced through

selective breeding efforts for species such as *Steinernema feltiae* [15] and *Steinernema carpocapsae* [16, 30]. The research done to enhance host-seeking traits has relied solely on selective breeding and the genes that are implicated in these processes are unknown. Host seeking improvements were correlated with an increase in overall fitness [15, 16]. Unfortunately, the trade-off is a reduction in desiccation tolerance, affecting storage stability [15, 16]. The use of new genomic data may reveal how the genes controlling these traits are genetically linked and provide new strategies of enhancing these traits without compromising others.

Persistence is as important as infectivity for EPN efficacy. Desiccation tolerance is important for EPN persistence and production. EPN species that forage near the soil surface tend to have better desiccation tolerance [31]. Desiccation can induce EPN quiescence, which leads to a longer shelf life and may also contribute to their longevity in the soil [31]. Storage stability is essential for EPN longevity in the soil and in commercial production and distribution. Artificial selection and hybridization can enhance desiccation tolerance [32–34], but removal of selection pressure ultimately results in loss of the desired traits [32, 33]. Deep knowledge of EPN genomes [27, 28], especially the genetic regulatory networks controlling the traits, coupled with improvements in genetic engineering for EPNs may provide new means to stabilize enhanced traits.

Among the traits important for persistence, heat tolerance has been particularly well studied. Increased heat tolerance allows EPNs to be applied in regions that experience high levels of heat during certain seasons. The introduction of heat shock gene *hsp70A* from *C. elegans* to *Heterorhabditis bacteriophora*, was the first successful attempt at making transgenic EPNs [35]. The incorporation of this gene led to a significant increase in resistance to heat stress [36]. Unfortunately, these transgenic EPNs did not exhibit enhanced persistence in the field [37, 38]. This example highlights the necessity of having a comprehensive understanding of gene functions related to fitness and field efficacy. A variety of stress-response genes in the insulin/IGF-1 signaling pathway [27] and others are promising candidates for improving field efficacy of EPNs. Access to genomic information makes it feasible to study molecular mechanisms using techniques including genetic transformation and RNAi.

In *H. bacteriophora*, heat tolerance is highly heritable and can be improved through selective breeding. However, this is not the case for all EPNs [39, 40]. Additionally, selective breeding for heat tolerance can result in serious deterioration of other beneficial characteristics, such as host seeking, host-penetration, virulence, longevity, and reproduction potential [41]. With increased understanding of the genetic basis of heat tolerance, researchers may be better able to design strategies for such improvements. The recently available EPN genomes may help in these efforts.

There are several caveats to acknowledge. One caveat is that enhancing desired characteristics may not directly translate into improved performance in the field as was seen in the *hsp70A* example [38]. Little is known about how these genes function when an EPN is released in the field. The nuances found in these dynamic environments are difficult to replicate in the lab. Another caveat is that the methods of enhancement need to be further developed in this system. Genetic transformation techniques in EPNs are still in their

infancy and further research with these techniques will eventually yield more consistent and effective results.

## The Genome of *Heterorhabditis bacteriophora*

The genome sequence of *H. bacteriophora* revealed numerous genes putatively implicated in parasitism and survival that could be manipulated for field applications [27]. It has also raised many questions since the 77 Mb genome contains more than 10 000 genes encoding proteins of unknown function (Table 1). The obligate association of *H. bacteriophora* with the bacterium *Photorhabdus luminescens* has shaped the architecture and content of the nematode genome as it relies on the bacteria for nutrient acquisition and metabolism [42]. The bacteria also serve as a means by which the insect's immune response is overcome [4, 27, 42]. *P. luminescens* produces an arsenal of enzymes including proteases to overcome host immunity, degrade host tissues and make them available for the developing nematodes. Further, the bacteria prevent opportunistic fungi and other bacteria from making use of the nutrient-rich insect cadaver. Certain species of EPNs such as *H. bacteriophora* rely on bacteria to overcome host immunity and kill the host, whereas other EPNs such as *Steinernema carpocapsae* are lethal even without their bacteria and therefore may be reliant on the bacteria for nutrient acquisition or sequestration of host resources from opportunistic soil microbes [43, 44]. However, it is the nematode that must locate hosts, gain entry into the hemolymph, and persist in the soil until a host is found, leaving plenty of room for genetic improvements to enhance field efficacy.

For example, in *C. elegans* dauers (analogous to steinernematid infective juveniles), the Insulin/IGF-1 signalling pathways serve as regulators for development [45]. The roles of these pathways include development of dauer stage and adult stage, longevity, stress resistance, and even innate immunity. The genes and proteins of these signalling pathways are thought to perform similar functions in *H. bacteriophora* [45]. The 19 genes of the insulin/IGF-1 signalling pathway are conserved in *H. bacteriophora* [27] and could be potential candidates for genetic manipulation to enhance longevity and/or stress resistance leading to better field efficacy (Figure 3).

Another potentially important gene family for enhancement of *H. bacteriophora* is the G protein-coupled receptor (GPCR) family. There are at least 82 predicted GPCRs in the *H. bacteriophora* genome [27] (Table 1). GPCRs are important in field efficacy because some of them are sensory receptors functioning in olfaction and host-seeking behaviour [46–48]. As noted above, host seeking is a highly heritable trait and has been temporarily improved through selective breeding [16, 30]. GPCR abundance and diversity could be linked to the niche inhabited by a nematode. In the case of EPNs, putative olfactory receptors could be used to enhance host seeking or potentially adjust host specificity. Increasing our knowledge of how GPCRs are used in host-seeking behaviour could be critical to improving or altering the host seeking abilities of EPNs, thus influencing field efficacy.

Genes that function in the symbiotic association between EPNs and the insect-pathogenic bacteria they carry could be used to enhance the biological control potential of EPNs. *H. bacteriophora* seems to have a reduced or modified immune response compared to *C.*

*elegans* [49]. It has far fewer C-type lectin domain-containing proteins (9 in *H. bacteriophora* compared to 133 in *C. elegans*), which function in the immune response of *C. elegans* to bacterial infection [49, 50]. This may be related to the association between *H. bacteriophora* and *P. luminescens* [51]. However, it is not yet known to what extent environmental bacterial infection might impair the efficacy of EPNs as biocontrol agents and more research needs to be done on this subject. If it is demonstrated that bacterial infection diminishes EPNs field efficacy against insect pests, this could be another area worth investigating further.

EPN secreted proteases are known to influence penetration of the nematode into the host hemolymph [52], tissue degradation of insect hosts [53], as well as immune suppression [54], and could be used to increase the field efficacy of EPNs in biocontrol. *H. bacteriophora* has fewer than 30 predicted protease and protease inhibitors in its secretome [27] (Table 1). This may reflect the nematode's reliance on *P. luminescens* for immune suppression and tissue degradation of the insect host. It is possible that host-killing by EPNs could be improved simply by adding additional copies of genes already present, similar to the transgenic inclusion of multiple endogenous cuticle-degrading proteases in entomopathogenic fungi [55].

The genomic sequence of *H. bacteriophora* provides a long list of candidate genes that could be used to improve infectivity and/or survival and this list will be refined as our understanding of the underlying biology increases. The successful application of tools from *C. elegans* (e.g. transformations and RNAi) [56] in *H. bacteriophora* coupled with this archive of genetic information is expected to lead to significant advances in the application of molecular genetics to improve the field efficacy of EPNs in biological control.

## The Genome of *Steinernema carpocapsae*

The genomes of *Steinernema carpocapsae* and four congeners (*S. feltiae*, *S. glaseri*, *S. monticolum*, and *S. scapterisci*) have recently been sequenced and annotated. Analyses of these genomes revealed numerous genes that could be involved in parasitism by EPNs and are candidates for use in programs to improve traits for biological control [28]. Similar to what has been found in the *H. bacteriophora* genome, more than 10 000 predicted proteins (~37% of the predicted proteome) seem to have no orthologues in other animals or even other nematodes [28]. Studying the function of these orphan proteins could reveal genes important for infection or survival and persistence and thus be useful to future transgenic endeavours in EPNs. Comparing sequenced EPN genomes (Table 1) confirms that they are similar in size but differ considerably in nucleotide prevalence (G+C content), which may affect the application of recombinant DNA techniques for genetic enhancement. Gene expression and regulation are affected by codon usage preferences [57], which has implications for technology transfer from *C. elegans* to the EPNs [56], with techniques developed in *C. elegans* potentially being more easily applied to *H. bacteriophora* due to their closer ancestry and similar nucleotide prevalence (Table 1) [27, 28, 58]. This needs to be further explored experimentally as there are not many reports of molecular techniques developed in *C. elegans* being applied to EPNs.

In contrast to the *H. bacteriophora* genome, steinernematids have a large variety of predicted proteases and protease inhibitors with signal peptides (Table 1). A multi-genome comparison revealed *Steinernema*-specific expansions of serine and metalloproteases [28]. Proteases and protease inhibitors are an important group of proteins for investigation in future selection and recombinant studies since they are known to be important in invasion and host-killing for *Steinernema* [52, 54]. Proteases in steinernematids have been shown to play an important role in suppressing insect host immunity as well as tissue degradation [54, 59–61]. There are functional studies showing that protease inhibitors play a role in nematode evasion of host immunity [62, 63], and genome analysis revealed that several families of protease inhibitors are expanded in steinernematids. One provocative possibility is that host range and specificity of EPNs may be influenced by their repertoire of secreted products and that using genetic transformation, the host range and/or specificity could be altered by the addition or removal of certain secreted products from the secretome. Not enough is known about the evolution of insect immunity, but as more genomes are being studied it seems that insect immunity could differ dramatically between orders and that niche partitioning among EPNs could be based on individual species' abilities to overcome or avoid the immune response of certain hosts [64, 65].

Fatty acid- and retinol-binding (FAR) proteins are another interesting gene family that was expanded in the genomes of steinernematids [28]. FAR proteins are thought to play a key role in parasitism by functioning in the sequestration of host retinoids as well as by contributing to immune evasion or suppression, though their exact functional role is not well understood [66, 67]. FARs seem to be involved in nematode parasitism of animals, insects, and plants [67–69], which makes understanding their mechanistic function important for both biocontrol as well as disease treatment and prevention.

The availability of genomic sequence from multiples species of *Steinernema* provides many candidate genes and gene families that could be used to improve infectivity and/or survival. It also highlights the importance of more mechanistic studies of EPN biology and the need for molecular tools to be more commonly applied in EPN research [56].

## Methods for Trait Improvement

As mentioned above, artificial selection and genetic engineering are the two main options for trait improvement in EPNs (Figure 2). Artificial selection does not require an understanding of genetic mechanisms underlying selected traits. Having been done for thousands of years with crops and livestock, individuals with certain traits are selected and crossed. This generates new cultivars/breeds with improved or desired traits. We can now employ genomic tools to understand what genetic changes introduced by domestication and artificial selection are actually behind the selected traits. This knowledge, combined with genetic engineering has resulted in more efficient production of enhanced traits or novel combinations in many systems [70] (Figure 2).

There are several tools and traits that will aid in the effort of enhancing EPNs for better field efficacy: (1) advanced genomic tools to identify the genes underlying desired traits; (2) genetic tools that can be applied to modify genes; (3) short generation time of EPNs and



their ability to be cultured *in vitro* and *in vivo*; and (4) a large collection of EPN species and strains with rich genetic diversity to select from (Figure 2).

The first step in genomics-assisted breeding of EPNs is the identification of desired traits and the species or strains with those traits. For a single trait, a species exhibiting the desired characteristic should be utilized. For example, *S. monticolum* is described as a cold-adapted EPN and may be a good species for studying cold tolerance or developing cold-adapted strains [71]. Genomic tools can be used to identify trait-associated genes or DNA markers, which in turn can facilitate the identification of genetically useful strains (Figure 2). It is important to ensure however, that the traits have been appropriately analyzed for their effect on field efficacy. Analysis should include the effect of these traits in the target field environment and not just in laboratory conditions.

Once traits have been identified, the next step is crossbreeding and selection. Similar to traditional breeding, strains of EPNs bearing desirable traits can be crossed. Over several generations the progeny should exhibit improvement compared to the founding population. Genomic information such as DNA markers can be used to evaluate the molecular effects of selection. The combined use of traditional breeding and genomic tools may shorten the time required for obtaining superior lineages of EPNs.

The third step is confirmation of trait retention, and improved field efficacy. This involves both lab and field-tests to confirm that the new progenitor strains perform better than the founding population. If the new populations prove to be superior they can be further propagated and used. Multiple selected strains with improved traits (as represented by DNA markers) but with variable genetic backgrounds should be maintained and inter-crossed to prevent trait deterioration [17, 18].

In addition to genomics-assisted breeding, genome sequences of EPNs can also be used for direct genetic modification, which may include mutagenesis, transgenesis, and targeted gene modification. Mutagenesis might generate novel genetic variations, yielding more effective genes than those that exist. One common practice is to do random mutagenesis followed by selection [72]. This can accelerate the selection process, thereby allowing for the analysis of larger ranges of genetic variation than are found in nature. Successful gains in desired traits may then be useful themselves or in combination with other techniques such as transgenesis. Transgenes could be derived from the same species (intraspecific), different species (interspecific), or even non-nematode organisms. Because releasing transgenic organisms into the environment remains controversial, transgenic nematodes may not be the first option for EPN trait improvement. One alternative is utilization of CRISPR/Cas9, a targeted gene editing procedure allowing for direct changing of specific alleles. CRISPR-mediated gene targeting can generate defined modifications in specific genes that mimic natural alleles [73]. Genetically modified EPNs generated by this process are more likely to be publically accepted since the final strains only contain modified alleles instead of genes from other organisms. Of course, the prerequisite of successful CRISPR-mediated gene targeting is the identification of genes that control the traits under selection.



Along with the advanced bioengineering tools and the availability of genomic information, it is essential to remember the importance of genetic diversity in EPN improvement. One reason is that large genetic diversity is an invaluable natural resource to select for useful traits and the underlying genes. Another reason is that one may need to employ integrated biocontrol using a collection of EPN strains and/or species to better control multiple insect pests in one application, rather than trying to develop a magic bullet.

## Concluding Remarks

The availability of the genomic sequence data and putative proteomes provides a large number of genes that could be useful in increasing the infectivity of EPNs. We highlighted proteases, protease inhibitors, FAR proteins, and GPCRs as potential targets for improvement, though there are certainly many more genes and gene families waiting to be discovered in these species that could be exploited. There are also many genes that could be used to increase infective juvenile persistence and survival in the soil. Several known stress-tolerance genes like heat shock proteins, trehalose-related molecules and pathways, as well as their orthologs and paralogs that have been expanded in EPN genomes, remain to be functionally tested. EPN research is burgeoning with possibility, but much remains intractable without the application of more molecular tools [56]. The field advanced significantly with the sequencing of these genomes, but whether this will lead to actual improvements in the field efficacy of EPN biocontrol remains to be seen.

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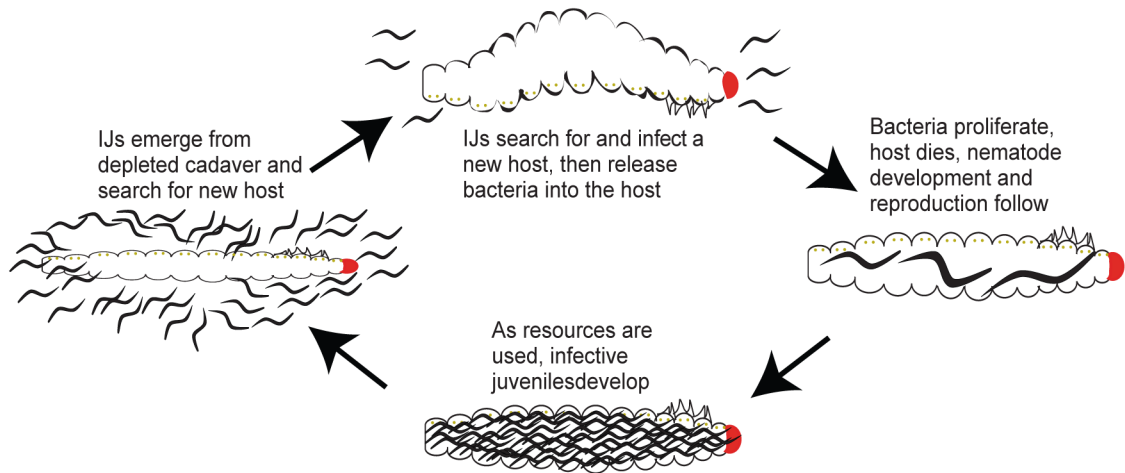
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### Trends Box

- Entomopathogenic nematodes (EPNs) have been used in biological control but improvement is needed to realize their full potential as an alternative to chemical pesticides.
- The genome sequence of six species of EPNs are now available and more are being sequenced.
- As we increase our knowledge of the genes underlying traits important for the field efficacy of EPNs the genomic data will be more useful in improving EPNs as biocontrol agents.

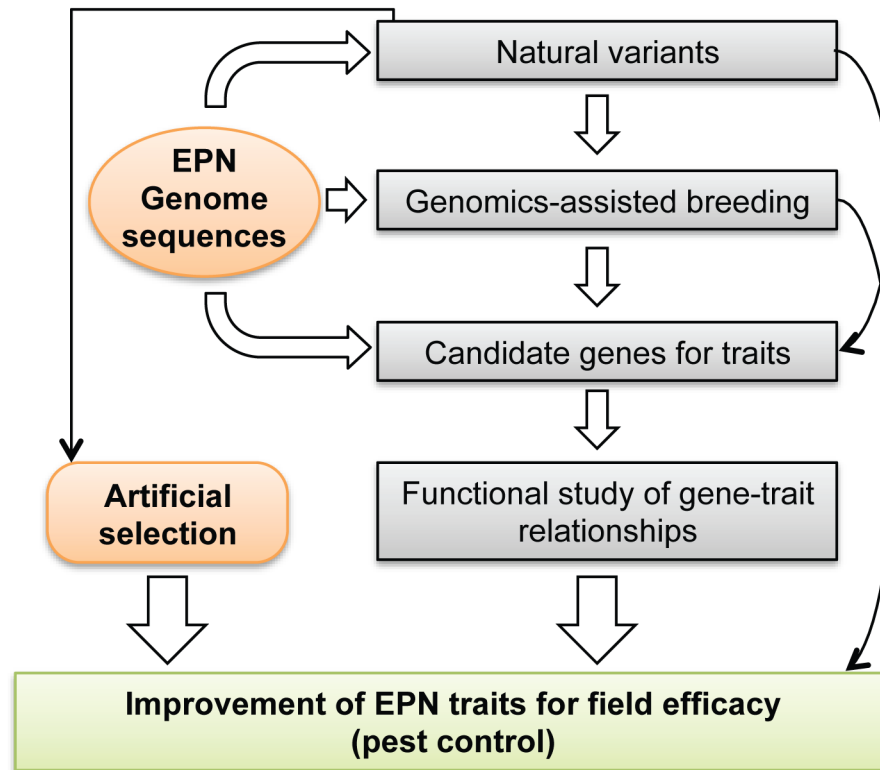
## Entomopathogenic Nematode Lifecycle



**Figure 1. The life cycle of entomopathogenic nematodes**

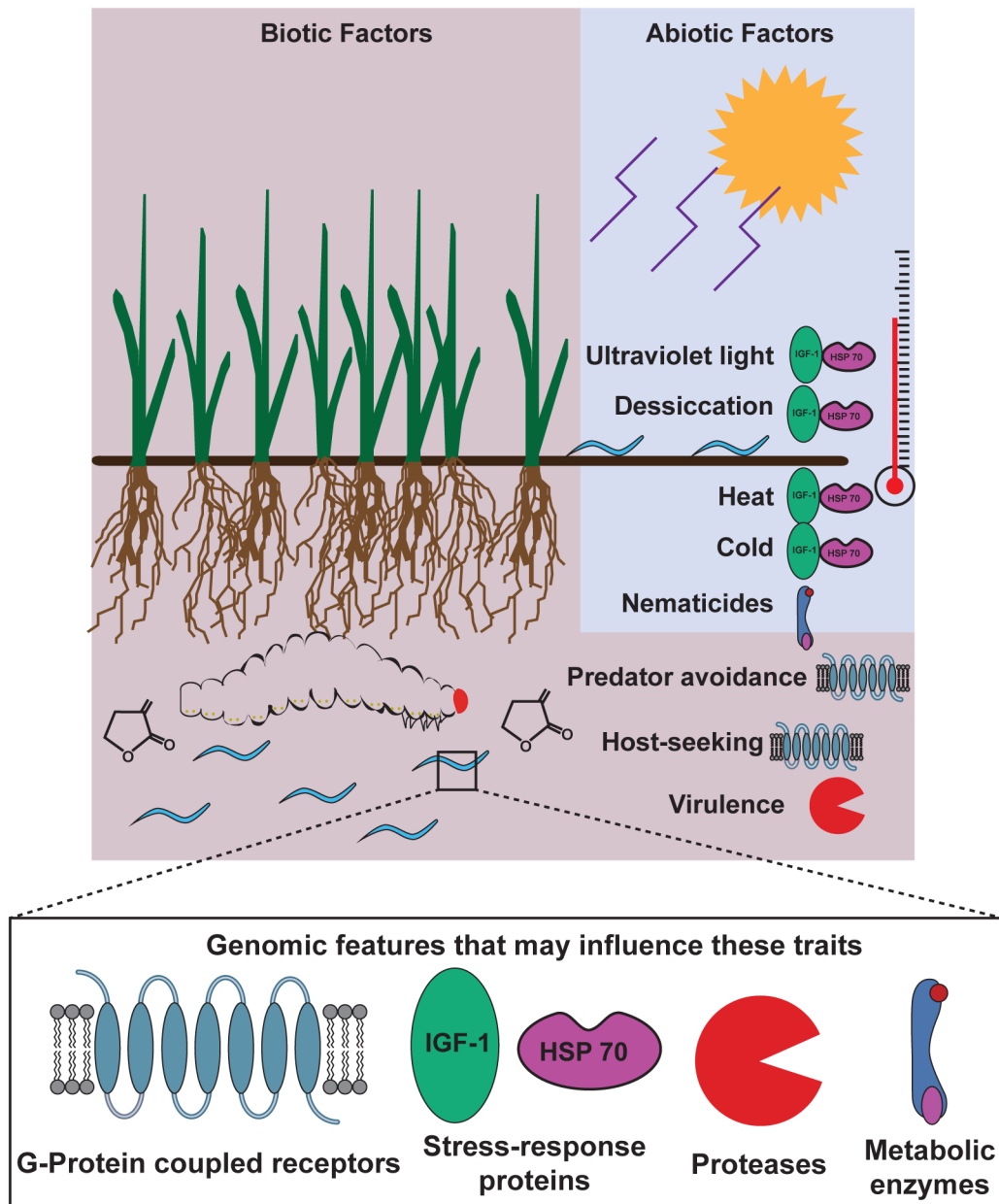
The infective juvenile (IJ) stage is a developmentally arrested third larval stage and is the only free-living stage of entomopathogenic nematodes (EPN); all other stages exist exclusively within the host. EPN IJs carry symbiotic bacteria and search for potential insect hosts. They enter a host, gain access to the hemolymph, and release their bacterial symbiont. The symbiont helps overcome host immunity and facilitates nutrient liberation from insect tissues. The nematodes develop and reproduce in the resulting nutrient rich environment until population density is high and resources begin to deplete, at which point new IJs develop and disperse, carrying the symbiotic bacteria to new hosts. Adapted from [2].





**Figure 2. Schematic illustration of how to improve entomopathogenic nematode traits for enhancing field efficacy**

The recently sequence genomes of entomopathogenic nematodes (EPN) can be used for selecting candidate genes that influence desirable traits and for identifying the diversity of natural gene variants for artificial selection and genomics-assisted breeding. Genome-wide association analysis of natural variants and strains obtained by genomics-assisted breeding could result in finding the candidate genes underlying certain traits which, when further validated by functional study of gene-trait relationships, may facilitate the improvement of EPN traits for enhanced field efficacy. Genomics-assisted breeding can be used to improve EPN traits without knowing specific gene-trait relationships. Artificial selection can be used to improve EPNs without previous knowledge of genomes or gene functions.



**Figure 3.** Biotic and abiotic factors that are important for the field efficacy of entomopathogenic nematodes and could be improved using classical genetics or molecular techniques.

**Table 1**

Main features of the recently sequenced genomes of entomopathogenic nematodes.

|   | <i>H. bacteriophora</i> | <i>S. carpocapsae</i> | <i>S. scapterisci</i> | <i>S. feltiae</i> | <i>S. glaseri</i> | <i>S. monticolum</i> |
|---|-------------------------|-----------------------|-----------------------|-------------------|-------------------|----------------------|
| Genome size (Mb)                                  | 77.0                    | 85.6                  | 79.4                  | 82.4              | 92.9              | 89.3                 |
| N50 (bp)  | 312328                  | 299566                | 90783                 | 47472             | 37444             | 11556                |
| Number of scaffolds                               | 1263                    | 1578                  | 2877                  | 5839              | 7515              | 14331                |
| Number of predicted genes                         | 21250                   | 28313                 | 31378                 | 33459             | 34143             | 36007                |
| G+C content (%)                                   | 32.2                    | 45.5                  | 48                    | 47                | 47.6              | 42                   |
| Number of putative GPCR <sup>a</sup>              | 82                      | 604                   | 731                   | 883               | 806               | 690                  |
| Number of putative proteases with signal peptides | 19                      | 268                   | 357                   | 267               | 248               | 423                  |
| Number of putative FAR <sup>b</sup> proteins      | 3                       | 41                    | 42                    | 43                | 54                | 38                   |

<sup>a</sup>GPCR; G protein-coupled receptor.

<sup>b</sup>FAR; fatty acid- and retinol-binding.