25 Genetic Improvement of Entomopathogenic Nematodes for Enhanced Biological Control

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25.1 Introduction

The world loses 32.1%, on average, of all crops due to herbivory by pests (mostly insects), viruses, weeds and crop diseases, with insect pests being a major driver of this loss (Oerke, 2006). Insecticidal nematodes of the genera *Heterorhabditis* and *Steinernema* are highly pathogenic and are used as biocontrol agents of numerous insect pests. Due to the many desirable traits of these entomopathogenic nematodes (EPNs), they have been commercialized on several continents. However, lack of consistent efficacy in the field prevents these nematodes from being more widely used; thus, researchers have been working to enhance their efficacy against arthropod pests under field conditions for decades. Two main strategies have been employed to improve EPN field efficacy and increase their usefulness in agriculture. The first is to isolate and identify new EPN species and/or populations (*e.g.*, Hunt and Nguyen, 2016). One line of reasoning is that endemic EPN species or populations will be adapted to local environmental conditions and to native pests, and may provide superior control compared to newly introduced species or populations of EPNs (Gaugler, 1988; Gaugler *et al.*, 1997a; Hiltpold, 2015). Considerable efforts have been spent surveying to extract new EPN isolates, which may lead to increased genetic variation and to the development of new nematode strains. Consequently, the first strategy to improve EPN efficacy relies on isolation and/or breeding of EPNs for enhanced insect pest suppression (see Subramanian and Muthulakshmi, Chapter 26, this volume). Traits typically targeted by this approach include increased tolerance to temperature extremes, ultraviolet light and desiccation, as well as higher host-seeking ability, virulence and resistance to nematicides. Improving these traits in EPNs has been done primarily by classical genetic techniques such as breeding and selection. However, the improved trait is not always stable, and trait gains can sometimes be lost once the selective pressure is removed (Glazer, 2014). Moreover, selection of some traits can sometimes lead to inadvertent reduction of others, or even loss of fitness (Gaugler *et al.*, 1989, 1990; Burnell, 2002). There is also the concern of inbreeding depression or other means of fitness loss during EPN mass production or as a result of continuous laboratory culture (Bilgrami *et al.*, 2006; Chaston *et al.*, 2011). Hence, the second major EPN improvement strategy, which is to use modern genetic and molecular tools to improve certain traits through genetic engineering. These tools have not yet been fully employed to improve EPN field efficacy in biological control, but genetic engineering remains a major strategy none the less (Burnell, 2002; Glazer, 2014, 2015). Progress has been made toward tool development and technology transfer from the *Caenorhabditis elegans* community,

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but the application of modern techniques to improve EPN efficacy is still in its infancy.

EPNs have been studied for and used in biocontrol programmes for more than 70 years. *Steinernema glaseri* was described in 1929 (Steiner, 1929) and was heavily studied for its potential to control the Japanese beetle (*Popillia japonica*). This nematode was quickly adopted by state-wide programmes in the 1940s and 1950s (Girth *et al.*, 1940; Glaser *et al.*, 1940; Cory and Langford, 1944, 1955; Fleming, 1968). *Steinernema carpocapsae* was identified as one of the most promising biocontrol agents because of its high virulence, broad host range, amenability to mass production on a commercial scale, and because it is exempt from government registration (Gaugler *et al.*, 1989). Many of the factors that make *S. carpocapsae* appealing as a biocontrol agent are also true of other steinernematid and heterorhabditid species. But clearly understanding the biology of both the pests to be controlled and the potential biocontrol agents is important. For example, biological control of white grubs, the root-feeding larvae of scarab beetles, will be different from a successful biocontrol programme targeting mole cricket pests (and indeed they are) (Shapiro-Ilan *et al.*, 2002; Frank and Walker, 2006). These pests have different life cycles and immune responses, and have co-evolved with different pathogens. White grubs have developed defence mechanisms that include infrequent carbon dioxide output, a dense peritrophic membrane, sieve plates over their spiracles, frequent defecation and certain behavioural and immune defences (Shapiro-Ilan *et al.*, 2002). To control white grub populations effectively using EPNs will require either using nematode species that have adapted to parasitizing white grubs or modifying an established EPN population through selection or genetic means to include characteristics that allow them to find and infect white grub larvae effectively in the field. It has been shown that cruise foragers like certain *Heterorhabditis* species and *S. glaseri* are better adapted to finding and infecting sedentary soil-dwelling insects such as white grubs than ambush foragers like *S. carpocapsae* (Gaugler *et al.*, 1997a; Lewis, 2002). Controlling mole crickets on the other hand requires a different approach, since they seem to be refractory to infection from many species of EPNs (Dillman *et al.*, 2012a), but instead are susceptible to *Steinernema scapterisci*, which is reported to be a cricket specialist (Nguyen and Smart, 1990, 1991). So while our understanding of pest insects and EPNs continues to increase, we are constantly discovering and describing new species of EPNs that may be better suited to controlling some pests than the currently used biocontrol agents. In this chapter, we discuss the strategies and future directions of genetic improvement of EPNs for biocontrol.

25.2 Traits for Improvement

There are several factors that limit the efficacy of control afforded by EPNs in the field. Broadly, these can be separated into two categories; infectivity and persistence. Infectivity includes the cascade of events beginning with host seeking and ending with the emergence of new infective juveniles (IJs) from a nutrient-depleted insect cadaver. Persistence includes all of the traits that help the EPNs survive and deal with the physical, chemical and biological components of the environment into which they are placed; everything from heat and desiccation to predator avoidance. There are also additional concerns regarding commercial production and shelf life (for more details see Askary and Ahmad, Chapter 13, Laznik and Trdan, Chapter 30, this volume), but in this chapter we will focus on traits that directly influence their performance in the field.

25.2.1 Infectivity

EPN infectivity can be influenced at every step of the infection process. First, the nematodes perform host-seeking behaviours to locate a suitable host, then they must obtain entry into the haemolymph, release their mutualistic bacteria, overcome the host immune response, kill the host, liberate host tissues and nutrients into a useable form, develop, reproduce and, eventually, when cadaver resources become limited, the rising generation of nematodes must acquire or associate with their symbiont and emerge as IJs to repeat the cycle again (Kaya and Gaugler, 1993; Lewis *et al.*, 1995, 2006). Any part of this process, and other parts not mentioned (*e.g.*, Hunt and Nguyen, 2016), could potentially be targeted for genetic improvement and could lead to a higher level of infectivity, thus increasing the efficacy of EPN biocontrol. There have already been a significant number of studies done to investigate selective improvement of host-seeking behaviours and infectivity (e.g. Gaugler *et al.*, 1989; Gaugler and Campbell, 1991; Grewal *et al.*, 1993; Tomalak, 1994; Peters and Ehlers, 1998; Hiltpold *et al.*, 2010; Bal *et al.*, 2014; Vadnal *et al.*, 2017). However, modern genetic

techniques such as genetic transformation have not yet been used to enhance any part of the cascade leading to infection.

25.2.2 Persistence and survival

EPN persistence and survival is greatly influenced by the ability of a nematode to withstand desiccation and a range of temperatures. Industrial production of EPNs relies on desiccation tolerance as a means of prolonging the life of nematodes for storage and shipment. Improvements for desiccation and heat tolerance so far have relied on selective breeding as a means for enhancing survival.

Because EPNs are limnoterrestrial, like all terrestrial nematodes, they are extremely sensitive to desiccation and so desiccation tolerance is essential in prolonging the life of EPNs, not only to increase shelf life for the purpose of distribution but also to ensure their persistence in the field. Low water content induces quiescence in nematodes, and also ensures that bacterial and fungal growth is prevented. However, desiccation is a harsh treatment that results in a high mortality rate. Selection for nematodes that can withstand the desiccation process has successfully produced strains of EPNs that can survive the process with a high rate of survival (Nimkingrat *et al.*, 2013a). Along with selective breeding, hybridization has also proven to be a powerful tool in enhancing desired traits such as desiccation and heat tolerance. Some species of nematodes are more adept at surviving dry conditions and a wider range of temperatures than others. Using these particular strains of EPN for hybridization, followed by selective breeding, has proven to be successful in producing new, more highly tolerant EPN lines (Nimkingrat *et al.*, 2013b). However, there are indications that the desired genes involved with desiccation tolerance are carried by heterozygous individuals. For species of nematodes that can reproduce asexually, these traits are easily retained by maintaining the nematodes in liquid culture. For gonochoristic species (which reproduce sexually) – such as many species of *Steinernema* – maintaining a population of heterozygous individuals is not possible (Ehlers, 2001).

In addition to selective breeding, the process of slow desiccation allows for adaptation. It has been shown that allowing for adaptation can be equally as influential as selective breeding.

There are indications that a slower desiccation process provides enough time for adaptation that results in an increase in survivorship (Nimkingrat *et al.*, 2013a). Additionally, heat tolerance has shown to be improved in individuals that were allowed time to adapt to dry environments (Salame *et al.*, 2010).

Heat tolerance is also another trait that has strongly flexible genetic components. This means that through selective breading these traits can be enhanced so that the EPN lines can withstand a broader range or higher range of temperatures. The heritability of heat tolerance is relatively high for some species of nematodes (such as *Heterorhabditis bacteriophora*), but lower in others (Bal *et al.*, 2014). Through selective breeding, heat tolerance can be improved; however, laboratory selection for these attributes has been shown to have deleterious effects on other desirable characteristics (Chaston *et al.*, 2011).

Inbreeding can have depressive effects on many desired traits. Some characteristics that are most highly affected include heat tolerance and fecundity. However, trait deterioration can be overcome with outbreeding. Outbreeding utilizes an outside line of EPNs that is mixed with the existing lines for breeding, and this method has been shown to be successful at restoring trait deterioration due to inbreeding (Chaston *et al.*, 2011).

Dispersal is another trait that can be selected for in order to increase survivorship and persistence. Selection for dispersal can increase the distances EPNs will travel to find a new host, as well as the speed at which they can travel. Much like heat and desiccation tolerance, there are trade-offs that can arise when selecting for enhanced dispersal, such as decreased reproductive potential and a reduced rate of nictation (Bal *et al.*, 2014). However, these particular trade-offs, unlike the trade-offs for heat and desiccation tolerance, appear to be behaviourally based. Out of the population of individuals that have an inclination to move faster and disperse farther, the majority are males (Bal *et al.*, 2014). However, due to the asymmetry of the sex ratio in this population, the reproduction potential is reduced. Additionally, these *sprinter* individuals have reduced nictation frequencies. Although the ability to nictate is not lost, these individuals have adopted a more cruiser-type method of finding a new host. Unlike the trade-offs that occur in selection for heat and desiccation tolerance, these reductions in reproduction potential and nictation frequency are not something that can be fixed with outbreeding, as they are behavioural aspects that are linked closely with breeding for the characteristic of enhanced dispersal (Bal *et al.*, 2014).

Overall selective breeding and hybridization have proven to be powerful tools in assisting with enhancements of EPN characteristics such as heat and desiccation tolerance and dispersal. Inbreeding can have deleterious effects, leading to reduced fitness, but can be overcome with outbreeding to restore the desired traits. However, the issue of maintaining desired characteristics will prove to be the biggest challenge for species of EPNs that reproduce sexually. Yet this particular issue may prove to be overcome through the utilization of molecular methods for genetic improvement.

25.3 Molecular Methods for Genetic Improvement

The idea of using recombinant DNA research to improve biological control has been around in the literature since at least the mid-1980s (Beckendorf and Hoy, 1985). The simplified idea is that once a useful gene is identified, one that could enhance the persistence or field efficacy of biological control agents, this gene could then be added to other species to confer similar advantages (Hoy, 1992). This has already been done successfully in several systems, especially in agricultural systems where genetic engineering is widespread and increasing. For example, the bacterium *Bacillus thuringiensis* has been studied extensively for its insecticidal activity and has been shown to produce many toxins that are highly effective at killing insect pests. Some of the insecticidal toxins from *B. thuringiensis* have been inserted into the genomes of many important agricultural crop plants to reduce crop loss and damage by insect herbivory (Vaeck *et al.*, 1987; Glare and O'Callaghan, 2000; Romeis *et al.*, 2006). Entomopathogenic fungi are another group where genetic engineering has led to enhanced biocontrol of insect pests. Cuticle-degrading enzymes and toxins are important for fungal infection of insects, many of which are encoded by single genes that are amenable to genetic manipulation (St Leger and Wang, 2010). Multiple copies of a cuticle-degrading protease gene were inserted into the genome of the entomopathogenic fungus *Metarhizium anisopliae* and were expressed constitutively, leading to reduced survival in infected insect hosts (St Leger *et al.*, 1996).

Genetic enhancement has benefitted from descriptive exploration of natural proteins and gene products. Myriad arachnid, scorpion and bacterial proteins and toxins have been considered for the genetic enhancement of biocontrol using genetic engineering (Edwards and Gatehouse, 2007; Whetstone and Hammock, 2007). Scorpion toxins have even been inserted into entomopathogenic fungi and have improved their ability to kill insect hosts (Wang and St Leger, 2007; Pava-Ripoll *et al.*, 2008). Certainly, more candidate biocontrol genes and toxins will come to light as we continue to discover and sequence more species. However, natural proteins and genes are not the only resource available for genetic enhancement as synthetic genes and proteins have also been used. For example, some of the chitinase genes in the entomopathogenic fungus *Beauvaria bassiana* do not have recognizable chitin-binding domains. So researchers have constructed novel hybrid chitinase genes where they fused endogenous functional chitinase genes from *B. bassiana* with chitin-binding domains from other species, which resulted in increased killing of insect hosts by the transgenic fungi (Fan *et al.*, 2007). These examples from other systems open exciting possibilities for the genetic improvement of EPNs, even if researchers only repeat what has been done in entomopathogenic fungi or plants, and with some creativity, perhaps even greater improvements can be realized.

The development of molecular and genetic tools for EPNs and technology transfer from the powerful genetic model *C. elegans* has been slow. Many of the powerful tools that allow for genetic engineering (genetic transformation) and the functional characterization of genes (RNAi) were developed in *C. elegans* 20 or more years ago. (Kimble *et al.*, 1982; Fire *et al.*, 1991; Mello *et al.*, 1991; Broverman *et al.*, 1993; Mello and Fire, 1995). There are encouraging examples of successful genetic transformation of EPNs in the literature (Hashmi *et al.*, 1995a, 1997, 1998; Vellai *et al.*, 1999), but these results were published 15 or more years ago and researchers in the field have not developed these methods further, nor have transgenic EPNs become commercially available. Hashmi *et al.* (1995a, 1998) reported transforming *H. bacteriophora* with a copy of the *C. elegans hsp70A* gene and that this resulted in dramatically increased heat tolerance in the transgenic nematodes under laboratory conditions. The transgenic nematodes had normal growth and development and did not appear to differ in virulence from the parental strain (Hashmi *et al.*, 1998). Although the transgenic nematodes and parental strain did not differ in persistence in the field (Gaugler *et al.*, 1997b; Wilson *et al.*, 1999), these studies have gone

a long way in showing that transgenic techniques can be applied to EPNs successfully.

Steinernema feltiae has also been transformed successfully, given the stress resistance gene trehalose-phosphate synthase 1 from yeast (Vellai *et al.*, 1999). These transgenic nematodes showed an increase in stress tolerance, further demonstrating the potential of molecular methods in improving EPN traits. Surprisingly, there have not been additional published reports of transgenic success in EPNs. Certainly, there is interest in the field to use molecular genetics to explore gene function in EPNs and to enhance their efficacy in biocontrol, but perhaps the perceived difficulty of these techniques has prevented further developments. There are now multiple ways to develop transgenic strains of nematodes, including microinjection of genetic constructs into the gonad (Evans, 2006) and microparticle bombardment (Praitis *et al.*, 2001). Although microinjection has been used to make transgenic EPNs (Hashmi *et al.*, 1995a), this technique has not been adopted by many EPN researchers. Perhaps microparticle bombardment, which is done on many nematodes and with multiple genetic constructs at the same time, will have more success in the EPN community.

Being able to introduce exogenous or modified genes into EPNs in a consistent and reliable manner is paramount to our ability to employ molecular methods in genetic improvement of EPNs in biocontrol. Other techniques, such as RNAi, will be extremely useful in determining the molecular function of the genes (Fire *et al.*, 1991, 1998). As with genetic transformation, although RNAi has been applied successfully in *H. bacteriophora* (Ciche and Sternberg, 2007), it has not been adopted by the field at large nor has it been developed further. Before we can make significant progress using molecular methods for genetic improvement of EPNs, these techniques need to be developed and adopted by the EPN community of researchers. Progress has been made toward developing these tools with the sequencing of the *H. bacteriophora* genome and multiple *Steinernema* congeners (Bai *et al.*, 2013; Dillman *et al.*, 2015). The huge amount of data released by these sequencing projects both regarding gene presence and expression should further enhance and encourage transgenic approaches by providing a large list of candidate parasitism and survival genes that could be used to enhance EPNs for biological control.

25.3.1 Genomic analyses of *Heterorhabditis bacteriophora*

The 77 Mb *H. bacteriophora* genome sequence has revealed numerous putative parasitism and survival genes that could be manipulated by transgenic approaches (Table 25.1) (Bai *et al.*, 2013). But it has also raised many questions since the *H. bacteriophora* genome contains more than 10,000 proteins of unknown function. *H. bacteriophora*'s obligate association with the bacterium *Photorhabdus luminescens* has shaped the content of its genome as the nematode relies on the bacteria for nutrients and protection. The bacteria produce an arsenal of enzymes and proteases to overcome insect host immunity, degrade host tissues and make them available for the developing nematodes, and prevent opportunistic fungi and bacteria from making use of the nutrient-rich insect cadaver. Unlike *Steinernema*, axenic (bacteria-free) *H. bacteriophora* nematodes are unable to kill insect hosts, suggesting that the bacteria do most of the heavy lifting on host entry (Han and Ehlers, 2000; Eleftherianos *et al.*, 2010). *H. bacteriophora* may rely mostly on *Photorhabdus* for dealing with the insect host post-infection, but it is still the nematode that must locate hosts to infect, gain entry into the haemolymph and persist in the soil until a new host is found, leaving

Table 25.1. Comparison of the sequenced entomopathogenic nematode genomes as previously reported. The *Steinernema monticolum* genome was not included. (From Bai *et al.*, 2013; Dillman *et al.*, 2015).

	Heterorhabditis bacteriophora	*Steinernema carpocapsae*	*Steinernema scapterisci*	*Steinernema feltiae*	*Steinernema glaseri*
Genome size (Mb)	77.0	85.6	79.4	82.4	92.9
N50 (bp)	312,328	299,566	90,783	47,472	37,444
No of scaffolds	1,263	1,578	2,877	5,839	7,515
No of predicted genes	21,250	28,313	31,378	33,459	34,143
GC content (%)	32.2	45.53	47.98	46.99	47.63

plenty of room for genetic improvements to enhance field efficacy. Most of the insulin/IGF-1 signalling pathway genes were found in the *H. bacteriophora* genome (Bai *et al.*, 2013). These genes play a critical role in dauer formation and are important regulators of stress resistance and innate immunity in *C. elegans*, and are thought to perform similar functions in *H. bacteriophora*. The 19 genes found to be conserved in the insulin/IGF-1 signalling pathway are all potential candidates for genetic manipulation or enhancement, as increasing IJ longevity and/or stress resistance may lead to better field efficacy and pest control.

Another gene family explored in the *H. bacteriophora* genome is the G protein-coupled receptor (GPCR) family. There are at least 82 predicted GPCRs in the *H. bacteriophora* genome, and these are important because they could be sensory receptors functioning in olfaction and host-seeking behaviour (Robertson and Thomas, 2006; Thomas and Robertson, 2008; Srinivasan *et al.*, 2013). The abundance and diversity of GPCRs can reflect the niche inhabited by a nematode, and in the case of EPNs, putative olfactory receptors are of high value as they could be used to enhance host seeking or adjust the specificity of hosts being sought. Increasing our knowledge of how GPCRs are used in host seeking could be critical to improving or altering the host seeking of EPNs.

Any genes that play a role in the symbiotic association between EPN and the insect-pathogenic bacteria they carry could potentially 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*. It has far fewer C-type lectin domain-containing proteins (9 compared to 133), which function in the immune response of *C. elegans* to bacterial infection (Schulenburg *et al.*, 2008; Bai *et al.*, 2013). This reduction in C-type lectin domain-containing proteins may be related to the association between *H. bacteriophora* and *P. luminescens* (Ciche *et al.*, 2008). But it is also not known to what extent bacterial infection might impede on the efficacy of EPNs as biocontrol agents. More research needs to be done, but the immune response of EPNs is yet another area that could be harnessed to improve field efficacy against insect pests.

Secreted products are another area that is rife with potential for genetic enhancement of EPNs. The field efficacy of EPNs will benefit from our increased understanding and characterization of the existing secreted products and their role in invasion and infection. One can imagine improving host killing by EPNs simply by adding additional copies of genes already present, similar to the transgenic inclusion of multiple endogenous cuticle-degrading proteases in entomopathogenic fungi (St Leger *et al.*, 1996). Another possible route of improvement is by the addition of new secreted products, like the transgenic addition of a scorpion venom gene into the genome of the entomopathogenic fungus *M. anisopliae* (Pava-Ripoll *et al.*, 2008). *H. bacteriophora* has fewer than 30 predicted protease and protease inhibitors in its secretome (Bai *et al.*, 2013). The presence of such a small number of putatively secreted proteases and protease inhibitors may reflect the heavy reliance of the nematode on *P. luminescens* for immune suppression and tissue degradation of the insect host. However, there are non-canonical secretion signals that are poorly understood and it is possible that *H. bacteriophora* has a larger secretome than is predicted by the abundance of signal peptides (Bennuru *et al.*, 2009, 2011). EPN-secreted proteases are known to influence penetration of the nematode into the host haemolymph (Abuhatab *et al.*, 1995), tissue degradation of insect hosts (McKerrow *et al.*, 2006), as well as immune suppression (Balasubramanian *et al.*, 2009).

The genomic sequence of *H. bacteriophora* provides a long list of candidate genes that could be used to improve infectivity and/or survival. The successful transformations and use of RNAi in *H. bacteriophora*, coupled with this archive of new genes to be studied, is expected to lead to significant advances in the application of molecular genetics to improve the field efficacy of EPNs in biological control.

25.3.2 Genomic analyses of *Steinernema*

The draft genomes of five species of *Steinernema* (*S. carpocapsae*, *S. feltiae*, *S. glaseri*, *Steinernema monticolum* and *S. scapterisci*) have recently been sequenced and annotated, and have revealed numerous genes that could be involved in the entomopathogenic lifestyle and could be used in programmes to improve traits important for biological control (Table 25.1) (Dillman *et al.*, 2015). 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 with other animals, or even other nematodes (Dillman *et al.*, 2015). Determining the function of these orphan proteins in EPNs could yield insight into genes important for infection or survival and

persistence, and thus be useful to future transgenic endeavours in EPNs. The genomes of these EPNs are similar in size but differ considerably in nucleotide prevalence (G+C content), which may influence the application and use of recombinant DNA techniques for genetic enhancement. Codon usage preferences can influence gene expression and regulation (Rao *et al.*, 2013), which could affect technology transfer from *C. elegans* to the EPNs, with technology developed in the *C. elegans* model being applied more easily to *H. bacteriophora* due to their closer ancestry and similar nucleotide prevalence (Table 25.1) (van Megen *et al.*, 2009; Bai *et al.*, 2013; Dillman *et al.*, 2015), although this needs to be further explored experimentally.

In contrast to the *H. bacteriophora* genome, the sequenced species of *Steinernema* have an abundance of predicted protease and protease inhibitors with signal peptides. The genome sequence revealed *Steinernema*-specific expansions of serine and metalloproteases (Dillman *et al.*, 2012b, 2015). Proteases are known to be important in invasion and host-killing for *Steinernema*, making the repertoire of proteases and protease inhibitors an important group of proteins for investigation in future selection and recombinant studies (Abuhatab *et al.*, 1995; Balasubramanian *et al.*, 2009). Proteases in steinernematids have been shown to play an important role in suppressing insect host immunity, as well as tissue degradation (e.g. Balasubramanian *et al.*, 2009, 2010; Toubarro *et al.*, 2009, 2010). Protease inhibitors are also expanded in the sequenced steinernematids, and there are functional studies showing that protease inhibitors play an important role in nematode evasion of host immunity (Milstone *et al.*, 2000; Zang and Maizels, 2001), although the mechanism remains poorly understood. One provocative possibility is that host range and specificity of EPNs may be influenced by their armoury of secreted products, and that using 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 (Elsik, 2010; Gerardo *et al.*, 2010).

Fatty acid- and retinol-binding (FAR) proteins are another interesting gene family that has been highlighted in the genomes of *Steinernema*, as they seem to be expanded in these species (Dillman *et al.*, 2015). 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, although their exact functional role is not well understood (Garofalo *et al.*, 2002; Kennedy *et al.*, 2013). They are particularly interesting because they seem to be involved in nematode parasitism of animals, insects and plants (Hao *et al.*, 2010; Iberkleid *et al.*, 2013; Kennedy *et al.*, 2013), which makes understanding their mechanistic function important for both biocontrol as well as disease treatment and prevention.

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 have mentioned proteases, protease inhibitors, FAR proteins and GPCRs, although there are certainly many more to be mined from these data. There is also an abundance of genes that could lead to increased IJ persistence in the soil, as well as increased survival. In addition to genes involved in the insulin/IGF-1 signalling pathway, there are known stress-tolerance genes like heat-shock proteins, trehalose-related molecules and pathways, as well as all of the orthologues and paralogues that have been expanded within the EPNs. What remains is for these genes to be functionally tested, perhaps using biochemical or RNAi techniques, and applied in recombinant DNA experiments. The field has been advanced by the sequencing of these genomes, but whether this leads to actual improvements in the field efficacy of EPN biocontrol remains to be seen.

25.4 Troubleshooting

As in crop breeding (e.g. Abd-Elgawad, 1991), success in the genetic improvement of EPNs for enhanced biological control generally depends on three main phases. These are hereditable genetic variation of the desired trait(s), adequate screening and improvement techniques, and the capacity of the improved strain in terms of its efficacy, fitness and stability in laboratory, greenhouse and, more importantly, under field conditions. Eventually, to materialize the net gain of such an improvement process, its cost should be justified by the advantages or profits obtained (e.g. Hoy, 1985).

Although selective breeding and mutagenesis have been classical approaches for genetic improvement of EPNs, they often have substantial problems

in some or all of the above-mentioned categories. One difficulty is that genetic refinement of EPNs to enhance their biological control potential through selective breeding is likely only if the desired alleles are found in the gene pools of the examined nematode populations. For example, insufficient genetic variation in resistance to ultraviolet light (UV) resulted in rejecting selective breeding as an option to improve UV tolerance in *S. carpocapsae* (Gaugler *et al.*, 1989). It is well established that sexual reproduction, mutations and the recombination of linked genes (crossing over) are major tools of the phenotypic variability of EPNs in nature. While sexual reproduction offers recombinations between genes located in different chromosomes, crossing over can perform the recombination of alleles present on the same chromosome. Therefore, broad surveys of diverse habitats to isolate novel EPNs to widen the genetic variation and consequently develop new nematode strains are considered excellent troubleshooting tools. Selective breeding is the preferred approach to improve a polygenic trait where each gene encodes only a small effect on the same phenotype. Optimally, the selected improvements will have high heritability (mathematically calculated as h^2). If the targeted trait has low heritability (h^2), then, a programme centred on the introduction and selection of new mutations affecting this trait would be preferable (Glazer *et al.*, 1991).

Nevertheless, many researchers (e.g. Gaugler *et al.*, 1990; Hastings, 1994; Burnell, 2002; Grewal *et al.*, 2011; Glazer, 2014, 2015) reported that traits improved in this way were not stable and that the phenotypes selected for tended to revert gradually to the unselected state following relaxation of the selection pressure. A wise strategy to troubleshoot these problems is to let nature perform the selection process, when possible. For instance, EPN strains endemic to areas with climate extremes will be naturally acclimated through selection to survive and reproduce in those conditions. Interestingly, EPN strains with these phenotypes have been extracted in programmes aimed at collecting cold-tolerant and warm-tolerant strains (Glazer *et al.*, 1996; Mracek *et al.*, 1998; Burnell, 2002; Abd-Elgawad and Nguyen, 2007). If natural selection is not applicable, the default strategy, to hinder the reverse of the targeted trait, is to cryopreserve the improved strains and to reapply selection pressure at constant intervals (Burnell, 2002).

Another issue that can result from applying artificial selection for enhancement of nematode strains is the occurrence of unintentional alteration of traits that may affect the fitness of the improved nematode strain adversely. Sometimes, the genes encoding for the targeted trait do not segregate independently. It is possible that some targeted genes have unknown linkage, which may lead to such problems. Therefore, more knowledge and tools for genetic improvement of EPNs are required. Certainly, modern genetic techniques such as RNA sequencing (RNA-seq) and transcriptomics can contribute to our understanding of these processes and provide needed genetic and molecular information (Glazer, 2014). For example, exposure of *H. bacteriophora* to desiccation stress could lead to increased desiccation tolerance. On relaxation of selection pressure, the tolerance gained was lost again during *in vivo* production, although this desired tolerance was positively retained in liquid-cultured EPNs (Anbesse *et al.*, 2013). Since the transcriptome is continually changing, RNA-seq can be utilized to detect a snapshot of RNA existence and quantity from a genome at a fixed moment in time (Chu and Corey, 2012), which may enable us to follow and understand the molecular basis of a beneficial EPN trait. Nevertheless, such reported results (e.g. Anbesse *et al.*, 2013) cannot be interpreted reliably and conclusively through a single technique, but a combination of genetic approaches to improve EPNs as bioinsecticides holds promise for solving this and other related issues. In this respect, other modern genetic techniques such as those related to genome editing, e.g. transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats that rely on a protein called Cas9 (CRISPR-Cas9), may also be used to modify traits (Glazer, 2014). For instance, TALENs are synthetic restriction enzymes that can cut DNA strands at a specific sequence. Transcription activator-like effectors (TALEs) can be engineered quickly to bind practically any desired DNA sequence (Boch, 2011). By combining such an engineered TALE with a DNA cleavage domain (which cuts DNA strands), one can engineer restriction enzymes that are specific for any desired DNA sequence. When these artificial enzymes are inserted in to the intended cells, they can be utilized for genome editing in situ (https://en.wikipedia.org/wiki/Transcription_activator-like_effector_nuclease).

The continuous progress in developing molecular tools should be directed to fundamental and applied EPN genetics, creating novel useful approaches while fine-tuning current methodology.

For example, fine-tuning of transgenic techniques can be applied successfully to EPNs through trial and error, as may occur in fixing the appropriate settings in order to pull needles for microinjection of EPNs (Hashmi *et al.*, 1999). On the other hand, Hashmi *et al.* (1995b) could validate a new and efficient genetic transformation system for EPNs, using arrays of micromechanical piercing structures, which is quick and easy to use. Such modern and other novel genetic techniques are desperately needed to address the challenges concerning improvement of EPNs for enhanced biocontrol of arthropod pests.

25.5 Conclusions and Future Prospects

The increase in both EPN species descriptions and in general EPN research is exciting and bodes well for the future of biocontrol using EPNs, and is encouraging for the prospects of genetic improvement of EPNs. Historically, the significant strain improvements made to EPN biocontrol have come through the discovery of new species or through classical genetics. However, there are promising reports of successful transgenic methods being applied in EPNs, but they remain few and have not been followed up by more recent studies (Hashmi *et al.*, 1998; Vellai *et al.*, 1999). Genetic improvement of EPNs via selection will continue to be important; however, transgenic organisms have become more common and they allow for improvements that simply are not possible with conventional methods. The advances that have been made and the continued development of molecular tools will be used to address difficult applied and fundamental questions.

Better field efficacy is needed if EPNs are to provide pest control at a level that is competitive with other means. Ultimately, this problem is likely to be solved as EPN biology continues to be a vibrant and growing field of research, attracting more and new researchers to this field. This continued growth and enthusiasm for EPN biology will be achieved by emphasizing not only the applied aspects of EPN research but also their incredible usefulness as models for basic biology (Akhurst and Dunphy, 1993; Emelianoff *et al.*, 2008; Dillman and Sternberg, 2012). EPNs remain a powerful system for the study of evolution, symbiosis, parasitism and development. They are also useful models of animal parasitism, since they are easier to work with than most vertebrate-parasitic species. There remain many questions that are unknown or underexplored, leaving EPN biology a field that is pregnant with possibility and that has plenty of room for more investigators to apply their skill and creativity to studying their interesting biology.

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