Population Genomics of Benzoxazinoid Resistance in Entomopathogenic Nematodes

Inaugural dissertation

of the Faculty of Science,

University of Bern

presented by

Vera Ogi

Supervisors of the doctoral thesis:

Prof. Dr. Matthias Erb, Institute of Plant Sciences, University of Bern

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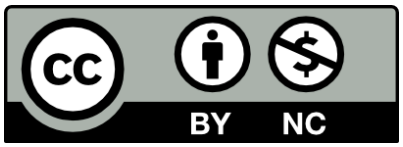

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Abstract

The increasing global population and the need for enhanced food production present significant challenges to modern agriculture. Traditional methods, such as expanding arable land and extensive use of chemical pesticides, are no longer viable due to biodiversity conservation concerns and the negative impacts of chemicals on ecosystems. This has led to the exploration of alternative pest control strategies, including the use of entomopathogenic nematodes (EPNs). These nematode-bacterium symbiotic complexes can infect and kill insect pests and have been used in agricultural biocontrol for decades. However, their generalist nature limits their effectiveness against specialist pests such as the western corn rootworm (WCR), *Diabrotica virgifera virgifera*, which sequesters maize-derived benzoxazinoids (BXs) to defend itself from natural enemies, including EPNs.

In this thesis, **we used *Heterorhabditis bacteriophora* as an EPN model to explore the evolutionary, ecological, and genomic factors that influence its ability to overcome the plant defense compounds BXs**. The thesis first provides a **literature review** of EPN adaptations to environmental stressors, and highlights the underlying genetic and biochemical mechanisms. **Chapter 1** reports a de-novo chromosome-scale assembly and annotation of the *H. bacteriophora* genome. It reveals the unique genomic features that distinguish EPNs from other nematodes, and provides insights into their unique adaptations for insect parasitism. **Annex I** further explores the chromosomal structure of *H. bacteriophora*, uncovering the presence of holocentric chromosomes despite unusual transposable element distribution. **Chapter 2** focusses on the interaction between *H. bacteriophora* and its bacterial symbiont *Photorhabdus*, examining the genomic and phylogenetic differences between susceptible and resistant EPN isolates exposed to BXs. Population genomics revealed regions of the EPN genome associated with BX resistance. **Chapter 3** presents a large-scale experimental evolution study where EPN isolates were exposed to BX-sequestering WCR larvae. The results showed a rapid evolution of BX resistance in previously susceptible isolates, and identified key genetic loci associated with this resistance.

This work advances the understanding of EPNs as biocontrol agents, providing novel insights into their genomic architecture and their potential for overcoming plant chemical defenses. These findings offer valuable avenues for improving the precision and efficacy of EPNs in agricultural pest management.

General introduction – ***Review published in Journal of Invertebrate Pathology, 2023, 107953.***

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Introduction – Stress tolerance in entomopathogenic nematodes: Engineering superior nematodes for precision agriculture

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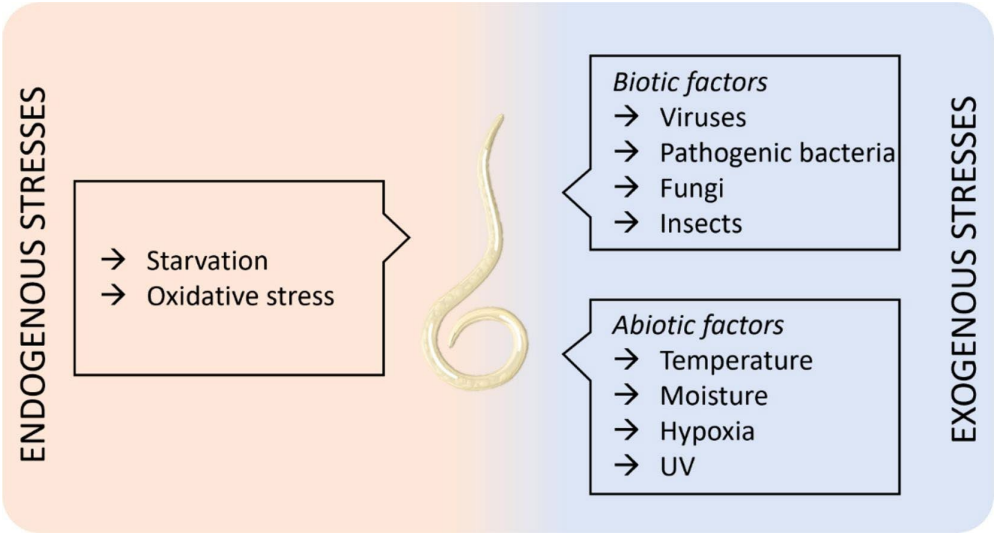
Abstract

Entomopathogenic nematodes (EPNs) are soil-dwelling parasitic roundworms commonly used as biocontrol agents of insect pests in agriculture. EPN dauer juveniles locate and infect a host in which they will grow and multiply until resource depletion. During their free-living stage, EPNs face a series of internal and environmental stresses. Their ability to overcome these challenges is crucial to determine their infection success and survival. In this review, we provide a comprehensive overview of EPN response to stresses associated with starvation, low/elevated temperatures, desiccation, osmotic stress, hypoxia, and ultra-violet light. We further report EPN defense strategies to cope with biotic stressors such as viruses, bacteria, fungi, and predatory insects. By comparing the genetic and biochemical basis of these strategies to the nematode model *Caenorhabditis elegans*, we provide new avenues and targets to select and engineer precision nematodes adapted to specific field conditions.

Graphical abstract



1. Introduction

Sustainable strategies to increase crop productivity are urgently required to ensure food safety and food security worldwide (Vågsholm et al., 2020). Biological control is a promising and environmentally friendly avenue to increase food production by decreasing herbivore-associated crop losses.

Animal pests are responsible for 8-16% crop losses annually (Birch et al., 2011; Oerke, 2006). Among these pests, root feeding insects are particularly damaging (Agrawal, 2011; Anbesse et al., 2013; Hunter, 2001; Johnson and Murray, 2008; Kergunteuil et al., 2016). For example, the western corn rootworm, *Diabrotica virgifera virgifera* Le Conte, causes damage and control costs exceeding two billion USD annually in the USA alone (Gray et al., 2009; Wechsler and Smith, 2018). Wireworms, larvae of click beetles (Coleoptera: Elateridae), inflict potato yield losses and control costs of about six million USD (Vernon et al., 2009). The greyback grub, *Dermolepida albohirtum*, causes about 28 million USD losses annually in Australian sugar industry (Chandler, 2002). While soil-dwelling herbivores are out-of-sight, their populations can be controlled by biocontrol agents such as entomopathogenic nematodes (EPNs) (Grewal et al., 2005; Koppenhöfer et al., 2020).

Entomopathogenic nematodes (EPNs), belonging to the Heterorhabditidae and Steinernematidae families, are obligate pathogenic roundworms that can infect and kill soil-living arthropods (Poinar, 2018; Shapiro-Ilan et al., 2014; Weischer and Brown, 2000). EPNs shelter a community of

endosymbiotic bacteria (Machado et al., 2018; Ogier et al., 2020; Pervez et al., 2020; Poinar and Grewal, 2012; Sajnaga and Kazimierczak, 2020). Infective juvenile (IJ) nematodes penetrate their host through natural apertures or by puncturing the cuticle (Bedding and Molyneux, 1982; Castelletto et al., 2014; Ciche and Ensign, 2003; Dowds and Peters, 2002). The EPN cuticle can contribute to suppressing and evading host immunity (Brivio et al., 2004; Brivio et al., 2002; Dunphy and Webster, 1987). Additionally, EPNs release proteins that induce tissue damage, toxicity, enzymatic inhibition, and immunosuppression (Burman and Pye, 1980; Chang et al., 2019; Han and Ehlers, 2000; Kim et al., 2017; Lu et al., 2017; Toubarro et al., 2013; Toubarro et al., 2009). Finally, EPNs release their bacterial symbionts by regurgitation or defecation (Ciche et al., 2006; Ciche and Ensign, 2003; Martens et al., 2004; Poinar, 1966). The bacteria produce digestive enzymes, immunosuppressors, and toxins, which ultimately kill the host within a few days (Brivio and Mastore, 2018; Ciche and Ensign, 2003; Clarke, 2020; Duchaud et al., 2003; Gaugler and Kaya, 1990; Kaya and Gaugler, 1993). EPNs then feed on the bacteria and reproduce in their insect host until resource depletion (Ciche and Ensign, 2003). At this stage, the nematodes produce new IJs that leave the body and seek for a new host (Gaugler and Kaya, 1990; Grewal and Georgis, 1999; Zhang et al., 2021). (Figure 1)

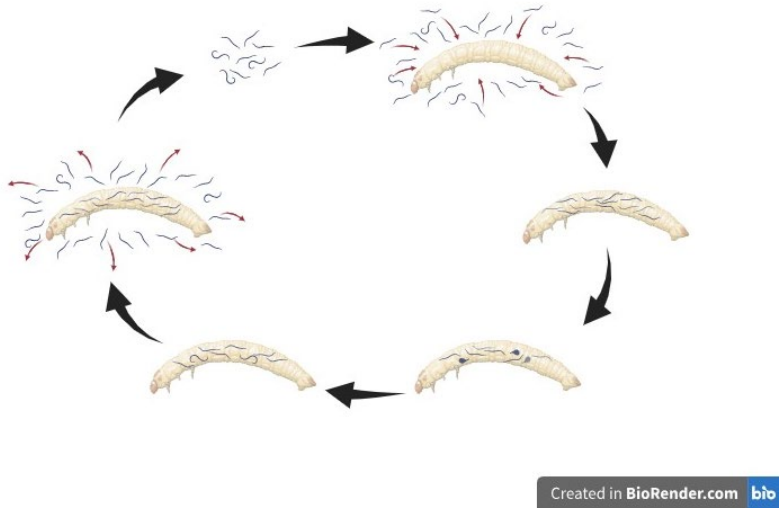


Figure 1. Life cycle of entomopathogenic nematodes (EPNs). The life cycle of the EPNs consists of the infective juvenile (IJ) stage, which is spent between hosts. In this state the EPN does not feed or develop, but they move through the soil matrix looking for the next host insect (cruiser) or wait for another insect to come by (ambusher). Generally, EPNs of the genus Heterorhabditis are considered to be ambushers and Steinernema to be cruisers. Once the EPN has located a new host, they enter either through natural orifices in the host body or through the cuticle, where they release their symbiotic bacterium. The bacterium in turn releases toxic compounds into the host body, which leads to the death of the host insect, at which point the bacteria will multiply and the EPNs will feed on them and develop into their sexual morphs and start a reproductive period of multiple generations depending on the host size. Once the host insect has been completely

consumed by the bacteria, the EPN will once again produce a generation of IJs, which will leave the cadaver of the host and restart the cycle.

EPNs have evolved several strategies, ranging from ambush to cruising, to locate and/or to increase probabilities of encountering preys (for review see Zhang et al., 2021). Their high virulence, together with the ease of mass production (Ehlers, 2001), suggest them as a biological agent of high potential for controlling soil-dwelling pests (Gaugler and Kaya, 1990; Kaya and Gaugler, 1993). To make use of this potential, however, it is crucial to attain high consistency of EPN's impact on herbivore populations that is independent of environmental conditions and pest characteristics, and robust to changes in application strategies (Gaugler, 1988; Griffin, 2012; Khatri-Chhetri et al., 2011; Koppenhöfer et al., 2020; Shapiro-Ilan et al., 2006a). This lack of understanding brings an urgent need to identify and characterize nematode traits that enhance their success in different conditions to generate superior EPNs.

Traits conferring superior efficacy include attributes increasing EPN persistence in soil, host encountering probability, infectivity success, and multiplication potential (Shapiro-Ilan et al., 2012; Shapiro-Ilan et al., 2006b). Traits involved in EPN commercial suitability, such as EPN production, formulation, and shelf-life, should also be considered for applied perspectives (Georgis and Gaugler, 2002; Glazer, 2015; Vernon and van Herk, 2022), but are not under the scope of this review. Although the identification of relevant traits by comparing EPN populations of different geographical origins or through artificial selection, hybridization or genomics-assisted breeding offers valuable insights, the functional characterization of genes encoding for specific traits remains a milestone to be achieved in developing genetic improvement programs (GIPs), and ultimately superior nematode isolates adapted to specific field conditions and pest targets.

Multiple genomes of EPNs and their endosymbionts are available to characterize the genetic pathways and gene families underlying specific traits (Table 1). Initial resources, such as the *H. bacteriophora* and *H. indica* draft genomes that are 77 Mb (~16'000 annotated protein coding genes) and 91 Mb (~10'000 annotated protein coding genes) long respectively, are available for comparative genomics (Bai et al., 2013; Bhat et al., 2021; McLean et al., 2018). *Steinernema* genomes have been assembled for multiples species including *S. carpocapsae*, *S. diaprepesi* and *S. feltiae* (Table 1). While the dissection of the genetic pathways involved in EPN biology and stress tolerance are still at an early stage, extensive knowledge is available from the model nematode *Caenorhabditis elegans*. *C. elegans* is a free-living nematode and lacks the molecular machinery to find and infect a host, and

133 originates from a narrow environmental background, rendering direct comparisons with EPNs
134 challenging. However, the knowledge gained from the model nematode may provide some avenues
135 to accelerate EPN research and genetic improvement in stress tolerance.

136 Table 1: List of genomes available in the two genera of EPNs, including indicators of quality (#scaffolds, N50)

137

	Species	Assembly level	#scaffolds/ contigs	Bp [Mb]	scaffold/contig N50 [kb]	GC percent	Annotation	Accession	Reference
Heterorhabditis	*bacteriophora*	scaffold	1'240	77.0	312.3	33.0	yes	GCA_000223415.1	
	indica	scaffold	3'549	91.3	216.8	38.0	no	GCA_020740425.2	(Bhat et al., 2021)
Steinernema	*carpocapsae*	chromosome	16	84.5	7'400.0	45.5	yes	GCA_000757645.3	(Serra et al., 2019)
	carpocapsae	scaffold	347	84.6	1'240.0	45.7	yes	NA	(Rougon-Cardoso et al., 2016)
	diaprepsi	contig	35'417	118.2	11.5	45.0	no	GCA_013436035.1	(Baniya et al., 2020)
	feltiae	contig	4'678	121.6	60.4	46.0	no	GCA_007213375.1	(Fu et al., 2020)
	feltiae	scaffold	5'834	82.5	47.6	46.5	no	GCA_000757705.1	(Dillman et al., 2015)
	glaseri	scaffold	7'510	92.8	37.4	47.5	no	GCA_000757755.1	(Dillman et al., 2015)
	khuongi	contig	8'783	81.8	46.1	47.5	no	GCA_016648015.1	(Baniya and DiGennaro, 2021)
	monticolum	scaffold	14'252	89.2	11.6	41.5	no	GCA_000505645.1	(Dillman et al., 2015)
	scapterisci	scaffold	2'864	79.7	91.1	47.5	no	GCA_000757745.1	(Dillman et al., 2015)

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In this review, we provide an overview of EPN traits that modulate nematode tolerance to endogenous and exogenous stressors and thus their persistence in soil as IJs. We highlight the current knowledge about the involved genetic pathways underlying stress resistance in EPNs and in *C. elegans*. Finally, we emphasize some of the limitations associated with the breeding of superior EPNs and advocate mitigation plans for effective biocontrol strategies.

2. Endogenous stresses

2.1. Nutritional stress tolerance

IJs are nonfeeding organisms that rely on internal lipids (mainly triacylglycerols) and glycogen reserves for survival (Grewal and Georgis, 1999; The *C. elegans* Research Community). Neutral lipids represent between 24 and 31% of the nematode dry weight (Patel and Wright, 1997a). In IJs of *H. bacteriophora* and *H. megidis*, unsaturated fatty acids represent respectively 57% and 62% of the detected fatty acids. Interestingly, steinernematid species contain relatively more saturated fatty acids, as the latest can reach up to 70% of the lipid pool (Patel and Wright, 1997a; Selvan et al., 1993b; Selvan et al., 1993a). Among the 18 fatty acids detected in *Steinernema* species, oleic acid (C18:1n-9), palmitic acid (C16:0), and stearic acid (C18:0) decline over storage time (100 days), suggesting that these fatty acids are preferentially used as energy suppliers (Patel and Wright, 1997a). On the other hand, glycogen reserves in steinernematids ranges from 8% to 18% dry weight (Patel and Wright, 1997b) and their level seem to decline more slowly than lipids in young IJs, but tend to decline faster after lipid depletion. Thus, it is hypothesized that glycogen might be the alternative source of energy after lipids reserves depletion in aged IJs. This was experimentally shown in *S. carpocapsae,* as the EPN maintained its infectivity potential at low lipid levels when glycogen levels were high (Wright et al., 1997). Infectivity later markedly decreased with decline in glycogen reserves. IJ reserves are critical factors shaping the nematode survival, stress tolerance, motility, behaviour, and ultimately infectivity (Abu Hatab and Gaugler, 1997; Fitters and Griffin, 2006; Grewal and Georgis, 1999; Griffin and Fitters, 2004; Hass et al., 2002; Jagdale and Gordon, 1997; Patel and Wright, 1997b; Qiu and Bedding, 2000). Although the role of neutral lipids and glycogen as energy storage for IJs seems established, manipulative studies using the available information from *C. elegans* (e.g., 471 putative genes involved in the lipid metabolism, fatty acid pathway (Rappleye et al., 2003; Watts and Ristow, 2017), should be undertaken to disentangle their specific roles in IJ nutritional stress tolerance.

2.2. Oxidative stress tolerance

Oxidative stress is caused by an imbalance between reactive species (RS) production and antioxidant defenses (Sies, 2018; Sies, 1985). The production of RS, including reactive oxygen species (ROS) and reactive carbonyl species (RCS), results from the reduction-oxidation reactions associated with the use of oxygen (Halliwell and Gutteridge, 2015; Pamplona and Costantini, 2011). For instance, ROS are mostly produced by the electron transport system of mitochondria during ATP production (Zorov et al., 2014). As highly reactive molecules, RS can disrupt the cellular metabolism and their overproduction results in cell death (Frijhoff et al., 2015; Nanette and Tim, 2013; Sies, 2018; Sies, 1985; Zorov et al., 2014). RS are detoxified through several mechanisms, such as conjugation (e.g., to glutathione), oxidation (e.g., by aldehyde dehydrogenases), or reduction (e.g., by aldoketoreductases) (Detienne et al., 2018; Hulbert et al., 2007; Pamplona and Costantini, 2011; West and Marnett, 2006). In *H. bacteriophora*, IJ tolerance to H_2O_2 exposure correlates with their lifespan in sand (Sumaya et al., 2017). The crossing of two isolates and mutagenesis further revealed that tolerance to oxidative stress can be enhanced (Sumaya et al., 2018). These results are consistent with studies conducted in *C. elegans* demonstrating the role of the oxidation level on nematode lifespan. For instance, silencing antioxidant enzymes such as catalases (CTL-2), thioredoxins (TRX-1), peroxidases (PRDX-2 and PRDX-3), or glutathione-S-transferases (GST-5, GST-10) accelerates aging and decreases lifespan (Ayyadevara et al., 2007; Ha et al., 2006; Jee et al., 2005; Miranda-Vizuete et al., 2006; Oláhová et al., 2008; Petriv and Rachubinski, 2004). Noteworthy, not all RS detoxification enzymes are associated with nematode lifespan and some of them have tissue-specific functions (for a review in *C. elegans* see Shields et al. 2021). The impact of RS and their detoxification machinery on *C. elegans* is well advanced, however, how oxidative stress shapes EPN IJs persistence in soil remains elusive and should be further investigated.

3. Abiotic stresses

3.1. Low temperatures

EPN juveniles can persist in the soil for over a year in continental climates (Kurtz et al., 2007) and can cope with cold temperatures (Ali and Wharton, 2013). For instance, *S. monticolum* is adapted to ptolerate temperature as low as 5° C (Koppenhöfer et al., 2000). Yet, EPN tolerance to low temperature and the underlying mechanisms are species- and likely even isolate-specific (Godina et al., 2022; Grewal et al., 1994; Jagdale and Grewal, 2003).

IJs can withstand and acclimate to falling temperatures by lowering their metabolism activity (Ali and Wharton, 2013; Brown and Gaugler, 1996). While IJs/dauer nematodes already have a hypometabolism, IJs subjected to cold conditions further slacken their metabolism, as reflected by the slower use rate of their lipid reserves (Andaló et al., 2011). In another example, *H. megidis* IJs decrease the production of proteins involved in metabolism and protein synthesis (e.g., ribosomal proteins), and *S. carpocapsae* decreases the production of proteins involved in intermediary metabolism and oxidative phosphorylation (Jagdale and Grewal, 2003). Reducing the metabolism and protein synthesis, as in *H. megidis*, may be an effective strategy to prevent proteotoxic stress and to spare energy for the cruiser nematodes (Lillis et al., 2022). Decreasing intermediary metabolism and oxidative phosphorylation may limit energy loss and reactive oxygen species (ROS) production (Lillis et al., 2022).

Additionally, EPNs can enhance their tolerance to low temperatures by producing trehalose, a natural disaccharide (α-d-glucopyranosyl-1,1-α-d-glucopyranoside) involved in resistance to freezing in numerous organisms (Ali and Wharton, 2015; Grewal and Jagdale, 2002; Jagdale and Grewal, 2003; Kandror et al., 2002; Liu et al., 2019; NDong et al., 2002; Virgilio et al., 1994). For instance, cold shock induces the transient activity of the trehalose-6-phosphate synthase (T6PS), trehalose accumulation, and cold tolerance in *S. carpocapsae* and *H. bacteriophora* (Jagdale et al., 2005; Pellerone et al., 2003). Trehalose protects the cells from thermal injury by stabilizing proteins in their native state and preventing denaturation or aggregation (Erkut et al., 2011; Hottiger et al., 1994; Jain and Roy, 2009; Singer and Lindquist, 1998), decreasing damage by oxygen radicals (Benaroudj et al., 2001), and stabilizing lipid membranes (Crowe et al., 1998a; Crowe et al., 1984; Leslie et al., 1994). In EPNs and in *C. elegans*, two trehalose phosphate synthases, Tps-1 and Tps-2, were reported (Depuydt et al., 2014; Murphy et al., 2003; Sonoda et al., 2016). In *C. elegans*, the two isoforms are under the control of one transcription factor (DAF-16) (Depuydt et al., 2014; Murphy et al., 2003; Sonoda et al., 2016). Trehalose is likely released in the intestines and transported via the glucose transporter FGT-1 over the plasma membrane into the pseudocoel (Feng, 2010; Kitaoka et al., 2013). Finally, trehalose and late embryogenesis proteins can promote bioglass formation and enhance EPN tolerance to freezing temperatures (Qiu and Bedding, 2002). Understanding the regulation of trehalose production may offer promising targets to enhance cold tolerance in EPNs.

Some EPN species further respond to cold stress by producing stress and detoxification proteins (Lillis et al., 2022). Incubating EPNs at 9°C promotes the production of dehydrogenases, thioredoxins, glutathione-S-transferases (GSTs), catalase, and oxidoreductases in *S.*

carpocapsae (Lillis et al., 2022). In nematodes, the antioxidant-, detoxification-, and unfolding protein response-pathways are regulated by the transcription factor SKN-1, a critical player for nematode longevity (Choe et al., 2012; Ewald et al., 2015; Gillet et al., 2017).

In *C. elegans*, low temperatures induce epidermal-specific autophagy (Chen et al., 2019). Autophagy is a process that maintains intracellular homeostasis by degrading and recycling cytoplasmic components through a lysosomal pathway (Deter et al., 1967; Levine et al., 2011; Megalou and Tavernarakis, 2009; Mizushima, 2007; Palmisano and Meléndez, 2019). The genetic and molecular pathways involved in autophagy are well characterized in the model nematode species and are likely to be conserved across the different nematode clades (Fu et al., 2022; Palmisano and Meléndez, 2019). The product of PAQR-2 detects low temperatures and upregulates poly-unsaturated fatty acids, gamma-linolenic acid, and arachidonic acid, which in turn increases the ratio between unsaturated and saturated fatty acids and increases epidermal autophagy (Chen et al., 2019; Svensk et al., 2013). Silencing genes such as *bec-1, let-512, or epg-1* in *C. elegans* shortens the nematode lifespan when exposed to low, but not to ambient, temperatures (Chen et al., 2019; Liang et al., 2012; Takacs-Vellai et al., 2005). Interestingly, in their study, Lillis et al. (2022) noted that an autophagy-related protein accumulated over storage time in *H. megidis*, and that the protein levels increased faster at 9°C than at 20°C, suggesting a possible role of autophagy in cold tolerance in EPNs.

Additional cold-tolerance mechanisms were described in *C. elegans*, including sensing by transient receptor potential (TRP) channels, activation of a PKC-2/$Ca2^{+}$-sensitive protein kinase C, activation of DAF-16/FOXO, or phosphorylation (Okahata et al., 2022). Whether similar mechanisms also exist in EPNs remains to be investigated.

3.2. Elevated temperatures

Elevated temperatures (>32 °C) have detrimental effect on EPN reproduction, growth, survival and pathogenicity (Grewal et al., 1994; Kung et al., 1991; Zervos et al., 1991). However, some species have been isolated from hot deserts (Edgington et al., 2011; Glazer et al., 1991; Kusakabe et al., 2019). This shows that some EPNs have evolved adaptive mechanisms to tolerate elevated temperatures. Up to date, whether the variation in tolerance is genetically determined or plastic remains unclear.

Efforts have been made to elucidate genetic information linked to high temperature tolerance. For example, comparing *H. bacteriophora* isolates with contrasting heat highlighted significant

downregulation of transcript levels in the heat tolerant (EN01) than in the heat sensitive (Grofit) nematodes (Levy et al., 2020). EN01 transcripts include glycerol kinase (GK), fatty acid desaturase (FAD), and a zinc finger protein (ZFP). Down regulation of TRE (trehalose)-stress responding in Grofit while the heat shock proteins (HSPs) and related pathways were up regulated in of both natural variants. HSPs catalyze the unfolding of either native or denatured proteins enabling the cell to eliminate or renature proteins damaged by high temperature (Candido et al., 1989). High genetic variation in one of the family protein, HSP70, has long been reported using the Restriction Fragment Length Polymorphism (RFLP) among 6 different high temperature surviving EPN species from different geographical locations (Hashmi et al., 1997). However the involvement of these HSPs in heat tolerance is not well understood (Glazer, 1996). In other studies, heat stress causes accumulation of trehalose as well as a shift in enzyme activities in the trehalose metabolism (T6PS and trehalase). For example, the activity of T6PS and trehalase increases and decreases, respectively, during the heat shock (Jagdale et al., 2005; Jagdale and Grewal, 2003). Trehalose accumulation might be an adaptive way to cope with thermal stress or preparation for desiccation stress that will subsequently follow due to evaporation. The genetic manipulation and or modification to attain heat tolerance in EPNs is possible through continuous exposure of EPNs to heat stress. For instance, in one study, hybrids of *H. bacteriophora* (PS7 and H06 crosses), have increased their mean temperature tolerance from 38.5 to 39.2°C after four selection steps (Ehlers et al., 2005).

Although the genetic mechanisms of heat tolerance in EPNs are not clear yet, studies in *C. elegans* have shown that the model organism is equipped with systems involved in tolerating the adverse effects of high temperatures stress. The Heat shock transcription factor-1 (HSF-1) and the forkhead box O (FOXO) transcription factor DAF-16 upregulate chaperone expression after acute heat exposure (Park et al., 2017). According to previous studies, HSF1 targets genes encoding molecular chaperones, that are essential for protein folding, preventing misfolding, and restoring the native conformation of misfolded proteins. Thus HSF1 promotes stability by preventing protein aggregation and subsequent proteome imbalance (Li et al., 2017; Servello and Apfeld, 2020).

3.3. Desiccation

EPNs are soil organisms living in large soil pores on surface films of water or at relative humidity > 97% (Jung et al., 2012; Navaneethan et al., 2010; Neher, 2010). They possess a hydrostatic skeleton, inferring that the nematode body is supported by fluid pressure (Neher, 2010; Riddle et

al., 1997; Wallace, 1971). Low water content may thus drastically limit their motility and survival (Glazer, 2002; Grant and Villani, 2003; Kaya, 2018).

Nematodes have evolved behavioural and biochemical strategies to cope with unfavourable soil moisture levels. The model worm *C. elegans* can navigate towards more favourable environment following a gradient in soil moisture, a process referred to as hygrotaxis (El-Borai et al., 2016). The nematode perceives and responds to changes in water gradients as shallow as 0.03% (Russell et al., 2014). Hygrotaxis requires the activation of both mechanosensitive- and thermosensitive pathways (Hibshman et al., 2020; Russell et al., 2014). The mechanosensitive pathway relies on a conserved DEG/ENaC/ASIC mechanoreceptor complex in the FLP neuron pair in the nematode cuticle (Russell et al., 2014). FLP neurons are located below the epidermis and are likely sensitive to changes in cuticle stretch by hydration (Russell et al., 2014). The thermosensitive pathway is activated through cGMP-gated channels in the AFD neuron pair (Russell et al., 2014). The relevance of thermosensing during the detection and response to dry environment was suggested to reflect the drop of temperatures due to water evaporation in moister soil layers (Russell et al., 2014). Signalling pathways involving a patched related protein (DAF-6), a notch ligand (OSM-11), and mitogen-activated protein kinases (MAPK) were further suggested to mediate the nematode behaviour (Banton and Tunnacliffe, 2012). Interestingly, hygrotaxis depends on the nutritional status of the nematode, as dry conditions only trigger *C. elegans* directional movements in starved conditions (Russell et al., 2014). Although yet untested, similar mechanisms likely drive moisture perception and response in EPNs.

Under severe dehydration, EPNs enter in anhydrobiosis (Gal et al., 2005a). Anhydrobiosis is a reversible physiological process by which an organism loses up to 98% of its body water up to metabolism arrest, a state called cryptobiosis (Cooper and van Gundy, 1971; Womersley, 1981). EPNs can reach partial anhydrobiosis (decreasing oxygen consumption by ~80%, (Grewal, 2000a)), but not cryptobiosis, and are thus considered as quiescent anhydrobiotes (Gaugler and Kaya, 1990; Simons and Poinar, 1973; Womersley, 1990). Desiccation elicits a transient increase in EPN metabolism, prior to slowing it down to levels below the normal metabolic rate (Grewal, 2000b). The glycogen production decreases, as reflected by the down-regulation of the glycogen synthase (*gsy-1*), while trehalose and glycerol synthesis from existing glycogen and neutral lipid reserves increases (Behm, 1997; Crowe and Crowe, 1992; Gal et al., 2001; Qiu et al., 2000; Womersley, 1990). Trehalose progressively replaces water in membranes and contributes to maintaining the structures and stabilizing proteins (Behm, 1997; Crowe et al., 1998b; Crowe and Crowe, 1992; Elbein et al., 2003). In *S. feltiae*, desiccation triggers a two-fold increase in

trehalose contents (Solomon and Glazer, 1999). Additionally, the casein kinase (CK2) is induced and elicits the transcriptional activation of a nucleosome-assembly protein (NAP-1) through physical interaction (Gal et al., 2005b; Gal et al., 2003; Somvanshi et al., 2008). Osmoregulant molecules (e.g. produced by ALDH), antioxidants (e.g., Gg., DESC47, HSP40) are further synthesized and may further protect the cells from desiccation-induced damage (Gal et al., 2003; Solomon et al., 2000; Somvanshi et al., 2008).

While *C. elegans* dauer nematodes are true anhydrobiotes which can lose more than 98% of their water content (Erkut et al., 2011; Madin and Crowe, 1975), some of the genetic and molecular mechanisms underlying desiccation responses are likely to be similar to those of EPNs' (for review see Hibshman et al., 2020). These mechanisms include the production of trehalose (by trehalose 6-phosphate synthases TPS-1 and TPS-2), an upregulation of the glyoxylate shunt to synthesize succinate and malate (by the isocitrate lyase and malate synthase enzyme ICL-1), the synthesis of late embryogenesis abundant proteins (LEA proteins), heat shock proteins (F08H9.3 and F08H9.4), antioxidant enzymes (SOD-1, GPX-2, GPX-6, GPX-7, CTL-1), and the desaturation of fatty acids by fatty acid desaturases (FAT-3 through FAT-7) (Abusharkh et al., 2014; Braeckman et al., 2009; Erkut et al., 2016; Erkut et al., 2013; Gal et al., 2004). The accumulation of trehalose, heat-shock proteins, and LEA proteins is further associated with the formation of glasses in a dry state, a process referred to as vitrification, which stabilizes biomaterials and avoid free-radical oxidation, lipid phase transition and protein crystallization (Crowe, 2002; Crowe et al., 1998a; Sun and Leopold, 1997). Finally, LEA proteins act as molecular shields around proteins and prevent them from aggregating (Goyal et al., 2005; Wise and Tunnacliffe, 2004). EPN ability for water replacement, vitrification, and molecular shielding under desiccation may be targeted as promising trait for EPN selection (Hibshman et al., 2020).

Desiccation-induced quiescence can increase (e.g., *S. carpocapsae*) or decrease (e.g., in *S. feltiae*) EPN lifespan, but has no or limited effect on their virulence when stored at room temperature (25°C) (Grewal, 2000a; Grewal, 2000b, 1998; Matadamas-Ortiz et al., 2014). The differential effect of quiescence on EPN lifespan has been attributed to their foraging lifestyles. Ambushers (e.g., *S. carpocapsae*) may be more exposed to desiccation during nictation than mobile cruisers (e.g., *S. feltiae*) and may thus have been under higher selection pressure to tolerate desiccation stresses (Grewal, 2000a; Patel et al., 1997). However, a concomitant storage of desiccated EPNs at lower temperatures reduce EPN lifespan (Grewal, 2000a). This sharp effect may be explained by the costs associated with desiccation and low temperature tolerance costs (Grewal, 2000a).

Desiccation tolerance is increased in EPNs being exposed to dehydration gradually rather than rapidly (Simons and Poinar, 1973; Womersley, 1990). Preconditioning EPNs to sub-lethal dehydration levels elicit an adaptive response that enhances their tolerance to subsequent, stronger, desiccating conditions (Kung et al., 1991; Nimkingrat et al., 2013; Patel et al., 1997; Strauch et al., 2004). The potential of the adaptive response is species and isolate specific (Grewal, 2000a; Nimkingrat et al., 2013; Somvanshi et al., 2008). The selection of desiccation tolerant isolates was successful in several studies (Mukuka et al., 2010b; Mukuka et al., 2010a), although this trait is rapidly lost during *in vivo* mass production when the selection pressure is relieved (Anbesse et al., 2013; Mukuka et al., 2010c).

3.4. Osmotic Stress

Nematodes can experience osmotic stress due to fluctuations in soil moisture levels. In dry soil conditions, nematodes may experience hypertonic environments (higher solute concentration compared to the intracellular solute concentration). As the soil dries out, the increasing concentration of soil solutes creates an osmotic gradient that draws water out of the nematode's body. Conversely, in wet soil conditions (e.g., after intense precipitations, soil amendments, or irrigation), nematodes may encounter hypotonic environments. The lowering solute concentrations can result in water entering the nematode's body, leading to swelling and cell damage.

Entomopathogenic nematodes have evolved several strategies to cope with osmotic stresses, although the response seems to be isolate- and salt-specific (Edmunds et al., 2021; Feng et al., 2006; Finnegan et al., 1999; Liu et al., 2002; Nielsen et al., 2011; Qiu et al., 2000; Thurston et al., 1994; Yan et al., 2010). First, nematodes may move towards areas with more favorable conditions. This phenomenon was show in an experiment with *H. indica* with about 35% nematode movement towards control conditions as compared to 0% towards above 30 dSm^{-1} salinity levels (Nielsen et al., 2011). Second, the nematode's outer cuticle serves as a protective barrier against water loss in hypertonic environments. It helps reduce desiccation and prevents excessive water influx during hypotonic conditions, providing a degree of osmotic protection (Lee and Atkinson, 1976; Wright, 1998). Third, EPNs may regulate their internal osmotic balance by adjusting the concentration of solutes within their cells. They may accumulate sugars or amino acids to counterbalance the osmotic gradients and maintain water balance (Chen et al., 2006). Ultimately, EPNs can enter partial anhydrobiosis rendering EPNs largely resilient to extreme conditions (Chen and Glazer, 2004; Finnegan et al., 1999; Glazer and Salame, 2000; Lamitina et al., 2004; Thurston et al., 1994; Wright, 1998). This osmotic dehydration involves the synthesis of

protectants, mostly glycerol and trehalose, from lipids and/or glycogen (Gal et al., 2004; Gal et al., 2001; Qiu et al., 2000). In EPNs, osmotic dehydration induces numerous genes involved in the production of osmoprotectants and proteins. Osmotic dehydration induced LEA protein (LEA), (NAP-1) and Casein kinase 2 (CK2) genes in *S. feltiae* (Gal et al., 2003). LEA-1 is essential for tolerance to osmotic stress in *C. elegans,* as silencing the gene normally expressed in the body wall muscle resulted in a specific and significant reduction in worm survival during induction of desiccation, osmotic and heat stress (Gal et al., 2004). Osmotic dehydration further induced the production of heat shock protein 60, coenzyme q biosynthesis protein, inositol monophosphatase, fumarate lyase, Actin, Proteasome regulatory particle (ATPase-like), GroEL chaperonin, GroES co-chaperonin and Transposase family member (Chen et al., 2005; Gal et al., 2003). Overall, the molecular processes involved in osmotic stress tolerance seems to be similar in EPNs and C. elegans (see for instance (Lamitina et al., 2004)(Possik et al., 2015; Rasulova et al., 2021; Solomon et al., 2004; Yu et al., 2017) for *C. elegans*). The advantages of osmotic dehydration to enhance EPNs resilience to different stresses can be exploited for optimizing EPNs formulations (Chen and Glazer, 2005; Kagimu et al., 2017; Qiu et al., 2000).

3.5. Hypoxia

Nematodes are aerobic organisms. They use oxygen to fuel mitochondrial energy production and to ensure a proper metabolism. In soil, oxygen levels are naturally lower than ambient levels (21%) due to root- and microbial respiration and/or soil water contents, and ranges between 0.5 and 21% (Félix and Braendle, 2010; Grant and Villani, 2003). Hypoxia (<1% oxygen for worms like *C. elegans*) limits the cell mitochondrial respiration and energy production, disrupts proteostasis (Fawcett et al., 2015). EPN tolerance to hypoxia is highly variable among species and isolates (Burman and Pye, 1980; Grewal et al., 2002; Kour et al., 2021; Morton and García-del-Pino, 2009). For instance, the survival rate of different *H. bacteriophora* isolates ranges from 10 to 90% after four day exposure to hypoxia (Grewal et al., 2002). Nematodes have evolved behavioural and physiological strategies to prevent hypoxia-induced detrimental effects on their virulence, longevity, and survival.

Nematodes can perceive environmental oxygen levels and migrate up or down its concentration gradients. While this behaviour remains elusive in EPNs, it has been investigated in *C. elegans* (Choudhry and Harris, 2018; Gray et al., 2004; Kitazume et al., 2018; Kumar, 2016). Interestingly, the oxygen sensing neurons (AQR, PQR, URX, BAG) involved in *C. elegans* repellence from elevated (21%) levels of oxygen do not mediate the nematode response to hypoxia (Zhao et al., 2022). Instead, the sensory neurons ADL and ASH seem to interact with signals from neurons

429 producing 3',5'-cyclic guanosine monophosphate (cGMP), a major second messenger, to
430 modulate the nematode response (Zhao et al., 2022). Additionally, mitochondria are critical in
431 sensing and responding to hypoxic conditions. Hypoxia triggers a reversible oxidation of a
432 cysteine residue (Cys366) in the mitochondrial complex I subunit NDUF2.1, causing a decrease
433 in the complex activity (Onukwufor et al., 2022). This decrease in turn modulates the production
434 of ROS in a site-specific manner, a process sufficient to elicit a behavioural response to hypoxia
435 (Kaelin and Ratcliffe, 2008; Onukwufor et al., 2022). In parallel, the conserved oxygen-activated
436 prolyl hydroxylases (PHDs) can also act as oxygen sensors and activate the hypoxia adaptation
437 program (Bruick and McKnight, 2001; Darby et al., 1999; Epstein et al., 2001; Ivan et al., 2002;
438 Kaelin and Ratcliffe, 2008; Trent et al., 1983). In *C. elegans*, a single family member of PHD is
439 known (EGL-9) (Epstein et al., 2001). Under hypoxic conditions, the lower PHD activity leads to
440 the inhibition of the glutamate receptor (GLR-1) recycling, itself inducing a roaming behaviour,
441 and to the upregulation of the hypoxia inducible factor (HIF-1) and its target genes, including
442 genes involved in the energy metabolism, pathophysiological processes, cell differentiation, and
443 apoptosis (Dengler et al., 2014; Kumar, 2016; Liu et al., 2012; Ma et al., 2012; Park and Rongo,
444 2016; Powell-Coffman, 2010; Shen et al., 2006; Vora et al., 2022). Finally, the nuclear hormone
445 receptor NHR-49 is required to orchestrate a *hif-1* independent response to hypoxia, including
446 detoxification, and autophagy genes (Doering et al., 2022). The orchestrated response to low
447 oxygen levels enables the nematode to switch from mitochondrial respiration to anaerobic
448 glycolysis while maintaining its homeostasis (physiological functions), and ultimately support
449 the nematode survival (Doering et al., 2022; Shih et al., 1996).

450 3.6. Ultra-violet (UV) tolerance

451 UV light exposure elevates the mutation rates in the genome. EPN IJs can suffer from UV
452 irradiation, although an important variability between isolates and between studies should be
453 noted. For example, UV radiations drastically decreased IJ virulence and survival in *S.*
454 *carpocapsae* in three out of four studies (Fujiie and Yokoyama, 1998; Gaugler and Boush, 1978;
455 Jagdale and Grewal, 2007; Shapiro-Ilan et al., 2015). While UV exposure was similar in both
456 studies in terms of wavelength (254 nm) and exposure duration (7-10 min), several hypotheses
457 can explain the apparent discrepancy. First, the UV irradiation intensity is a crucial parameter to
458 consider. Studies in *C. elegans* demonstrate that UV irradiations at 20 J/m^2/min triggers severe
459 stresses, while irradiations at 10 J/m^2/min elicit limited toxicity (Wang et al., 2010). Second, pre-
460 exposure to a mild, sub-lethal, stress can elicit an adaptive response of the organism, leading to
461 increased tolerance to higher stress levels (Yanase et al., 1999). When compared within the

same study, steinernematids have a higher tolerance to UV exposure than heterorhabditis (Shapiro-Ilan et al., 2015).

Several UV tolerance mechanisms were elucidated in *C. elegans*. The model nematode can perceive and avoid UV light through (at least) four sensory neurons (ASJ, ASK, AWB, ASH) (Bargmann, 2006; Ward et al., 2008). ASH and ASK neurons express a seven-transmembrane protein (LITE-1) UV light receptor (Gong et al., 2016) whose activation triggers the release of a neurotransmitter, glutamate (Ozawa et al., 2022). Glutamate receptors (*glc-3*, *mgl-1*, *mgl-2*) are expressed only in ASH and ASK (glutaminergic neurons) (Ozawa et al., 2022). The mechanisms involved in ASJ and AWB activation in response to UV light remain yet more elusive. Using similar technologies than in *C. elegans* (e.g, neuron ablation, genetically inserted synapses, RNAi) will contribute to better understanding whether the same cellular and molecular mechanisms are involved in EPNs perception and behavioral responses to UV irradiations. Upon exposure to UV, animal cells activate surveillance mechanisms responsible for cell cycle arrest, DNA repair, and apoptosis in case of elevated DNA damage (Bailly and Gartner, 2013; Fortunato et al., 2021) (Bailly and Gartner, 2013; Fortunato et al., 2021). These cellular mechanisms have been extensively studied in *C. elegans* and their underlying pathways have been mostly elucidated (for review see (Canchaya et al., 2003; Elsakrmy et al., 2020). Because DNA damage response pathways are highly conserved in animals (Clancy, 2008), it is likely that EPNs undergo similar processes upon UV exposure. Understanding the impact of UV light on EPN biology is critical when using inundative or foliar pest control strategies.

4. Biotic stresses

4.1. Viruses

To date, no viral pathogen is known to infect EPNs in soil, although the presence of bacteriophages has been described in the nematode endosymbiont genomes (Canchaya et al., 2003; Kaya et al., 1998). Accumulating genomic resources such as RNAseq data will likely identify a diversity of viruses interacting with EPNs. Several viruses are indeed known to infect *C. elegans* (Félix et al., 2011). The nematode anti-viral response includes RNA interference (RNAi), RNA uridylation, and intracellular pathogen response (IPR) (for review see (Martineau et al., 2021). The natural variation in *C. elegans* resistance to viral infections revealed the crucial role of the dicer related helicase DHR-1 protein, a pattern recognition receptor (PRR) that mediates the intracellular pathogen response (IPR) program (van Sluijs et al., 2021). Whether and which

493 viruses can infect EPNs, and which markers are critical for EPN immune response remains to be
494 elucidated.

495 4.2. Bacteria

496 Soil bacteria can attach to nematodes' cuticle (Adam et al., 2014; Topalović et al., 2019)
497 Pathogenic bacteria have been isolated from free-living nematodes (Schulenburg and Félix,
498 2017), plant pathogenic nematodes (Adam et al., 2014; Elhady et al., 2017), and EPNs (Bajaj and
499 Walia, 2005; Enright et al., 2003). For instance, the screening of EPN soil isolates identified
500 *Pasteuria* sp. on the cuticle and inside *S. pakistense* IJs (Bajaj and Walia, 2005). In addition to
501 that, recently, several other bacteria were washed-off from soil borne nematodes´ cuticle and
502 most of them proved to be pathogenetic against *Galleria mellonella* larvae (Loulou et al., 2023).
503 The entomopathogenic bacterial strains isolated by the authors include *Lysinibacillus fusiformis,*
504 *Kaistia sp., Alcaligenes sp., Enterobacter sp., Klebsiella quasipneumoniae* subsp.
505 *quasipneumoniae, Bacillus cereus, Acinetobacter sp., Pseudomonas aeruginosa, and*
506 *Morganella morganii* subsp. *morganii* (Loulou et al., 2023). The presence of the endospore-
507 forming bacteria is associated with the abolition of virulence in EPNs (Bajaj and Walia, 2005). The
508 EPN immune response to bacterial agents remains unclear. In *C. elegans*, a pathogen attack is
509 detected through PRRs that detect pathogen-associated molecular patterns and/or
510 disturbances in cellular homeostasis and triggers cell autonomous and non-autonomous
511 responses (Martineau et al., 2021). Interestingly, *C. elegans* is able to detect and evade
512 pathogenic bacteria (Bai et al., 2013; Kim and Flavell, 2020; Schulenburg and Müller, 2004; Tran
513 et al., 2017). For example, the worm can detect and avoid bacterial products, such as the exolipid
514 serrawettin W2 from *Serratia marcescens* (Pradel et al., 2007). Understanding how EPNs detect
515 and respond to specific pathogens may provide target markers that enhance EPN survival in soil.

516 4.3. Fungi

517 Nematophagous fungi are common nematode enemies and can be classified as nematode-
518 trapping (predators), endoparasitic, or egg- and cyst- parasites (Lopez-Llorca and Jansson, 2007;
519 Pathak et al., 2017; Soares et al., 2018). The presence of nematophagous fungi in the soil can
520 decrease EPN populations and their biocontrol efficacy (Kaya and Koppenhöfer, 1996; Soares et
521 al., 2018). EPNs can protect themselves from the fungal predators/parasites through several
522 mechanisms. First, cruising nematodes with high mobility can move away from a fungal trap
523 (Bright et al., 2009; Karthik Raja et al., 2021). Second, heterorhabditid species conserve their
524 second-stage cuticle after moulting for some time, often until invasion into the insect, which

provides them an enhanced protection from fungal infection (Timper and Kaya, 1989). Third, EPNs can detect and avoid fungal chemical cues, although several fungal species have evolved to produce nematode attractants (El-Borai et al., 2011). Finally, EPN can learn cues associated with the presence of fungi and avoid them in subsequent exposure (Willett et al., 2017). In *C. elegans*, the detection of nematophagous fungi is achieved through mechanical and chemical perception, via the tyramine signalling (Maguire et al., 2011; O'Donnell et al., 2020). Fungus-induced cuticular damage and fungal polysaccharides further activate the worm immune system (e.g., production of antimicrobial peptides) via pattern recognition receptors (PRRs) (Martineau et al., 2021). EPNs with enhanced physical or immune defenses, as well as "educated" EPNs, may provide future avenues to treat fields with high nematophagous fungi density and ensure EPN efficacy.

4.4. Insects

Predatory collembolas, such as *Folsomia candida, Sinella curviseta*, or *Hypogasturas cotti*, prey on EPNs, reducing their biocontrol efficacy in natural conditions (Epsky et al., 1988; Gilmore and Potter, 1993; Hodson et al., 2012; Ishibashi et al., 1987; Read et al., 2006; Ulug et al., 2014). Although very few studies investigated the EPN defenses against insects, it can be hypothesized that nematodes and/or their endosymbionts, are producing and exuding a range of chemicals that repels their enemies.

5. Engineering superior nematodes

5.1. Artificial selection and genetic engineering methods

Understanding mechanisms that modulate EPN IJ persistence in soil will be key to develop "superior" (more resistant or tolerant) nematodes and thus to enhance pest biocontrol. Engineering superior EPNs can be achieved through artificial selection or genetic engineering (Lu et al., 2016). The short life cycle of EPNs and their ease of mass cultivation render them ideal to select traits of interest.

Artificial selection can be conducted by exposing EPNs to a chosen selection pressure (e.g., desiccation, low temperature) for several generations. Genetic traits that promote EPN fitness under the imposed stress may then be selected. Many studies successfully improved EPN tolerance to diverse stressors, such as cold (Koppenhöfer et al., 2000), plant secondary metabolite (Zhang et al., 2019), plant volatiles (Hiltpold et al., 2010), etc. Sequencing the genome of the first and last, selected, EPN generations may further allow to elucidate the genetic

pathways/markers involved in tolerance. However, as several pathways can be concomitantly involved in tolerance to one stress, it is judicious to split the initial EPN population in several sub-populations each ongoing artificial selection in parallel to compare the obtained results. Additionally, selecting several superior isolates in variable genetic backgrounds is recommended to prevent trait deterioration through inter-crossing (Bilgrami et al., 2006; Chaston et al., 2011). Not only have EPNs been selected under stressful conditions, but also their endosymbiontic bacteria alone. The best example was shown in an experimental evolution study where five *Photorhabdus* symbionts from different nematodes were selected in benzoxazinoids. The selected endosymbionts were later reintroduced to their host isolate and one of the selected bacteria significantly improved the infectivity of the host isolate against benzoxazinoid-sequestering western corn rootworm (Machado et al., 2020).

Genetic engineering tools include mutagenesis, transgene insertion, and genome editing tools. Mutagenesis can be performed by exposing nematodes to chemical agents such as ethyl methane sulfonate (EMS) (Liu et al., 2012; Sumaya et al., 2018). The induced DNA mutations may (or may not) lead to the appearance of a desired phenotype that can then be stabilized. Transgenes can be inserted into the gonads of adult hermaphrodites, resulting in extrachromosomal arrays that can be further incorporated into the genome (Mello et al., 1991). However, the insertion site of the sequences is not controlled in these assays (Praitis et al., 2001; Wilm et al., 1999). To palliate these limitations, several genome editing technologies were developed, such as zinc-finger nucleases (ZFNs), transcriptional activator-like nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) (Sugi, 2016). ZFNs and TALENs are engineered site specific nucleases that induce double strand breaks at desired loci (Wood et al., 2011). The breaks are then repaired by non-homologous end-joining, resulting in insertions and deletions at the site of interest (Wei et al., 2014; Wood et al., 2011). CRIPSR/Cas9 allows targeted genome editing by incorporating foreign nucleotides into the CRISPR locus of the host genome, resulting in the production of CRISPR RNAs (crRNAs) and to the sequence-specific cleavage of homologous target dsDNA by Cas endonucleases (Jinek et al., 2012). CRISPR/Cas9 can induce heritable changes in the nematodes' genome (Bortesi and Fischer, 2015; Chiu et al., 2013; Frøkjær-Jensen, 2013; Lo et al., 2013; Tzur et al., 2013).

5.2. Selection limitations

Engineering EPNs that are superior in tolerating some stresses can be associated with some limitations. First, the success of selection depends on the trait heritability (h^2) (Hartl and Clark,

1997). For instance, the heritability of oxidative stress tolerance is of h^2>0.9 in *H. bacteriophora*, implying a high probability for the tolerance trait to be transmitted to the next generation (Sumaya et al., 2018). Second, the selected traits may deteriorate in absence of the selective pressure (Anbesse et al., 2013; Chaston et al., 2011). Third, selecting specific traits may come at the expense of other traits relevant for EPN efficacy (persistence in soil, but also infectivity, reproduction, or storage ability). Neutral (e.g., between desiccation and freezing tolerance) (Sumaya et al., 2017), negative (e.g., between desiccation tolerance and EPN fitness) (Gaugler and Kaya, 1990), and positive (e.g., between heat-tolerance and longevity) (Grewal et al., 2002) correlations were reported. A better understanding of the genetic mechanisms underlying the different responses will be crucial in predicting the occurrence of possible cross-talks. Genetic improvement should always be associated with a thorough characterization of the EPN virulence, longevity, and reproduction abilities, ideally under field conditions (Bilgrami et al., 2006; Gaugler et al., 1989).

6. Conclusion

EPNs are promising biological control agents of numerous agricultural pests. Yet, their variable outcome in controlling pest populations in the field has hindered their use. Engineering superior EPNs with enhanced tolerance to endogenous and exogenous stresses encountered at the free-living stage (IJs) will likely promote their efficacy. Importantly, the selection of traits of interest should be performed according to the specific characteristics of the field and target pest. Using such a precision approach may reinforce the use of biological control in sustaining food production and food security.

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Study model

Heterorhabditis bacteriophora, a well-established EPN model organism to investigate the evolutionary, ecological, and genomic mechanisms underlying adaptation to plant specialized metabolites sequestered by their insect hosts

The Entomopathogenic nematode *Heterorhabditis bacteriophora*

Entomopathogenic nematodes (EPNs) are aquatic, soil dwelling nematodes which infect and kill insects (Koppenhöfer et al. 2020; Gaugler and Kaya 1990). They are representatives of two either of two genera, *Steinernema* and *Heterorhabditis*, respectively, which are the only uncontested members of the EPN clade, while several other nematode genera and species which are also known to parasitise insects, they differ in the fact that only EPNs kill their host within a few days after infection, whereas the other insect parasitic nematodes usually live within the living insect. The genus Heterorhabditis is associated with a symbiotic bacterium, *Photorhabdus*, which is an integral part of the life cycle and strategy of the nematode. Axenic Heterorhabditis individuals, while being able to a kill their host insect through a combination of physical damage and venom, are not able to successfully infect a host and therefore reproduce. But in combination with their symbiont, they can quickly and efficiently kill insect hosts, and this ability has been studied for their potential as natural pesticides 1980 and they have been actively used in a wide range of agricultural applications for a large part of them. However, despite this ecologically interesting and promising natural herbivore control agent, not much has previously been studied regarding their genome. A reference genome has been created by (Bai et al. 2013) and the annotation has been updated by (McLean et al. 2018). Yet, due to the fragmented state of the reference genome, it did not lend itself to more in-depth analysis of chromosomal regions and interactions, and not many studies have been conducted using it (Vadnal et al. 2017; Kenney et al. 2019) and

Maize, benzoxazinoids and the western corn rootworm

Maize is an important cereal crop grown widely, which contributes a lot of calories to human consumption, and is also used for animal feed. Maize has been cultivated agriculturally for around 9000 years, with a beginning in Mexico and continued expansion through the Americas. It has been introduced in Europe in the 1500 and is cultivated nearly globally (Rebourg et al. 2003). Maize, along with several other cereal crops such as wheat, produces a group of compounds

called benzoxazinoids (Robert and Mateo 2022). Benzoxazinoids are indole derived compounds which serve a double function, partly for nutrient acquisition, particularly iron, and partly as a defence compound (Wouters et al. 2016a; Hu et al. 2018a; Hazrati et al. 2020). They are stored spatially separate, with glucosides being stored in vacuoles which only encounter β-glucosidases upon tissue disruption, such as an herbivore attack, or upon release into the soil. Together, they then form reactive, unstable aglycones, which in turn spontaneously degrade into benzoxazolinones (Wouters et al. 2016b; Hu et al. 2018b). These compounds are toxic to herbivores but have the ability to chelate iron in the soil, thereby allowing the plant to more easily acquire essential nutrients for growth and reproduction (Zhou et al. 2018). In the herbivore, the resulting toxin can have anti-feeding, anti-digestive, or toxic effects (Wouters et al. 2016a). Benzoxazinoids are also known to have effects on the cellular level, as such they can interact with proteins and thereby impact the activity of digestive enzymes, and they also interact with DNA, as they can intercalate in there, thereby causing damage to the genetic material and affecting all downstream processes, such as transcription and translation and any proteins that are formed based on the damaged DNA (Hashimoto and Shudo 1996). These effects are a major problem for many herbivorous insects, but there are some that have adapted to these compounds and specialized in feeding on maize, such as the western corn rootworm (WCR, *Diabrotica virgifera virgifera*, Poinar). It is a maize specialist herbivore pest, whose larval stages feed on the roots of maize plants and whose adults can feed on leaves or reproductive organs of maize plants (cobs, silk etc) (Elliott et al. 1990). They originate in the same region as maize and have spread from Mexico throughout much of the Americas (Lombaert et al. 2018). Differently to maize they have only relatively recently been introduced to Europe, with first records occurring in the early 1990s, and have spread since then (Baca 1994). The WCR is an economically significant herbivore pest, causing >2 billion USD yearly in the US alone, both in yield loss and in pest management strategies (FAO 2017). They have developed many coping mechanisms to deal with conventional pest management strategies. They can circumvent crop rotation strategies, particularly the widely distributed maize/soybean rotation by feeding on soybean plants as adults and laying eggs in soybean fields so they can emerge in newly planted maize fields the following year (Miller et al. 2007). They have a high tolerance for chemical pesticides and genetically modified maize plants trigger adaptations in WCR which largely negate the benefits of the adaptations in what can be considered an evolutionary arms race (Meinke et al. 2021). Their coping strategy in response to BXs produced by maize is even more intricate, as they have evolved the ability to feed on maize and instead of being harmed by the toxins present therein,

they stabilize them through glycosylation and sequester them to use against their own enemies (Machado et al. 2020; Robert et al. 2017).

Interaction and evolutionary history

The ability of the very generalist EPNs to act in a wide range of insects makes them economically interesting, since they can we used to control a wide variety of herbivore pests. However, this same generality may lessen their effect on more specific threats. As such, the very specific defense the western corn rootworm employs through sequestration of plant specialized metabolites in their glycosylated form can be challenging for such a generalist natural enemy (Zhang et al. 2019). The state of the benzoxazinoids that is sequestered in WCR larvae is non-toxic, hence the possibility of employing this strategy, but the continued presence of the benzoxazinoids allows the insect to use it to protect itself against its own enemies, by de-glycosylating the compound when an attack is detected so the toxic form of the compound is released upon contact to an enemy (Robert et al. 2017). EPNs of the genus *Heterorhabditis* have been shown to have a significantly lower rate of infection in WCR larvae that were feeding on maize that contained BXs than maize that did not contain BXs, however, not all isolates of the species had the same infection success (Zhang et al. 2019). Particularly isolates collected in Mexico and the US showed a higher infection rate than isolates from other regions of the world, such as Europe. This is due to the shared evolutionary history of the organisms in the original range of both maize and the WCR. Maize has been globally produced for a long time, as mentioned above, but since the ability to sequester BXs is not very common, *H. bacteriophora* from other regions of the world have not been exposed to the same level of selection pressure to develop the ability to tolerate this toxin.

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Scope of the PhD thesis

Scope – Investigating the genomics and evolution of benzoxazinoid resistance in *Heterorhabditis bacteriophora*.

Over the course of my dissertation, I aimed to investigate a variety of aspects to the biology of EPNs in general by comparing them to other nematode species, and both the ecology, evolution and genomics of *H. bacteriophora* in particular. I created a de-novo assembled chromosome scale genome for *H. bacteriophora*, we conducted a large-scale guided evolution experiment focussing on the changes due to the presence of BXs and I conducted various population and comparative genomic analyses based on whole genome sequencing data of both different species of *Heterorhabditis* as well as different isolates of *H. bacteriophora*.

In **Chapter 1**, I de novo assembled and annotated a chromosome scale high quality reference genome for *H. bacteriophora* as genomes available prior to the beginning of this project were insufficient for the analysis planned. This genome massively improved the contiguity of previously assembled resources and allowed for more in-depth analysis of chromosomal structure and differences to other genomes (**Annex I**). We also investigate what makes EPNs unique among nematodes by comparing both known and confirmed genera of EPNs to other species of nematodes, including plant parasites, mammalian parasites and free-living ones.

In **Chapter 2**, we identified which *H. bacteriophora* isolates have a higher or lower tolerance to BXs and used the new reference genome to identify regions of the genome that show differentiation between susceptible and resistant populations. We also investigated the importance of the symbiotic bacterium in EPN speciation and the relative exclusivity of different species of *Photorhabdus* and *Heterorhabditis*. Chapter 1 and 2 were recently submitted to *Molecular Ecology* as one consolidated manuscript.

Chapter 3, consists of a large-scale experimental evolution experiment where both BX susceptible and resistant EPN isolates were exposed to BXs or a control treatment for several generations, followed by an analysis of the genetic changes occurring in differently samples that were adapted to the presence of BXs compared to those that underwent a control treatment.

To conclude, I will discuss the findings discovered in this project and their importance in the field, as well as explain what other experiments and analyses can be conducted in the future to delve even more deeply into the world of benzoxazinoid resistance in EPNs.

Chapter 1 – *Chapters 1 and 2 are submitted to Molecular Ecology as one manuscript*

Chapter I – A de-novo chromosome-scale genome of *Heterorhabditis bacteriophora* provides insight into EPNs

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Abstract

The growing nutritional demand of the world population poses great challenges to sustainable and productive agriculture. Entomopathogenic nematodes (EPNs) are an economically interesting alternative to traditional methods of pest control, despite poorly understood aspects of their biology and genomics. This study provides a comprehensive characterization of the genome of *Heterorhabditis bacteriophora*, the place of EPNs in nematode phylogeny and the genomic uniqueness of EPNs. We performed a *de novo* chromosome-scale assembly of the *H. bacteriophora* genome and compared it with other nematodes, highlighting syntenic orthologs and genome organization in EPNs. WE identified several orthologs that are unique to EPNs in the context of the sample, such as Striatin domain-containing proteins and RBR-type E3 ubiquitin transferase as well as Heparan-alpha-glucosaminide N-acetyltransferase and Glyco_trans_2-like domain-containing proteins. These insights show what makes an EPN unique through the challenges they face that are different from other nematodes.

Introduction

The continued provision of food to the ever-growing human population largely relies on our ability to maximise food production per area and reduce any losses of yield (FAO 2017), such as damaging and destruction of plants through insect herbivores (Birch et al. 2011; Oerke 2006). Entomopathogenic nematodes (EPNs) are parasitic nematodes that target and kill insects and present a sustainable and economically viable solution for controlling herbivores. By offering an environmentally friendly alternative to chemical pesticides, EPNs reduce the reliance on synthetic agrochemicals and contribute to more sustainable agricultural practices (Gaugler and Kaya 1990; Lu et al. 2016).

EPNs are naturally present in (agro)ecosystems as obligate parasites of insects and molluscs (Chen et al. 2004). EPNs count at least two independently evolved genera, *Heterorhabditis* and *Steinernema* (Kaya and Gaugler 1993; Burnell and Stock 2000), which host symbiotic bacteria (genus *Photorhabdus* and *Xenorhabdus* respectively) that support the infection and prove fatal to insects. Their ability to independently locate and infect hosts through chemical perception and to attract uninfected hosts through chemical signalling make them a promising natural and sustainable alternative for herbicides in the attempt to control insect herbivores . However, maximizing their efficacy requires in-depth knowledge about what makes them effective insect parasites (Westwood et al. 2010; Blaxter and Koutsovoulos 2015; Luong and Mathot 2019; Trejo-Meléndez et al. 2024).

Despite their economically interesting prospects, the genomic era of EPNs is in its infancy.-The genome of *Heterorhabditis bacteriophora* has been sequenced by (Bai et al. 2013) and has benefitted from an updated annotation by (McLean et al. 2018), although available resources are fragmented to such a degree that they hardly promote progresses in our understanding of its genomic and phenotypic evolution.

This study aimed at providing functional insights on the *H. bacteriophora* genome and adaptation to benzoxazinoids. Following whole genome sequencing, we provide insights into the genomic features associated with an entomopathogenic lifestyle.

Materials and Methods

Rearing of nematodes and insects

Thirty-five nematode isolates representing 6 different species of entomopathogenic nematodes (*Heterorhabditis bacteriophora*, *H. beicherriana*, *H. georgiana*. *H. zacatecana*, and *H. ruandica*) were included in this study (Supplementary Table 1). The different nematode isolates were identified using different molecular and morphological characters (Machado et al. 2021). For population maintenance and amplification, greater wax moth larvae (*Galleria melonella* L.) were infected with water suspension containing about 100 nematodes. Infected larvae were placed in White traps (White, 1927), from which the emerging nematodes were collected. Nematodes were immediately frozen in liquid nitrogen and stored at -80°C before further processing in the case of DNA extraction. For the infectivity assays, the emerging nematodes were collected in bacterial culture flasks at a concentration of 50-500 living nematodes per ml and stored at 4-8°C in liquid culture (tap water) for 2-4 weeks before use.

Eggs of the Western Corn Rootworm (WCR, *Diabrotica virgifera* virgifera (Poinar)), provided by Chad Nielson (USDA, US), were incubated at 25-28°C until hatching. They were then reared in soil and fed on Maize plants provided by Delley Seeds and Plants Ltd (DSP, Delley, Switzerland) of the line W22, and the corresponding mutant in the Benzoxazinoid pathway, bx1::W22 (Maag et al. 2016). They were raised on the corresponding diets until the larvae reached the L3 stage, at which time they were used for the infectivity assay.

DNA extraction, sequencing and genome assembly

High molecular weight DNA was extracted from a pool of ~350,000 nematodes using the Circulomics Nanobind Tissue Big DNA Kit (Circulomics, Baltimore, Maryland, United States), with the addition of buffer NL specifically for the extraction of DNA from nematodes, and deviations from the original protocol as follows: a volume of nematodes weighing less than 100 mg were combined with proteinase K and buffer NL to ensure optimal access to extract the highest quality DNA possible. The extracted DNA was sequenced at the Next Generation Sequencing centre of the University of Bern, using PacBio HiFi (Pacific Biosciences of California, Inc.) sequencing in a single SMRTcell (Pacific Biosciences).

The genome size expected from flow cytometry was validated using a k-mer decomposition approach in TAREAN (Novák et al., 2017) and used to predict the genome size in the assembly process. The genome sequencing data was assembled using the canu pipeline with an expected

genome size of 111 Mb (Ciche, 2007) and only using reads longer than 15000 bp (Koren et al., 2017).

Hi-C enables long-range scaffolding of short contigs. It uses the physical closeness within single chromosomes to infer contiguity between different contigs in the assembly (Burton et al. 2013). The crosslinking was done using formaldehyde to fix the organization of the genome and link nearby loci and the *Hind*III restriction enzyme was added to digest chromatin, and a Phenol-Chloroform extraction was performed. Sequencing of the crosslinked material was done using half a flow cell Illumina Sequencing at the NGS sequencing platform at the University of Bern. Contigs assembled from PacBio HiFi reads were scaffolded by crosslinked HiC data, using the juicer pipeline (Durand et al., 2016). The resulting assembly was assessed for completeness using BUSCO (nematoda_odb10, n = 3131) in nucleotide mode (Manni et al., 2021).

Genome annotation

Transposable elements were annotated using EDTA with default settings except for --anno 1 (Ou et al. 2019). The same method of TE annotation was also used for the reference genome of *C. elegans* to facilitate comparisons between the two genomes. Finer classifications of the TEs in *H. bacteriophora* were achived using TEsorter (Zhang et al. 2022). The divergence of TE copies from their consensus was estimated using parseRM (Mitra et al. 2013) on annotated TEs following Ou et al. (2019) and used for relative dating of transposition events according to Maumus and Quesneville (2014).

De novo annotation was done using MAKER (Cantarel et al. 2008), after having soft-masked TE annotations. In a first step, predictors in the form of a combination of RNAseq data (downloaded from ncbi for *H. bacteriophora* isolate m13e, under different stresses) and protein sequences from other nematode species (downloaded from uniport at 4.4.2022) were used. The RNAseq data was aligned to the assembly using Trinity (Grabherr et al. 2011), and then used as an input to MAKER along with the annotation data. In a second round, the evidence based-annotation was used for one pass of *ab-initio* annotation.

Comparative phylogenomics

For comparison purposes, genomes and protein sequences were retrieved from a range of 15 nematode species (*Globodera pallida, Bursaphelenchus xylophilus, Strongyloides ratti, Steinernema carpocapsae, Enterobius vermicularis, Loa Loa, Brugia malayi, Toxocara canis,*

Heterorhabditis bacteriophora, Teladorsagia circumcincta, Angiostrongylus cantonensis, Necator americanus, Caenorhabditis briggsae, Caenorhabditis remanei, Caenorhabditis elegans, Supplementary Table 2). The species were selected to represent a phylogenetically diverse selection of different parasitic life strategies (International Helminth Genomes Consortium 2019), including EPNs, plant parasitic nematodes (PPNs), other parasites (mammalian) as well as free-living nematodes.

To investigate similarities between these species, orthology between the annotations was examined using Orthofinder (Emms and Kelly 2019) identifying orthogroups present in all species. Orthofinder also created a phylogenetic tree from the protein sequences using STAG (Species Tree from All Genes) (Emms and Kelly 2018) and rooting it using STRIDE (Emms and Kelly 2017). This allowed orthofinder to use all data, including multi-copy orthologs, towards robust tree generation despite high levels of duplication within the genomes. The resulting tree was bootstrapped using iqtree (Minh et al. 2020) on the SpeciesTreeAlignment.fa output form orthofinder (settings: -st AA -m LG+I+G -B 1000).

To further analyse the orthogroups of interest without *C. elegans*-centred biases, an Hmmer scan (Finn et al. 2011) was conducted to find putative function for genes, where protein sequences obtained from the orthogroups genes were used as input and the basic settings were used with E-value cut-offs as Significant E-values of 0.01 for a sequence and 0.023 for a hit.

Results

A nearly complete *Heterorhabditis bacteriophora* genome

The genome of *H. bacteriophora*, strain m13e, was sequenced using PacBio Hifi (mean read length: 12,939 bp; coverage ca. 250x), assembled using Canu (Koren et al. 2017) and scaffolded by Hi-C using the Juicer pipeline (Dudchenko et al. 2018). Consisting of 226 scaffolds (N50: 11,8 Mb; Busco score: 93.6% complete), the resulting assembly reached a total size of 85 Mb, being close to the genome size estimated by k-mer decomposition analysis (105 Mb) as well as flow cytometry (i.e. 111.7 Mb; Ciche 2007). The seven largest scaffolds (>9 Mbp) (Figure 1) corresponding to the expected number of chromosomes according to Curran (1989).

A total of 13,832 copies of transposable elements (TEs) were annotated using EDTA (Ou et al. 2019), summing up to 12% of the chromosome-scale scaffolds. Consistent with the similarly repetitive genome of *C. elegans* (Cutter et al., 2009), abundant TEs in *H. bacteriophora* included a majority of Mutator DNA transposons (5.04%) and BelPao LTR retrotransopsons (~1.5%;

Supplementary Table 3). However, in contrast to *C. elegans* and its relatives (e.g. Woodruff and Teterina, 2020), TEs appeared particularly abundant in the centre of scaffolds rather than along chromosome arms in *H. bacteriophora* and showed evidence of recent transposition according to their divergence (Supplementary Figure 1 A&B). In particular, Mutator transposons appeared to have contributed more than 4 Mb of interspersed sequences in the distant past (i.e. ~10 MYA), whereas Gypsy LTR retrotransposons proliferated recently (i.e. ~4 MYA) and specifically comprised almost 2.5 Mb across centres of chromosomes in *H. bacteriophora*.

A total of 17,787 gene models containing on average 6.5 exons were supported by a combination of *ab initio* annotation, RNAseq-based evidence, and annotations from other nematodes, corresponding to 75 Mb and thus encompassing approximately 88% of the genome assembly. With this gene annotation, showing a score of 79.7% complete BUSCOs (based on the nematode dataset nematoda_odb10, n = 3131), the assembly presented 2040 additional genes as compared to previously released assemblies (Bai et al. 2013; McLean et al. 2018). The assembly presented here showed greatly improved contiguity, with a N50 of 11,8 Mb as compared to 312 kb of the prior version, supporting a nearly complete *H. bacteriophora* genome.

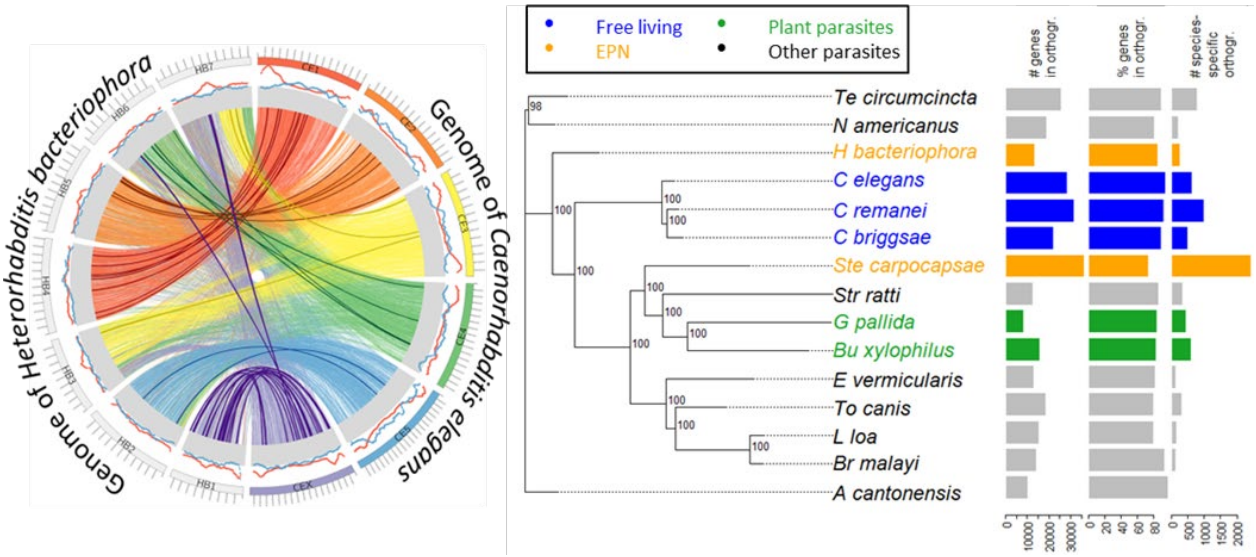

Figure 1: Comparative genomics of Heterorhabditis bacteriophora *and nematodes. A: Chromosome-scale assembly of the genome of* H. bacteriophora *(strain m13e) in comparison to* Caenorhabditis elegans*, with orthologous relationships shown as lines connecting their chromosomes, with blocks of five syntenic genes displayed in bold. Lines on the outer circle show the density of genes (blue) and transposable elements (red) along chromosomes. The short lines perpendicular to each chromosome represent the scale of the chromosomes in Mb. B: Phylogeny with bootstrap support (on tree nodes) based on orthologous genes of 15 species of nematodes (*Globodera pallida, Bursaphelenchus xylophilus, Strongyloides ratti, Steinernema carpocapsae, Enterobius vermicularis, Loa Loa, Brugia malayi, Toxocara canis, Heterorhabditis bacteriophora, Teladorsagia circumcincta, Angiostrongylus cantonensis, Necator americanus, Caenorhabditis briggsae, Caenorhabditis remanei, Caenorhabditis elegans*) coloured according to their lifestyle*

(black: mammalian parasites, blue: free living, orange: EPN, green: plant parasitic) showing the number of genes identified in the annotation, their percentage forming orthogroups and the number of species-specific orthogroups.

Genomic comparison with other nematodes reveals elements of pathogenicity

A coalescence-based phylogenetic tree based on gene trees from all orthologous groups among 15 mostly parasitic nematodes (Supplementary Table 2) placed *H. bacteriophora* as ancestral to a monophyletic clade including mammalian parasites, plant parasitic nematodes, and the other EPN *Steinernema carpocapse* as well as the clade of free-living *Caenorhabditis* nematodes (Figure 1B). The large phylogenetic distance between the two EPN species is consistent with the notion that entomopathogenicity evolved multiple independent times.

Orthologous genes annotated across this set of nematode species identified 266 orthogroups present exclusively in *H. bacteriophora* (Supplementary Table 4). Although only 17 orthogroups were specific to both EPN species as compared to other life strategies, most of the corresponding genes in *H. bacteriophora* showed no clear orthology in *C. elegans* (Supplementary Table 5). Accordingly, to infer their putative functions, we searched for protein domains with Hmmer (Finn et al. 2011) and found several candidates such as Striatin domain-containing proteins which are involved in calcium dependent phosphorylation or dephosphorylation and may function as scaffolding or signalling protein promoting tolerance of EPNs to the immune system of insects (Tanti et al. 2023). Similarly, RBR-type E3 ubiquitin transferase being involved in ubiquitin conjugation (Kodama et al. 2007) and Heparan-alpha-glucosaminide N-acetyltransferase involved in the lysosomal degradation of heparin sulfate may increase immune system tolerance (Schiff et al. 2020), whereas Glyco_trans_2-like domain-containing proteins involved in glycosyltransferase during the biosynthesis of sugars can play a role to cope with bacteria as food source in EPNs (Sobhanifar et al. 2016) (Supplementary Table 5)

Discussion

Entomopathogenic nematodes are an ecologically and economically interesting biological alternative to chemical pesticides. To better understand their biology and facilitate improvement in infection rates and host defense tolerance, we present a novel high quality, contiguous assembly and annotation of the EPN *H. bacteriophora*, as well as population genomic analysis into EPNs in general and benzoxazinoid resistance in particular.

The genome of Heterorhabditis *bacteriophora* and comparison with other nematodes

The genome of *Heterorhabditis bacteriophora* assembled here added more than 8 Mb to the previously available resources (Bai et al. 2013) and yielded an assembly of 85 Mb being resolved into seven large scaffolds corresponding to chromosomes. On top of considerably improved contiguity, this genome model is likely nearly complete, although its size is lower than estimated by flow cytometry or k-mer decomposition as could be expected from an organism containing symbionts or from collapsed rDNA and other repeats. This new resource opens avenues for detailed genomic investigations of *H. bacteriophora* and entomopathogenic nematodes in general, including the characterization of factors influencing their efficacy as natural pest control of economic and ecological importance. It further promotes our understanding of genome evolution among highly diverse nematodes and unlocks opportunities to assess evolutionary processes having shaped populations of *H. bacteriophora* in response to their bacterial symbionts as well as insect hosts, helping to unlock the potential of EPN genetics for pest control in sustainable agriculture (Maushe et al. 2023).

The organisation of the genome in *H. bacteriophora*, as seen from the distribution of both genes and TEs, is surprisingly different from *C. elegans*. Genes indeed appeared evenly distributed across whole chromosomes, contrasting with their lower density across distal regions of chromosomes in *C. elegans*. Even more contrasted, the TE density was highest in the centre of chromosomes in *H. bacteriophora*, whereas TEs are most abundant on the arms of chromosomes in *C. elegans*. Given that *C. elegans* is one of the most well-known examples of a species with holocentric chromosomes (Albertson and Thomson 1993), this questions the general assumption that chromosomes in *H. bacteriophora* are organized and segregate as in the model species (Carlton et al. 2022). Some nematode species such as *Trichinella spiralis* (Subirana and Messeguer 2013) have indeed been shown to be monocentric. The recent accumulation of Gypsy LTR retrotransposons in the centre of chromosomes in *H. bacteriophora* mimics their high density across non-recombining pericentromeric regions of e.g. plant chromosomes (Kumar and Bennetzen 1999; Wlodzimierz et al. 2023), suggesting that the distribution of kinetochore activity shall be further assessed among nematodes.

With *H. bacteriophora* being in distantly related to *Steinernema carpocapsae,* EPNs are confirmed as polyphyletic. Consistent with previous evidence that entomopathogenicity evolved multiple times in nematodes, evolution towards obligate insect parasitism has been postulated

to rely on the close association between nematodes and their symbiotic bacteria (Trejo-Meléndez et al. 2024). Beyond symbiotic interactions, gene families related to protease and protease inhibitor families have been associated with parasitism in Steinernematids (Dillman et al. 2015). Accordingly, genes encoding for Striatin domain-containing proteins and RBR-type E3 ubiquitin transferase were here shown to be specifically enriched among investigated EPNs, which both influence protein structure, as to potentially promote their tolerance to specific immune responses when infecting insects. One possible reason for a specific adaptation to insect immune systems is that the antimicrobial peptides are produced differently in insect than they are in mammals, which would explain a need for a change in the response (Sheehan et al. 2018). Other candidates for entomopathogenicity are Glyco_trans_2-like domain-containing proteins, whose involvement in the biosynthesis of sugars may be an adaptation to the high level of symbiosis with bacteria, where it could have an effect on the digestion of bacteria.

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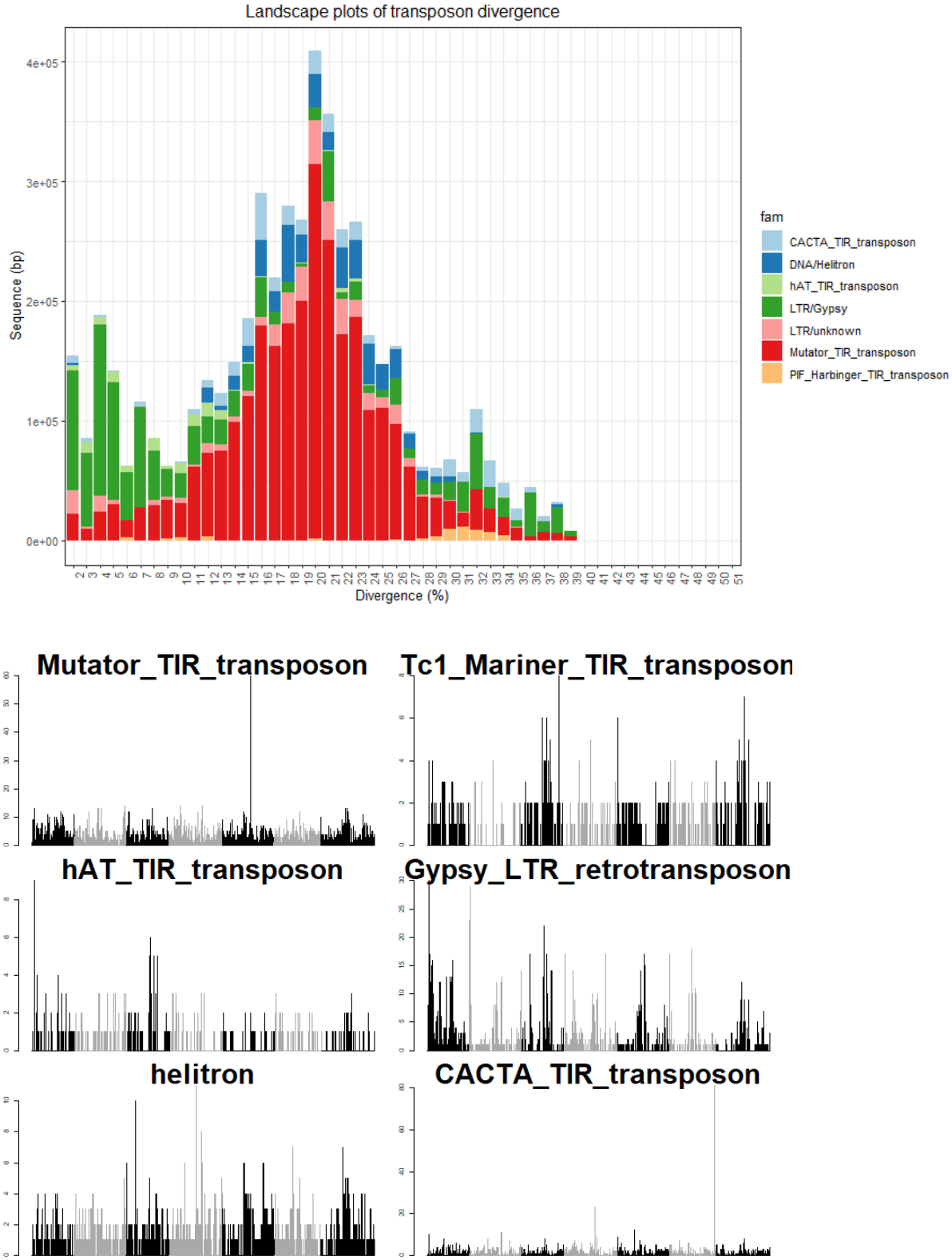
Supplementary information

Supplementary Figure



Supplementary Figure 1: Divergence and distribution of annotated TEs in Heterorhabditis bacteriophora. *A) Percentage of divergence between TE copies and the consensus sequence representative of their TE family as estimate of the time spent since transposition. B) Distribution of TE copies from different families as annotated along the chromosomes.*

Supplementary Tables

Supplementary Table 1: Isolates of Heterorhabditis *used in the study. Including information regarding if any infectivity assay has been conducted and if applicable is they were susceptible of resistant to benzoxazinoids.*

Isolate	species	country	Infectivity assessed	Susceptibility to benzoxazinoids
0943	*H. bacteriophora*	Turkey	Yes	Yes
Ma	*H. ruandica*	Rwanda	No	NA
Boj	*H. bacteriophora*	Iran	Yes	No
RW14Art	*H. ruandica*	Rwanda	No	NA
CN4e	*H. beicherriana*	China	No	NA
H06	*H. beicherriana*	China	No	NA
DE2	*H. bacteriophora*	Germany	Yes	No
DE6	*H. bacteriophora*	Germany	Yes	Yes
EN01e	*H. bacteriophora*	Commercial	Yes	Yes
Hb17	*H. bacteriophora*	Turkey	Yes	No
Hbbio	*H. bacteriophora*	Usa	Yes	No
HU2e	*H. bacteriophora*	Hungary	Yes	No
IL9e	*H. bacteriophora*	Australia	Yes	No
IR2e	*H. bacteriophora*	Iran	Yes	No
IT6e	*H. bacteriophora*	Italy	Yes	No
m13e	*H. bacteriophora*	Trinidad and Tobago	No	NA
MEX23	*H. bacteriophora*	Mexico	Yes	No
MEX20	*Heterorhabditis* sp	Mexico	No	NA
MEX32	*H. bacteriophora*	Mexico	Yes	No
MEX37	*H. bacteriophora*	Mexico	Yes	No

MG618b	*H. bacteriophora*	Switzerland	Yes	Yes
MEX40	*H. zacatecana*	Mexico	No	No
MEX41	*H. zacatecana*	Mexico	No	No
PT1e	*H. bacteriophora*	Portugal	Yes	No
RBP	*H. bacteriophora*	Spain	Yes	No
RW14	*H. bacteriophora*	Rwanda	No	NA
S12	*H. bacteriophora*	USA	Yes	No
Hbb	*H. georgiana*	USA	No	NA
S14	*H. bacteriophora*	USA	Yes	No
S15	*H. bacteriophora*	USA	Yes	No
S5P8	*H. bacteriophora*	USA	Yes	No
S7	*H. bacteriophora*	USA	Yes	No
TT01	*H. bacteriophora*	Trinidad_Tobago	Yes	Yes
VM1	*H. bacteriophora*	Spain	Yes	No
S8	*Heterorhabditis* sp.	USA	No	NA

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Supplementary Table 2: List of the species used for the orthofinder analysis, including the information regarding the quality of the assembly and the accession number.

Scientific name	Common name	Life strategy	Genome size	scaffolds	N50	# Genes	Accession
Globodera pallida	potato cyst nematode	Plant parasitic	119.6 Mb	173	2.2 Mb	7,870	GCA_023343765.1
Bursaphelenchus xylophilus	Pine wood nematode	Plant parasitic	78.3 Mb	11	12.8 Mb	15,884	GCA_904066235.2
Strongyloides ratti	NA	Parasite	43.2 Mb	135	11.7 Mb	12,446	GCA_001040885.1
Steinernema carpocapsae	NA	EPN	84.5 Mb	16	7.4 Mb	30,825	GCA_000757645.3
Enterobius vermicularis	Human pinworm	Parasite	150.1 Mb	19,832	20.6 kb	12,895	GCA_900576705.1
Loa Loa	African eye worm	Parasite	91.4 Mb	5,764	174.4 kb	15,040	GCA_000183805.3
Brugia malayi	lymphatic filariasis	Parasite	87.2 Mb	196	14.2 Mb	11,688	GCA_000002995.5

Toxocara canis	Dog roundworm	Parasite	317.1 Mb	22,857	375.1 kb	18,596	GCA_000803305.1
Heterorhabditis bacteriophora		EPN	85 Mb	226	12 Mb	17,787	GCA_000223415.1
Teladorsagia circumcincta	NA	Parasite	700.6 Mb	81,730	47.1 kb	26,258	GCA_002352805.1
Angiostrongylus cantonensis	Rat lungworm	Parasite	293.3 Mb	26,577	860.8 kb	10,314	GCA_009735665.1
Necator americanus	New world hookworm	Parasite	244.1 Mb	11,864	211.9 kb	19,663	GCA_000507365.1
Caenorhabditis briggsae	NA	Free living	108.4 Mb	638	727.9 kb	22,727	GCA_000004555.3
Caenorhabditis remanei	NA	Free living	130.5 Mb	186	21.5 Mb	26,296	GCF_010183535.1
Caenorhabditis elegans	NA	Free living	100.3 Mb	6	17.5 Mb	46,927	GCA_000002985.3

Supplementary Table 3: Statistics regarding the annotated TEs. A) TEs annotated through the use of EDTA, including LTRs, TIRs and non-TIRSs. The count shows how many times they appear in the genome, bpMasked shows how many base pairs are masked as TEs for future analysis, such as annotation. % masked shows what percentage of the genome is represented in such masked regions. B) Further classification of annotated TEs using TEsorter, including both Bel-Pao and Copia, which had not been identified using ETDA.

EDTA output

Class	Count	bpMasked	%masked
LTR	--	--	--
Gypsy	2,968	2,455,998	3.02
Unknown	857	557,579	0.69
TIR	--	--	--
CACTA	1,844	909,821	1.12
Mutator	5,707	4,095,822	5.04
PIF_Harbinger	88	36,545	0.04
Tc1_Mariner	552	313,681	0.39
hAT	434	187,400	0.23
nonTIR	--	--	--
Heliotron	1,382	1,012,416	1.25
Total interspersed	13,832	9,569,262	11.78

TEsorter output

Order	Superfamily	# of sequences	# of clades	# of full Domains

LTR	Bel-Pao	1211	0	92
LTR	Copia	84	1	0
LTR	Gypsy	869	4	9
LTR	Retrovirus	1	0	0
LTR	Mixture	2	0	0
LINE	Unknown	2	0	0
TIR	Tc1_Mariner	2204	0	0
TIR	hAT	70	0	0

Supplementary Table 4: GO term enrichment analysis for orthogroups only found in H. bacteriophora *and not in any of the other nematode species used in this study.*

-- biological processes_ _genes.txt --

	GO.ID	Term	Annotated	Significant	Expected	P-value
1	GO:1904780	negative regulation of protein localization to centrosome	10	1	0.01	0.0075
2	GO:0051228	mitotic spindle disassembly	10	1	0.01	0.0075
3	GO:0030970	retrograde protein transport, ER to cytosol	10	1	0.01	0.0075
4	GO:0071712	ER-associated misfolded protein catabolic process	10	1	0.01	0.0075
5	GO:0097352	autophagosome maturation	11	1	0.01	0.0083
6	GO:0034504	protein localization to nucleus	21	1	0.02	0.0158
7	GO:1902476	chloride transmembrane transport	23	1	0.02	0.0173
8	GO:0030433	ubiquitin-dependent ERAD pathway	24	1	0.02	0.0181
9	GO:0036498	IRE1-mediated unfolded protein response	41	1	0.03	0.0307

--
molecular_functions_genes.txt
--

	GO.ID	Term	Annotated	Significant	Expected	P-value
1	GO:0031593	polyubiquitin modification-dependent protein binding	14	1	0.01	0.011
2	GO:0005254	chloride channel activity	23	1	0.02	0.018
3	GO:0042802	identical protein binding	36	1	0.03	0.029

Supplementary Table 5: Results of the Hmmer search of 17 H. bacteriophora *genes that represent the 17 orthogroups that were found to be unique to EPNs.*

Gene name	#	code	characterization	E-value
hbac_00007187-RA	7	AOAON4T1Z9_BRUPA	Uncharacterized protein	1.6e-10
hbac_00004341-RA	28	W7XG84_TETTS	Uncharacterized protein	3.9e-14
hbac_00004291-RA	2280	A0A158R3L6_NIPBR	Striatin domain-containing protein	8.9e-26
hbac_00005379-RA	14	A0A498SNK3_ACAVI	Uncharacterized protein	2.0e-13
hbac_00002450-RA	273	A0A6G1R074_9TELE	Uncharacterized protein	1.6e-35
hbac_00000927-RA	40	AOAON4YNV9_NIPBR	Uncharacterized protein	2.9e-18
hbac_00004141-RA	23	A0A0R3PIMO_ANGCS	Uncharacterized protein	1.3e-36
hbac_00000741-RA	3	H3FD44_PRIPA	RBR-type E3 ubiquitin transferase	1.5e-08
hbac_00011793-RA	3296	A0A1D2MH46_ORCCI	Heparan-alpha-glucosaminide N-acetyltransferase	2.1e-69
hbac_00010881-RA	33	A0A016VRK7_9BILA	Glyco_trans_2-like domain-containing protein	4.2e-11
hbac_00011921-RA	6628	T1J5Y8_STRMM	Uncharacterized protein	3.8e-41
hbac_00007326-RA	7	AOAON4X407_HAEPC	Uncharacterized protein	4.6e-10
hbac_00000697-RA	26	A0A3P6R0L6_CYLGO	Uncharacterized protein	1.3e-14
hbac_00005144-RA	12	A0A016UBG3_9BILA	Uncharacterized protein	3.8e-17
hbac_00005179-RA	334	A0A3Q2UC95_CHICK	VWFD domain-containing protein	2.2e-100
hbac_00009700-RA	74	AOAOKODM32_ANGCA	Uncharacterized protein	5.2e-54
hbac_00012213-RA	15	AOAOKOG3U8_STRVS	DNA polymerase epsilon catalytic subunit	2.4e-20

Chapter 2 – *Chapters 1 and 2 are submitted to Molecular Ecology as one manuscript*

Chapter II – Plant toxin resistance of *Heterorhabditis bacteriophora* and the relationship with its symbiotic bacterium

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Abstract

The growing nutritional demand of the world population poses great challenges to sustainable and productive agriculture. Entomopathogenic nematodes (EPNs) are an economically interesting alternative to traditional methods of pest control, despite poorly understood aspects of their biology and genomics. This study provides an insight into the importance of the symbiotic relationship between an EPN and its symbiotic bacterium and the EPNs capacity to resist benzoxazinoids that are sequestered as defense compounds by an important insect pest. We performed phylogenetic analyses and genetic structure within *Heterorhabditis* characterized species being largely inconsistent with the delineation in the *Photorhabdus* endosymbiotic bacterial species, suggesting that *H. bacteriophora* have evolved partially independently of their endosymbionts. Population genomics analyses within *H. bacteriophora* identified genetic variation distinguishing between strains susceptible and resistant to benzoxazinoids. Genome-wide differentiation (F_{ST}) pointed to genomic regions related to deoxyribonucleotide biosynthetic processes, polypeptide N-acetylgalactosaminyltransferase activity and single-stranded DNA endodeoxyribonuclease activity that were shaped by strong selective pressures. Having identified candidate genes associated with insect pathogenicity and benzoxazinoid resistance, our findings provide a foundation for future work on the efficacy and infectivity of EPNs in pest management.

Introduction

The continued provision of food to the ever-growing human population largely relies on our ability to maximise food production per area and reduce any losses of yield (FAO 2017), such as damaging and destruction of plants through insect herbivores (Birch et al. 2011; Oerke 2006). Entomopathogenic nematodes (EPNs) are parasitic nematodes that target and kill insects and present a sustainable and economically viable solution for controlling herbivores. By offering an environmentally friendly alternative to chemical pesticides, EPNs reduce the reliance on synthetic agrochemicals and contribute to more sustainable agricultural practices (Gaugler and Kaya 1990; Lu et al. 2016).

EPNs are naturally present in (agro)ecosystems as obligate parasites of insects and molluscs (Chen et al. 2004). EPNs count at least two independently evolved genera, *Heterorhabditis* and *Steinernema* (Kaya and Gaugler 1993; Burnell and Stock 2000), which host symbiotic bacteria (genus *Photorhabdus* and *Xenorhabdus* respectively) that support the infection and prove fatal to insects. Their ability to independently locate and infect hosts through chemical perception and to attract uninfected hosts through chemical signalling make them a promising natural and sustainable alternative for herbicides in the attempt to control insect herbivores . However, maximizing their efficacy requires in-depth knowledge about what makes them effective insect parasites (Westwood et al. 2010; Blaxter and Koutsovoulos 2015; Luong and Mathot 2019; Trejo-Meléndez et al. 2024).

While *H. bacteriophora* has been shown to be effective in controlling pest herbivores (Hiltpold et al. 2010), its effectiveness can be reduced in specialist herbivores that sequester and use plant specialized metabolites (Robert et al. 2017). The Western Corn Rootworm (WCR, *Diabrotica virgifera virgifera*) is a devastating specialist herbivore, whose larvae specifically feeds on maize roots (Eben 2022). The larval stage of this specialist herbivorous beetle causes damages worth >2 billion USD a year (FAO 2017). Originating from southern North America (Mexico), WCR has adapted to maize defenses (Eben 2022; Robert et al. 2017), which include a group of indole-derived specialized metabolites called benzoxazinoids that act in both nutrient (iron) acquisition and defense (Robert and Mateo 2022). To circumvent these effects, WCR larvae have developed the ability to not only tolerate benzoxazinoids, but also to sequester and stabilize some of them, namely MBOA and HDMBOA-Glc, for use as toxic compounds against their own enemies, such as EPNs and their endosymbionts (Robert et al. 2017). MBOA is stabilized through glucosylation, and continuously exuded by the larvae as MBOA-Glc that is repellent for EPNs and sufficient to decrease infection (Zhang et al. 2019). HDMBOA-Glc is sequestered in the haemolymph and can be converted to MBOA upon EPN infection. Interestingly, *H. bacteriophora* strains that originate

in the same area of the world as maize and the WCR have developed the ability to cope with the presence of benzoxazinoids in their host (Zhang et al. 2019). In contrast, *H. bacteriophora* from other parts of the world such as Europe, where the herbivore was introduced relatively recently (~50 years ago) (Bažok et al. 2021), remain susceptible to benzoxazinoids (Zhang et al. 2019). Previous work has shown that, under laboratory conditions, susceptible EPNs can adapt to the presence of benzoxazinoids within as little as five generations (Zhang et al. 2019). The mechanistic and genomic basis of benzoxazinoid resistance in *H. bacteriophora* remains unclear (Zhang et al. 2019).

This study aimed at providing functional insights on the *H. bacteriophora* genome and adaptation to benzoxazinoids. Following whole genome sequencing, we provide insights into the genomic features associated with an entomopathogenic lifestyle. Furthermore, we investigate how *Heterorhabditis* species differ genetically and how much the speciation of the nematodes and their symbiotic bacteria influence each other. Finally, we compare the genomes of *H. bacteriophora* strains that are resistant and susceptible to benzoxazinoids to identify candidate genes involved in this important trait.

Materials and Methods

Rearing of nematodes and insects

Thirty-five nematode isolates representing 6 different species of entomopathogenic nematodes (*Heterorhabditis bacteriophora*, *H. beicherriana*, *H. georgiana*. *H. zacatecana*, and *H. ruandica*) were included in this study (Supplementary Table 1). The different nematode isolates were identified using different molecular and morphological characters (Machado et al. 2021). For population maintenance and amplification, greater wax moth larvae (*Galleria melonella* L.) were infected with water suspension containing about 100 nematodes. Infected larvae were placed in White traps (White, 1927), from which the emerging nematodes were collected. Nematodes were immediately frozen in liquid nitrogen and stored at -80°C before further processing in the case of DNA extraction. For the infectivity assays, the emerging nematodes were collected in bacterial culture flasks at a concentration of 50-500 living nematodes per ml and stored at 4-8°C in liquid culture (tap water) for 2-4 weeks before use.

Eggs of the Western Corn Rootworm (WCR, *Diabrotica virgifera* virgifera (Poinar)), provided by Chad Nielson (USDA, US), were incubated at 25-28°C until hatching. They were then reared in soil and fed on Maize plants provided by Delley Seeds and Plants Ltd (DSP, Delley, Switzerland) of the line W22, and the corresponding mutant in the Benzoxazinoid pathway, bx1::W22 (Maag

et al. 2016). They were raised on the corresponding diets until the larvae reached the L3 stage, at which time they were used for the infectivity assay.

Whole-genome sequencing within *Heterorhabditis*

A total of thirty-five nematode isolates were collected from locations all over the world, particularly from North-America and Europe (Supplementary Table 1). These isolates were molecularly and morphologically identified and assigned to the different species (*Heterorhabditis bacteriophora*, *H. beicherriana*, *H. georgiana*, *H. zacatecana* and *H. ruandica*).

DNA was extracted using the Analytik Jena DNA extraction kit according to the manufacturer's instructions with some adjustments, which are mainly using a pellet pestle motor to homogenize pelleted nematodes in a small amount of lysis buffer before adding the remaining buffer and continuing with the extraction. The extracted DNA was then used for PCR free library preparation and the libraries were 150 bp paired-end sequenced using Illumina sequencing at the NGS platform at the University of Bern. Sequencing was done on half a flow cell, resulting in a total of 44.4 gb data (~1.2 GB per isolate)

The whole genome sequences were aligned to the reference genome using gatk (Version 4.2.0.0) (McKenna et al. 2010). The aligned vcf file was then filtered using a combination of gatk (Version 4.2.0.0) and bcftools (Li 2011) as to only retain SNPs with a minimum depth >10, a minor allele frequency > 0.1 and less than 10% missing data. Filtering for neutral SNPs for some of the analysis was done using SNPeff (Cingolani et al. 2012)

Population genetic structure

The best likelihood phylogenetic tree for both the nematode and the symbiotic bacterium was created based on concatenated SNPs using RAxML, using the standard settings (GTRGAMMA) (Stamatakis 2014). To compare the phylogenetic trees of the nematode (host) and the bacterium (symbiont), the R package dendextend (Galili 2015) was used. The Baker's gamma index was estimated as a measure of association between the two phylogenies using the cor_bakers_gamma function. A permutation test was performed by randomly shuffling the labels on the nematode tree 100 times to assess the null distribution of Baker's gamma and checking if the observed nematode tree is significantly more associated with the symbiont tree than expected by chance. Visualization of the tanglegram was done in R using a combination of the dendextend package and base R.

The species of the symbiotic bacteria were defined based on a combination of 16S sequencing, housekeeping genes and whole genome sequencing (Machado et al. 2024). Association between nematode and bacterial species (particularly *H. bacteriophora* and *P. laumondii* was

determined using a X^2 test by comparing the number of *H. bacteriophora* isolates in symbiosis with *P. laumondii* subsp. *laumondii* and with other bacterial species to non-*bacteriophora* species in association with *P. laumondii* subsp. *laumondii* and with others. The same analysis was conducted for susceptible and resistant isolates of *H. bacteriophora* associated with *P-laumondii* subsp. *laumondii* and others.

For the nematode and the symbiotic bacterium, genetic variation was a posteriori partitioned among 2 to 7 and 2 to 10 K groups respectively, using STRUCTURE (Pritchard et al. 2000) with 10,000 BURNIN and 100,000 repetitions after Burnin using the admixture model. The best K was determined using the highest delta K produced by Structure Harvester (Earl and vonHoldt 2012) and the outputs of multiple runs of STRUCTURE were consolidated using CLUMPP (Jakobsson and Rosenberg 2007).

Isolation by distance between *Heterorhabditis* species was tested using Loiselle kinship coefficients (Loiselle et al. 1995) based on the first 10000 SNPs of each isolate estimated with Spagedi (Hardy and Vekemans 2002) as genetic distances and associated to Euclidean spatial distances based on longitude and latitude, using the R package 'vegan' (Oksanen et al. 2001).

Infectivity assays and identification of susceptible vs resistant isolates

To assess the infectivity of the different isolates of *H. bacteriophora*, an infectivity assay in WCR larvae containing benzoxazinoids or not containing benzoxazinoids was conducted. For this, 1.25 oz solo cups (frontier agricultural sciences) were filled with 3.5 g of autoclaved sand (LANDI Schweiz AG, Dotzingen, Switzerland). To each cup, 5 WCR larvae were added. For each isolate and each diet, 5 cups with 5 larvae were used, bringing the total number of larvae to 25 per treatment. To each solo cup, 50 infective juveniles were added (counted under the microscope, mean of 3 drops of 5 µl). To ensure similarity of treatment, it was made sure to add a total of 600 µl liquid to each cup, adding tap water to close the difference, as well as in control treatments. The solo cups were then incubated at 25°C in the dark until data collection. Data collection was done at days 3-7, by visually checking the larvae for infectivity (denoted by clear red colouring and stiffness of dead larvae)

The percentage of infected larvae at day 7 post infection was collected from different experiments. To account for potential differences in different experiments, at least 1 isolate was always used in multiple experiments, to ensure comparability. In 3 experiments that isolate was DE6, and in 2 IL9. Those data were them used to correct the other experiments to make sure that the same isolate always had the same mean. The data was then plotted in R, using the ggplot2

package (Hadley Wickham 2016). Infectivity data was also assessed on days 3-6, which any isolate showing significant (t-test) susceptibility to benzoxazinoids being considered for further experiments.

Genome scans

To assess genetic differences underlying resistance vs susceptibility to benzoxazinoids, genetic differentiation (F_{ST}) and divergence (d_{xy}) among populations were estimated based on SNPs using the PopGenome package (Pfeifer et al. 2014). F_{ST} and d_{XY} analysis between a group of susceptible and a group of resistant isolates was conducted in R using the PopGenome package (Pfeifer et al. 2014), with non-overlapping sliding windows of 5 biallelic SNPs and visualized in using the ggplot2 package (Hadley Wickham 2016). Outlier windows presenting higher values than the 3rd quartile plus 1.5 times the interquartile range of the complete dataset were identified for both F_{ST} and d_{xy} independently. Provided that differentiation can artificially inflate in regions of low diversity (Cruickshank and Hahn 2014), only windows presenting outlier values for both F_{ST} and d_{xy} were considered as candidate loci shaped by selection. Genes partly or completely encompassed by outlier windows were considered as putatively under selection and used in a GO term enrichment analysis as well as in a Hmmer search (Finn et al. 2011).

Go enrichment analyses

GO-term annotations available for *C. elegans* were used and assigned to other nematodes species using blast (Altschul et al. 1990). This annotation as then used to conduct the GO term enrichment analysis, using the topGO package on R with the standard weight correction (Alexa A 2023). We only considered GO-terms with a P-value of <0.05 for analysis.

Results

Co-evolution of *Heterorhabditis* nematodes and their *Photorhabdus* symbionts

A total of 35 *Heterorhabditis* isolates representing five species (*H. bacteriophora, H. beicherriana, H. georgiana*. *H. zacatecana*, and *H. ruandica*) were characterized with whole-genome sequence data mapped to the new reference genome (mean coverage ~18x). This resulted in 3,371,445 high-quality SNPs that were concatenated to infer a RAxML likelihood

phylogeny that highlighted *H. ruandica* and *H. zacatecana* as well as *H. georgiana* and *H. beicherriana* as sister species, whereas *H. bacteriophora* appeared as early diverged from the other species (Figure 2). Genetic differentiation using STRUCTURE on a subset of 332'941 neutral SNPs optimally grouped the different *Heterorhabditis* isolates into three genetically related clusters (optimal K = 3)(Earl and vonHoldt, 2012 (Pritchard et al. 2000)) (Supplementary Figure 1). More specifically, STRUCTURE clustered *H. beicherriana* and *H. georgiana* as well as *H. zacatecana* and *H. ruandica* together into two groups differentiated from *H. bacteriophora*, confirming phylogenetic insights and supporting their sister species status (Figure 2). Beyond the widespread *H. bacteriophora* presenting isolates in multiple regions, samples from other species were indeed geographically isolated from one another (Supplementary Figure 2, Supplementary Table 1), with *H. ruandica* (from Africa) and *H. beicherriana* (from Eastern Eurasia) appearing largely allopatric. Geographic isolation hence appears to poorly represent the extent of genetic differentiation between taxa.

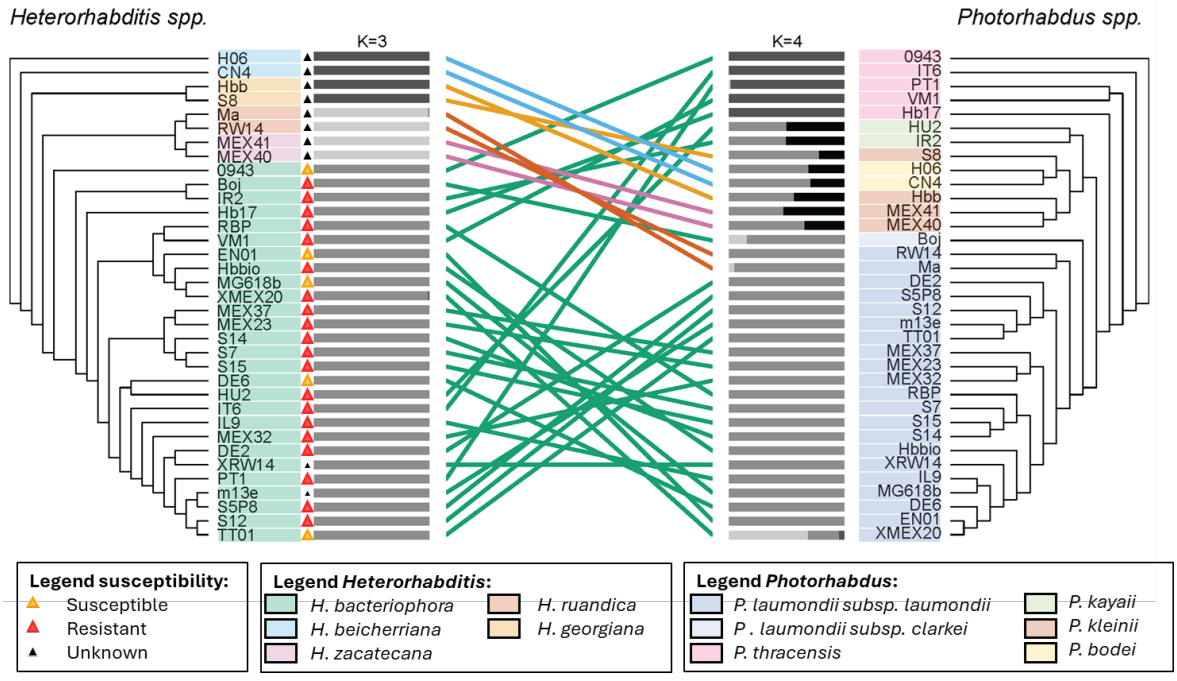

Figure 2: Tanglegram showing correspondence between the phylogenies of Heterorhabditis *spp. (left) and their symbiotic bacteria* Photorhabdus *spp (right).* Heterorhabditis zacatecana *(pink),* H. ruandica *(dark orange)*, H. beicherriana *(blue), and* H. georgiana *(light orange) are not sister species in the bacterial tree, unlike the nematode tree, but they are still clearly differentiated from* H. bacteriophora *(green). The triangles between the barplot and the nematode phylogeny represent the susceptibility of the given nematode isolate to benzoxazinoids. Barplots next to the tips of phylogenetic trees represent STRUCTURE results for the best K for both the nematode (K=3) and the bacterium (K=4, Supplementary Figure 3). The STRUCTURE result clearly distinguishes* H. bacteriophora *from other EPN species in the nematode phylogeny, whereas the bacterial phylogeny highlights more variation of symbionts within* H.

bacteriophora*, as well as several strains that seem ancestral to all other* H. bacteriophora *symbionts as well as the other* Heterorhabditis *symbionts*.

To assess the extent to which genetic differentiation among symbiotic bacteria associates with genetic differentiation among nematodes, we characterized genotypes of *Photorhabdus* by mapping raw sequencing reads to the genome of *Photorhabdus laumondii* subsp. *laumondii* (isolate TT01) to yield 79'816 SNPs of a minimum depth of 3. STRUCTURE analysis on the bacterial genotypes grouped them into four related clusters (optimal K = 4) (Supplementary Figure 3). The first genetic cluster included *P. thracensis* symbionts which are associated to *H. bacteriophora*. A second cluster included *P. kayaii* which are associated to *H. bacteriophora*, *P. bodei* which are associated to *H. beicherriana*, and *P. kleinii* which are associated to *H. georgiana* and *H. zacatecana*. A third cluster included *P. laumondii* subsp. *clarkei* and *P. laumondii* subsp. *laumondii* which are associated to *H. bacteriophora*. Lastly, the fourth cluster, closely related to the third cluster was represented by *P. laumondii* subsp. *laumondii* XMEX20 which is associated to *H. bacteriophora*.

Despite inconsistent genetic structure of the nematodes and their bacterial symbionts, a co-phylogenetic analysis highlighted that their phylogenies are more similar (Baker's gamma = 0.386) than expected by chance as assessed from a permutation test (P-value < 0.01)(Figure 2, Supplementary Figure 4). The monophyletic clade of *Heterorhabditis* species (i.e. *H. beicherriana*, *H. zacatecana*, and *H. georgiana*) was associated with more diverse symbionts also forming a monophyletic group (assigned to *P. kleinii* and *P. bodei*) being absent from *H. bacteriophora*, whereas symbionts associated with *H. bacteriophora* were included within a monophyletic group (assigned to *P. laumondii* subp. *laumondii*) as well as an early-diverging, paraphyletic group (assigned to *P. thracensis*). Although multiple *Photorhabdus* taxa were represented within *H. bacteriophora* (i.e. *P. laumondii* subsp. *laumondii, P. laumondii* subsp. *clarkei*, *P. thracensis* and *P. kayaii*), whereas the sister clades *H. ruandica* and *H. zacatecana* were associated with phylogenetically distant bacteria, the matching of *Heterorhabditis* and *Photorhabdus* phylogenies were consistent with specialized interactions and co-speciation having ruled diversification of the nematode and its bacterial symbionts (Půža and Machado 2024).

The genetic differentiation matching the delineation of nematode species that can be seen in both the phylogenetic and the STRUCTURE results is largely absent in the bacteria, although the in the samples in this study *H. bacteriophora* was significantly associated with *P. laumondii* subsp. *laumondii* (chi-squared = 4.3697, P-value = 0.03658).

Population genomics of *Heterorhabditis bacteriophora* and adaptation to host benzoxazinoid defenses

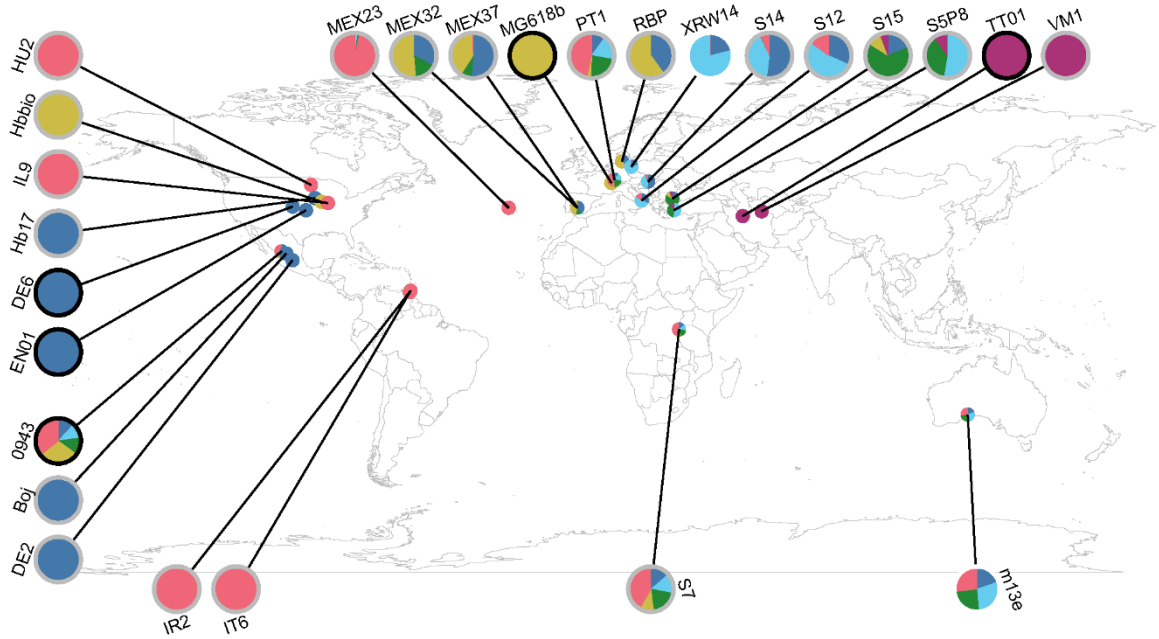

Figure 3: Distribution of genetic variation within Heterorhabditis bacteriophora. *Pie charts show allocation of each sample to the genetic clusters inferred by STRUCTURE at K=6 (Supplementary Figure 5), with the outer ring coloured according to the isolate being susceptible (black) or resistant (grey) to benzoxazinoids (Supplementary Figure 6). The absence of a ring indicates a lack of data.*

Within *H. bacteriophora*, the STRUCTURE analysis based on neutral SNPs for K=6 (i.e. the best K according to structure harvester; Supplementary Figure 5) showed mostly genetically pure groups in North America and across the easternmost part of our sampling area (Middle-East). In contrast, isolates from Central Europe were genetically heterogeneous, showing particularly strong admixture. A mantel test correlating the genetic distance and the geographic distance between all *H. bacteriophora* isolates showed no significant correlation ($p = 0.88$) consistent with non-equilibrium between genetic drift and gene flow, supporting Central Europe as a melting spot of genetic diversity within that species.

Infectivity assays assessing the extent to which infective juveniles of 25 *H. bacteriophora* isolates infect their WCR host (Supplementary Table 1, Supplementary Figure 6) distinguished isolates being susceptible vs resistant to benzoxazinoids. Only four isolates (DE6, EN01, TT01, 0943) were

clearly susceptible, as shown by their significantly lower infection rates in insects containing benzoxazinoids. An additional isolate, MG618b, was also considered to be susceptible to benzoxazinoids as it similarly showed lower infection in the three days following exposure to benzoxazinoids. Therefore, a total of five isolates, mostly sampled in Europe, were shown to be susceptible to benzoxazinoids (Supplementary Figure 6). These susceptible isolates were assigned to different STUCTURE clusters and were partly admixed from different genetic backgrounds (Figure 3). Most susceptible isolates were indeed genetically clustered with resistant ones (e.g. susceptible MG618b being closely related to the resistant Hbbio). Only the non-European isolate being susceptible (i.e. TT01, from Trinidad and Tobago) was assigned to a genetic cluster including only resistant ones such as PT1, S12 and S5P8.

Susceptible isolates presented diverse *Photorhabdus* bacteria being also sampled in resistant isolates, suggesting that bacterial species are uncoupled to benzoxazinoid resistance (chi-squared = 9.491e-32, P-value = 1). They further showed a restricted distribution across Central Europe (except TTO1), supporting that geography and the lack of long-term exposure to Maize and its WCR specialized herbivore may explain susceptibility to benzoxazinoids. Given its lack of strict association with the genetic structure, susceptibility to benzoxazinoids failed to result in genome-wide differentiation and hence likely evolved multiple times through changes at a few loci only.

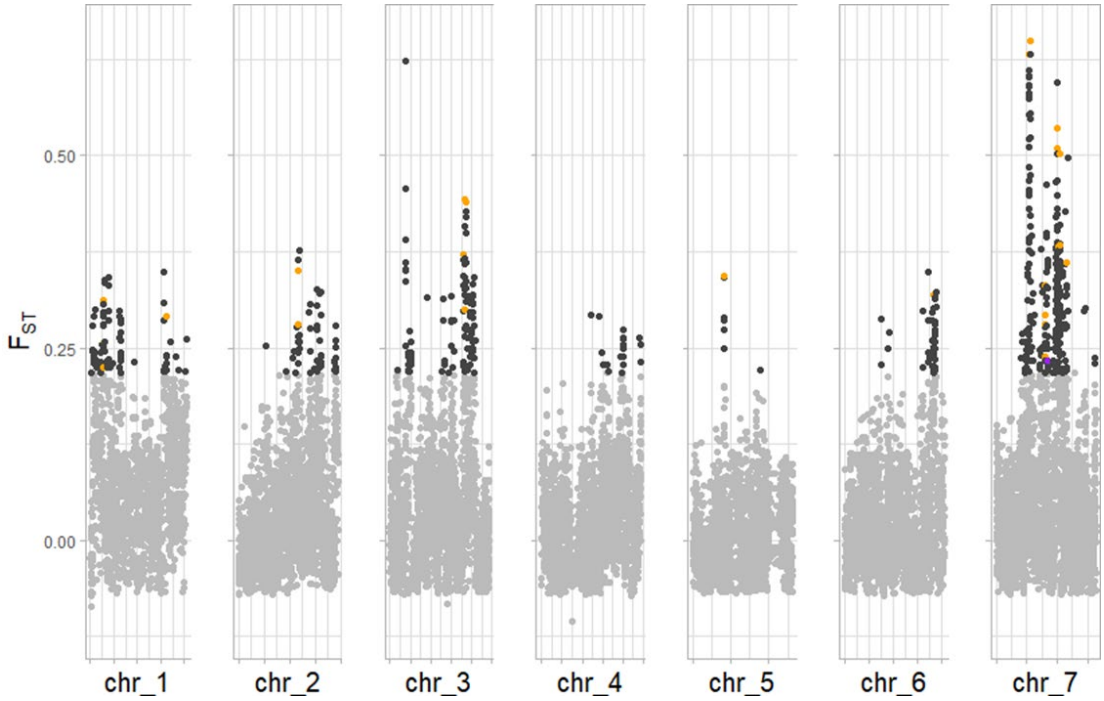

Figure 4: Genome-wide F_{ST} *estimated in non-overlapping windows of 5 SNP between isolates of* Heterorhabditis bacteriophora *stains being susceptible vs resistant to benzoxazinoids. Outlier* F_{ST} *windows (larger than the 3rd quartile plus 1.5 times the interquartile range) are depicted in black. Windows which are outliers both for* F_{ST} *and for* d_{XY} *(orange) represent windows that may be subject to strong selective pressure and reduced gene flow.*

To identify loci underlying benzoxazinoid resistance in *H. bacteriophora*, a genome scan assessed the differentiation (F_{ST}) and divergence (d_{xy}) in non-overlapping windows of five SNPs between susceptible and resistant isolates (Figure 4). A total of 19 windows were conservatively identified as both F_{ST} and d_{xy} outliers, supporting large frequency differences and sequence divergence that were likely shaped specifically by selection. The corresponding 42,208 bp were chiefly distributed on scaffolds 3 and 7, as well as a narrow peak on chromosome 5, and contained a total of 12 genes putatively involved in the resistance of EPNs to benzoxazinoids. Out of them, only 3 genes presented a high similarity to *C. elegans* genes and were subjected to a GO term enrichment analysis, highlighting a variety of enriched GO terms chiefly related to DNA maintenance and repair (Table 1). The three genes that had close relatives in *C. elegans* were putatively involve in ATP binding and the deoxyribonucleotide biosynthetic process (WBGene00004391), the polypeptide N-acetylgalactosaminyltransferase activity (WBGene00001630) and single-stranded DNA endodeoxyribonuclease activity (WBGene00003405).

313 *Table 1: GO terms that are enriched in genomic regions identified by the genomes scan (Figure 3) between isolates of*
314 Heterorhabditis bacteriophora *showing different susceptibilities to benzoxazinoids. Top: GO terms that are biological*
315 *processes, bottom: GO terms that are classified as molecular functions*

-- enriched biological processes --

GO.ID	Term	Annotated	Significant	Expected	P-value
GO:0000723	telomere maintenance	6	1	0.01	0.0068
GO:0007095	mitotic G2 DNA damage checkpoint signalling	7	1	0.01	0.0079
GO:0000724	double-strand break repair via homologous recombination	12	1	0.02	0.0135
GO:0006260	DNA replication	17	1	0.03	0.0192
GO:0036498	IRE1-mediated unfolded protein response	41	1	0.06	0.0458

-- enriched molecular functions --

GO.ID	Term	Annotated	Significant	Expected	P-value
GO:0004653	polypeptide N-acetylgalactosaminyltransferase activity	6	1	0.01	0.0072
GO:0030246	carbohydrate binding	29	1	0.05	0.0344

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Discussion

Entomopathogenic nematodes are an ecologically and economically interesting biological alternative to chemical pesticides. To better understand their biology and facilitate improvement in infection rates and host defense tolerance, we a population genomic analysis into EPNs in general and benzoxazinoid resistance in particular.

Heterorhabditis entomopathogenic nematodes have evolved independently of their endosymbionts

Whole-genome sequencing of multiple nematode isolates supported a genetic structure matching taxonomic species within the genus *Heterorhabditis*, being grouped into three genetic clusters: one composed of *Heterorhabditis ruandica* and *H. zacatecana*, the second one composed of *H. beicherriana* and *H. georgiana*, and the third one composed of *H. bacteriophora*. There is significant geographic isolation between the species of the first two groups. More specifically, *H. ruandica* has only been reported in Rwanda, and *H. zacatecana* has only been reported in Mexico. Similarly, *H. georgiana* has only been reported in the United States of America and *H. beicherriana* has only been reported in China and in the Caribbean islands. Hence, it is likely that these sister species are not allopatric populations, but have not yet sufficient time to diverge, consistently with *Heterorhabditis* being an evolutive young nematode group. An alternative explanation could be that there is extensive convergent evolution in this nematode group. Despite the limits inherent to the poorly known distribution of some of the isolates within the *Heterorhabditis* genus, the partitioning of genetic variation among investigated samples is overall consistent with *H. bacteriophora* being genetically isolated from their other species, despite their co-occurrence.

The genetic structure of sampled *Photorhabdus* symbionts demonstrates that the same species of nematode (*H. bacteriophora*) can live with different species of bacteria (i.e. *P. laumondii*, *P. kleinii*, *P. thracensis*) and that different species of nematodes can host the same species of bacteria (*H. bacteriophora and H. ruandica*). Such discrepancy suggests that the coevolution between nematodes and bacteria hardly resulted in highly specialized interactions and is likely inherent to the chiefly maternal transmission of symbionts among *Heterorhabditis* generations (Enright and Griffin 2004; Gaugler and Kaya 2018). Despite non-random association between nematode and their bacterial strains, symbionts have been regularly swapped following events such as admixture reported in *Heterorhabditis* and hinders confident predictions on members of the relationship (McMullen et al. 2017; Wang et al. 2023).

Entomopathogenic nematodes of the genus *Heterorhabditis* have evolved resistance to plant benzoxazinoids sequestered by some host insects

Isolates of *H. bacteriophora* being able to infect insects sequestering benzoxazinoid compounds that are toxic to them were sharing an evolutionary history with the western corm rootworm (WCR, *Diabrotica virgifera* subsp. *virgifera*) and maize (*Zea mays),* confirming insights from Zhang et al. (2019). Showing a higher rate of infection than isolates from regions with historically limited exposure to benzoxazinoids, resistant isolates of *H. bacteriophora* provide a valuable opportunity to assess the genetic basis of that phenotype.

As expected from their recent divergence, susceptible and resistant isolates of *H. bacteriophora* presented a lack of genome-wide genetic differentiation to be expected with incipient species or ecotypes, and rather indicated adaptation to benzoxazinoids having locally evolved through a few high impact changes in the genome. Accordingly, candidate loci highlighted by their extreme divergence between populations of susceptible and resistant isolates likely contained genes controlling tolerance to benzoxazinoids, and appeared enriched in GO terms associated with DNA maintenance and repair, as expected from benzoxazinoids interacting and modifying DNA under physiological conditions (Hashimoto and Shudo 1996). Similar mechanisms have been found to be important in the adaptation of the symbiotic bacterium to benzoxazinoids (Machado et al. 2020). Furthermore, the candidate genes involved in protein folding are likely relevant to remedy interference of benzoxazinoids with the structure of proteins.

Surprisingly, some isolates of *H. bacteriophora* lacking a long history of co-existence with the WCR and maize (e.g. IR2 and Boj, isolates from Iran) appeared strongly resistant to benzoxazinoids. Although recent adaptation following local cultivation of maize cannot be ruled out, resistant isolates of *H. bacteriophora* may have evolved in response to other insects and crops, such as wheat that also produces benzoxazinoids (Shavit et al. 2022). Although supporting an oligogenic architecture of resistance to benzoxazinoids, our indirect evidence must be functionally validated in order to ascertain their role and further understand how such a complex phenotype has evolved, considering also that different isolates may have developed adaptations on different genes. Furthermore, a more comprehensive sampling is necessary to shed light on the eco-evolutionary feedback promoting resistance to benzoxazinoids across the mosaic of abiotic conditions and communities to which *H. bacteriophora* is exposed.

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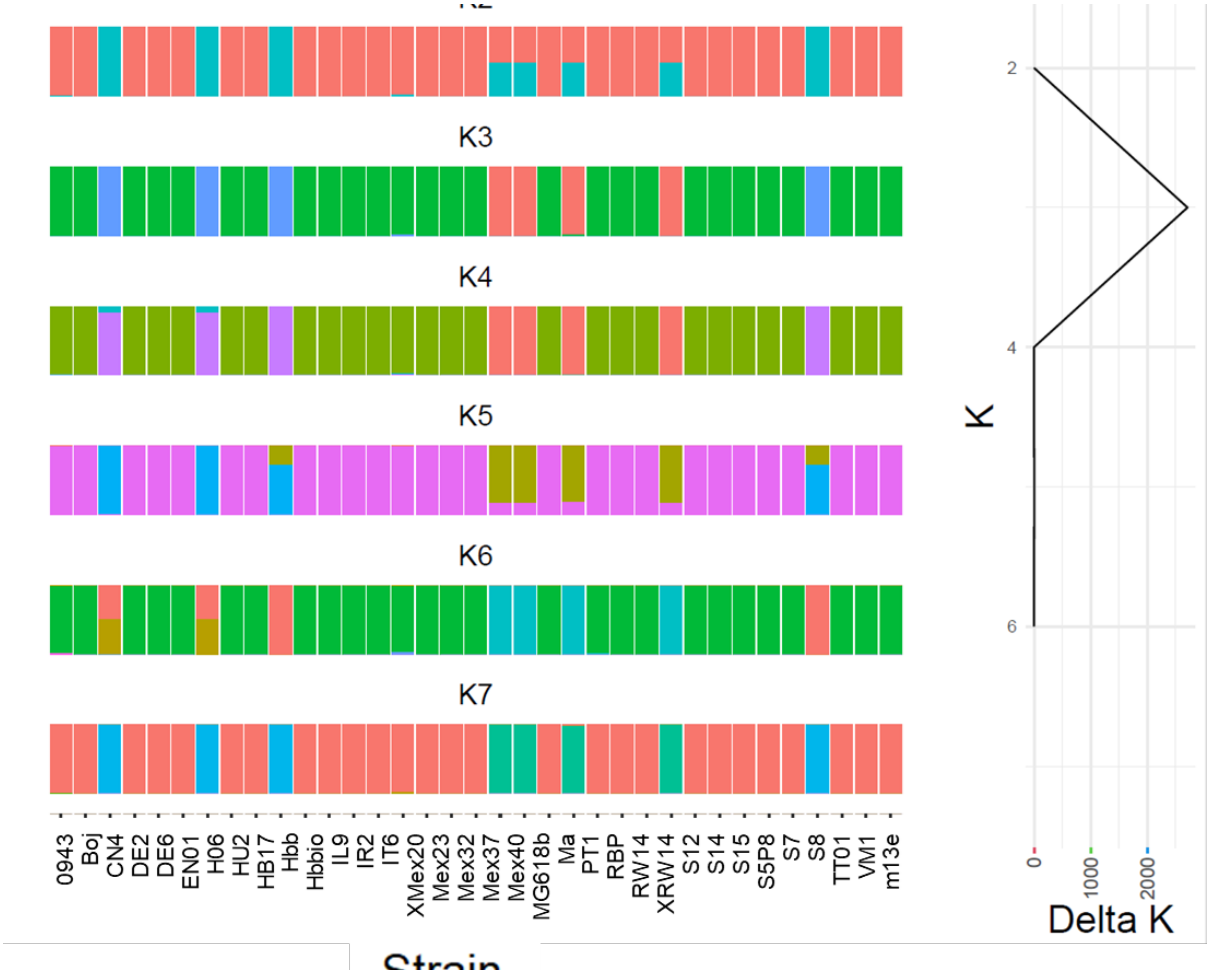
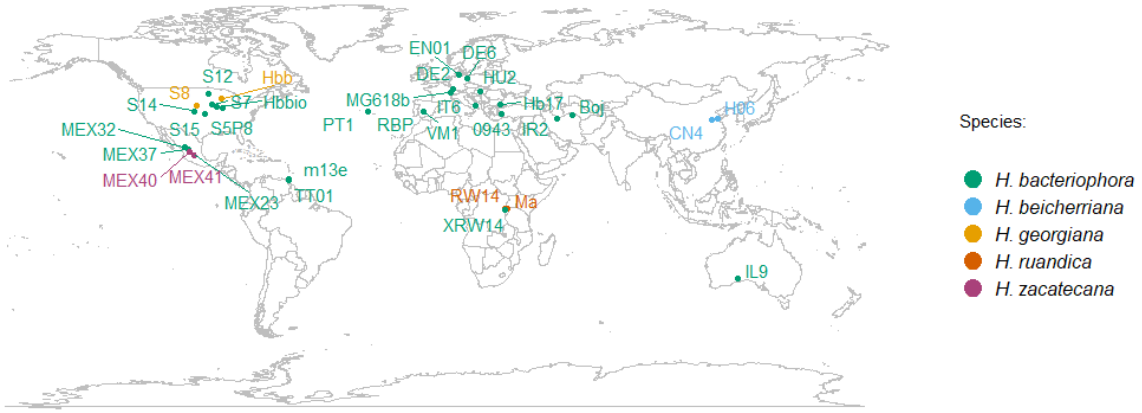
Supplementary material

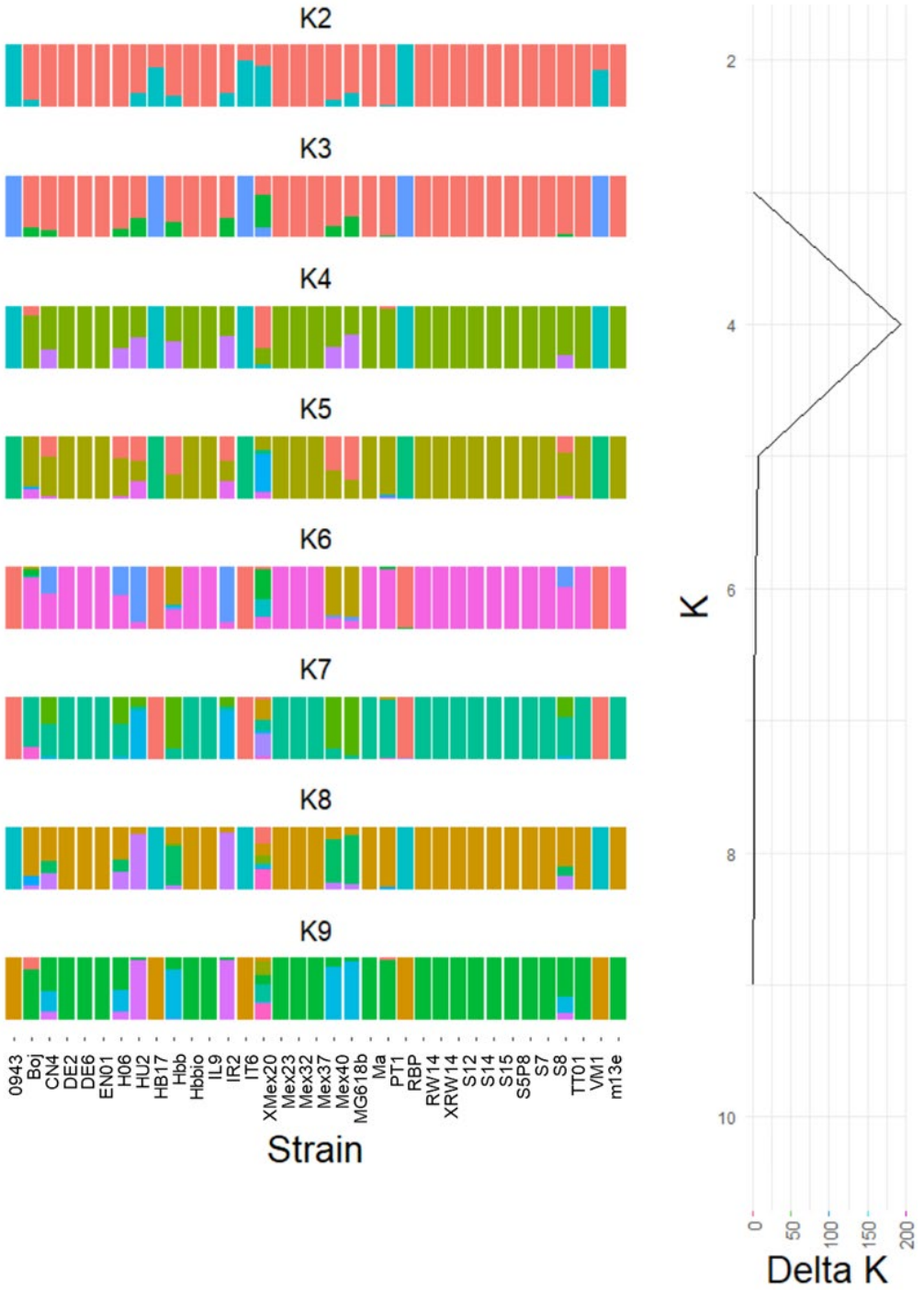
Supplementary Figures



Supplementary Figure 1: Results for different K for all isolates of Heterorhabditis available. On the right, the Delta K for the different K can be seen, with K=3 being the best.



Supplementary Figure 2: Geographic origins of all isolates of Heterorhabditis used in this study. Green = H. bacteriophora, *Blue* = H. beicherriana, *Yellow* = Heterorhabditis sp., *Orange* = H. georgiana, *Pink* = H. zacatecana, *Dark orange* = H. ruandica



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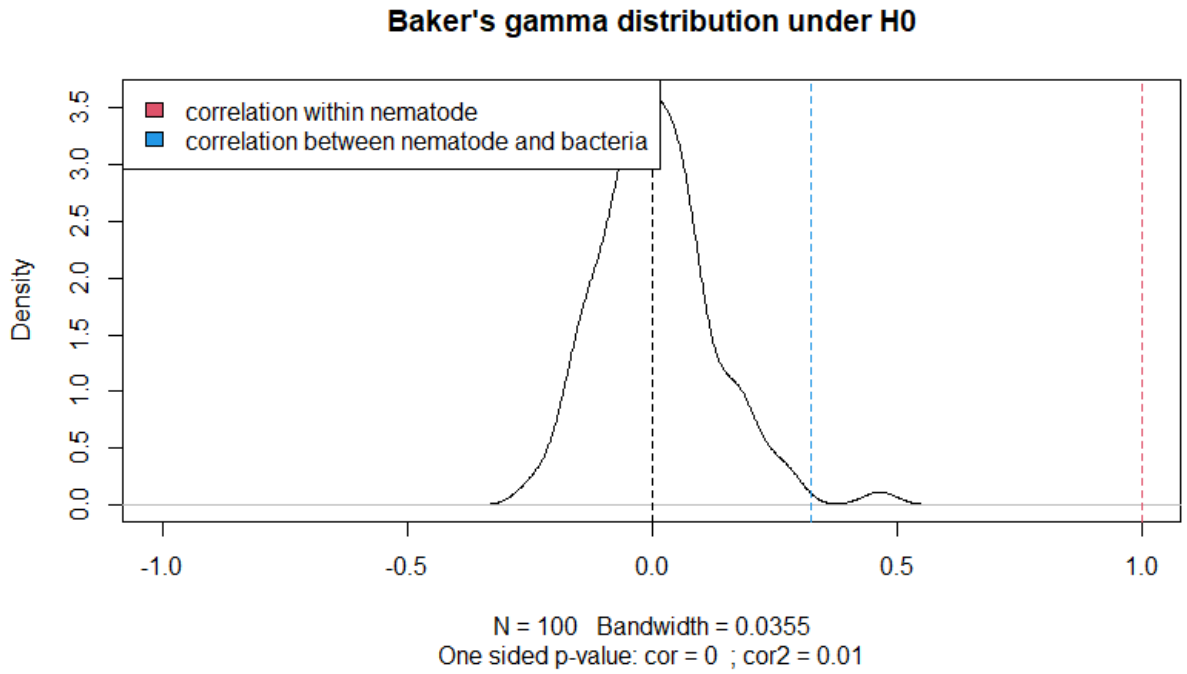
446 *Supplementary Figure 3: all K tested for the STRUCTUE analysis of the 35 strains of* Photorhabdus*. K2 to K9 were*
447 *tested, shown are the results of the clump analysis of 10+ runs for each K. on the right, the results of the evanno*
448 *method to determine the best K can be seen, here the highest Delta K was found for K=4.*

449



Supplementary Figure 4: the distribution of Baker's gamma values when permuting over the labels of one tree repeatedly. The vertical lines represent the important values, in red is the comparison of the same tree (i.e. same topology), in black is the zero value and in blue is the value found for the trees compared in the original tangelgram.

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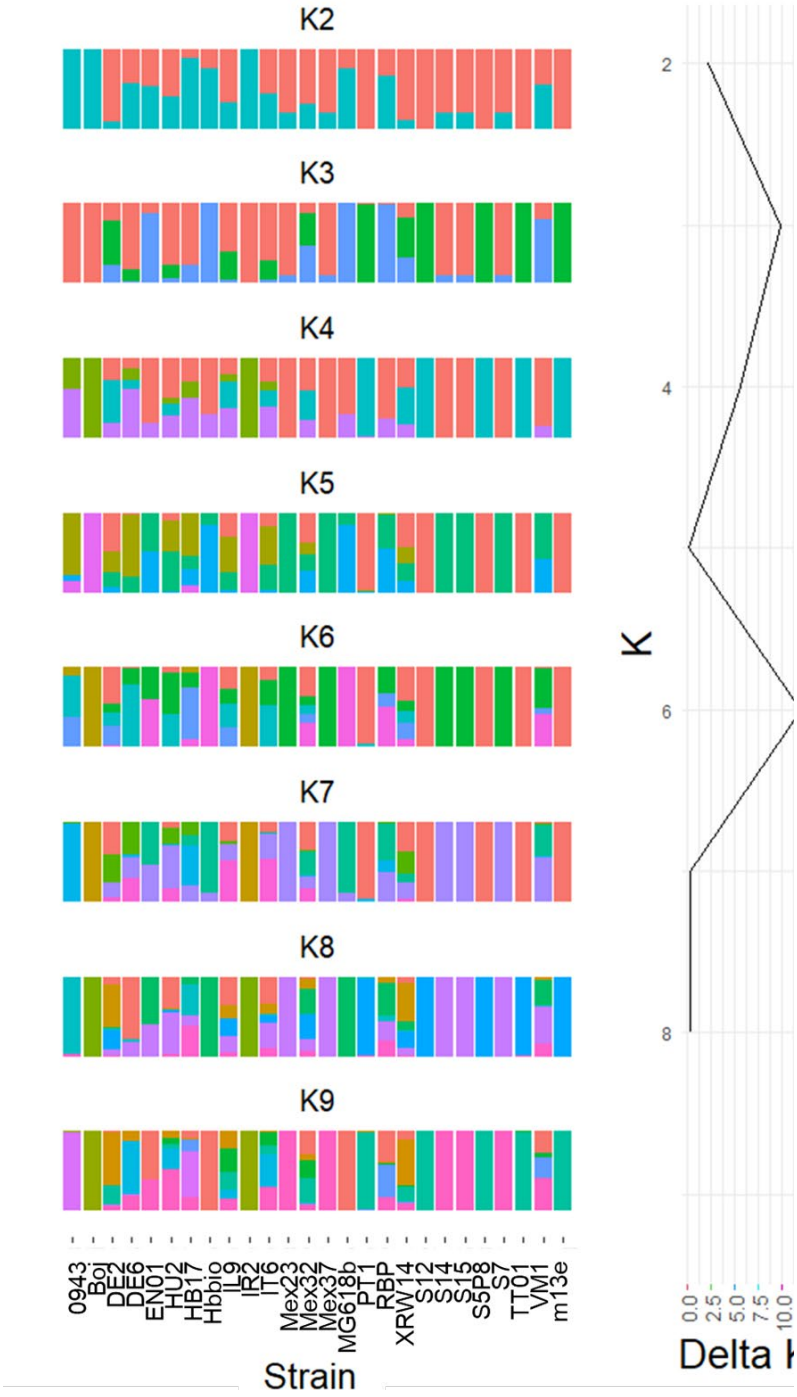
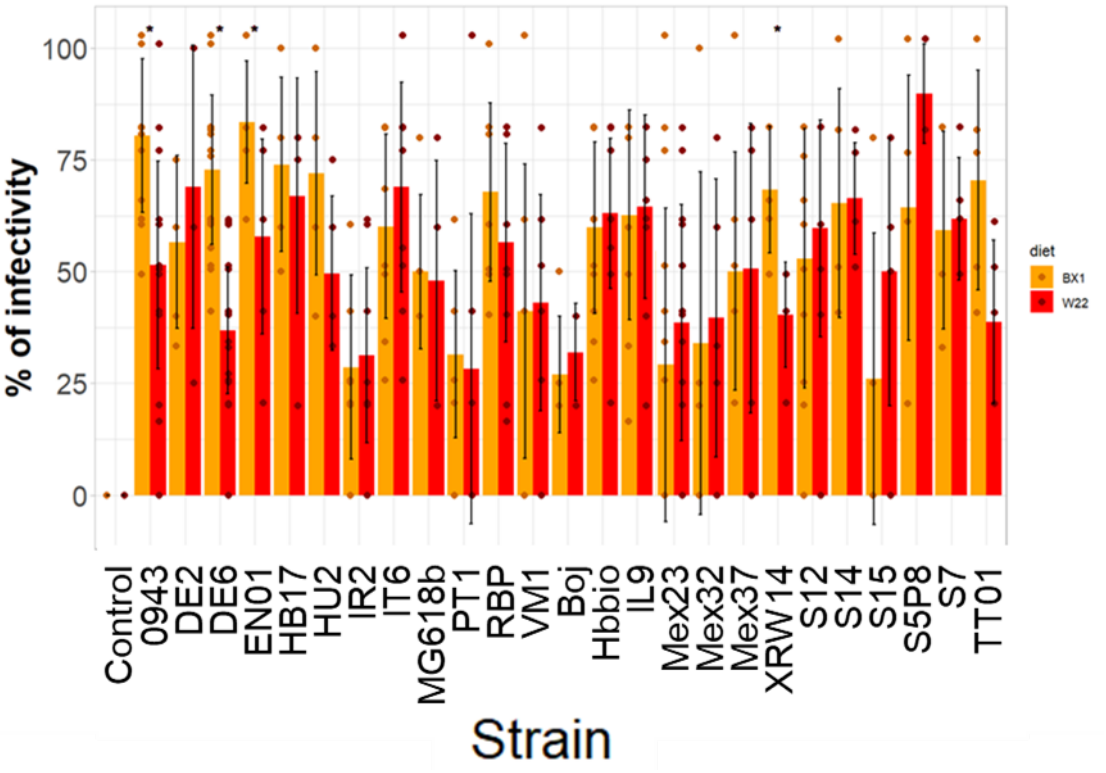


Supplementary Figure 5: all K tested for the STRUCTUE analysis of the 26 strains of H. bacteriophora. K2 to K9 were tested, shown are the results of the clump analysis of 10+ runs for each K. on the right, the results of the evanno method to determine the best K can be seen, here the highest Delta K was found for K=6



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459 *Supplementary Figure 6: Infectivity assay of different isolates of* H. bacteriophora.*in red is always shown the*
460 *infectivity in WCR larvae fed on Maize of genotype W22, in orange in those fed on bx1::W22, mutants in the*
461 *benzoxazinoid pathway which only produce small amounts of benzoxazinoids.*

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Supplementary Tables

Supplementary Table 1: Isolates of Heterorhabditis *used in the study. Including information regarding if any infectivity assay has been conducted and if applicable is they were susceptible of resistant to benzoxazinoids.*

Isolate	species	country	Infectivity assessed	Susceptibility to benzoxazinoids
0943	*H. bacteriophora*	Turkey	Yes	Yes
Ma	*H. ruandica*	Rwanda	No	NA
Boj	*H. bacteriophora*	Iran	Yes	No
RW14Art	*H. ruandica*	Rwanda	No	NA
CN4e	*H. beicherriana*	China	No	NA
H06	*H. beicherriana*	China	No	NA
DE2	*H. bacteriophora*	Germany	Yes	No
DE6	*H. bacteriophora*	Germany	Yes	Yes
EN01e	*H. bacteriophora*	Commercial	Yes	Yes
Hb17	*H. bacteriophora*	Turkey	Yes	No
Hbbio	*H. bacteriophora*	USA	Yes	No
HU2e	*H. bacteriophora*	Hungary	Yes	No
IL9e	*H. bacteriophora*	Australia	Yes	No
IR2e	*H. bacteriophora*	Iran	Yes	No
IT6e	*H. bacteriophora*	Italy	Yes	No
m13e	*H. bacteriophora*	Trinidad and Tobago	No	NA
MEX23	*H. bacteriophora*	Mexico	Yes	No
MEX20	*Heterorhabditis* sp	Mexico	No	NA
MEX32	*H. bacteriophora*	Mexico	Yes	No
MEX37	*H. bacteriophora*	Mexico	Yes	No
MG618b	*H. bacteriophora*	Switzerland	Yes	Yes

MEX40	*H. zacatecana*	Mexico	No	No
MEX41	*H. zacatecana*	Mexico	No	No
PT1e	*H. bacteriophora*	Portugal	Yes	No
RBP	*H. bacteriophora*	Spain	Yes	No
RW14	*H. bacteriophora*	Rwanda	No	NA
S12	*H. bacteriophora*	USA	Yes	No
Hbb	*H. georgiana*	USA	No	NA
S14	*H. bacteriophora*	USA	Yes	No
S15	*H. bacteriophora*	USA	Yes	No
S5P8	*H. bacteriophora*	USA	Yes	No
S7	*H. bacteriophora*	USA	Yes	No
TT01	*H. bacteriophora*	Trinidad and Tobago	Yes	Yes
VM1	*H. bacteriophora*	Spain	Yes	No
S8	*Heterorhabditis* sp.	USA	No	NA

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Chapter 3 – Unpublished work

Chapter III – Experimental adaptation of *Heterorhabditis bacteriophora* isolates to plant specialized metabolites.

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Abstract

The western corn rootworm (WCR), *Diabrotica virgifera virgifera*, is a major maize pest that sequesters benzoxazinoids (BXs), plant defense metabolites, to resist entomopathogenic nematodes (EPNs). While BXs enhance the defenses of the WCR, certain EPNs have evolved mechanisms to overcome these defenses, likely through genetic adaptation. This study examines the evolution of BX resistance in EPN populations using experimental evolution across five host generations of benzoxazinoid-containing WCR larvae and benzoxazinoid-deficient larvae, thereby selecting susceptible isolates to become resistant and having them remain susceptible respectively. Infectivity assays demonstrated that EPN isolates initially susceptible to BXs evolved resistance within three host cycles, achieving infectivity rates comparable to those in BX-deficient conditions. Whole-genome sequencing of EPN populations revealed significant genetic divergence between the ancestral (F_0) and evolved (F_5) populations, identifying key loci potentially associated with BX resistance. These findings highlight the capacity of EPNs to rapidly adapt to plant chemical defenses, providing new insights into enhancing the efficacy of biocontrol strategies against BX-sequestering pests like WCR.

Introduction

Insect pests are regulated by both bottom-up forces, such as plant defenses that reduce herbivore survival and reproduction, and top-down forces, including natural enemies like predators, parasitoids, and pathogens, which reduce herbivore abundance (Gripenberg and Roslin 2007; Cloyd 2020). These forces are critical determinants of ecosystem functioning and biodiversity, as they influence species interactions, population dynamics, and the balance between herbivores and their natural enemies (Price et al. 1980). Some specialized herbivores have evolved the ability to hijack plant defenses by sequestering toxic compounds, which they can repurpose for their own defense against natural enemies (Erb and Robert 2016). This strategy, while beneficial to the herbivores, can turn them into significant agricultural pests. Yet, natural enemies can still succeed in controlling herbivores and mitigating their negative impacts on plant health and yield (Hajek and Eilenberg 2018). Understanding how pest enemies have evolved to counter plant defenses being sequestered or utilized by insects could open new avenues for developing sustainable biocontrol strategies in pest management and to ensure food security.

Predators and parasitoids have evolved different strategies to resist or to tolerate plant defense metabolites. For example, it has been demonstrated that birds in Mexico that prey on monarch butterflies have become insensitive to the bitter-taste-plant cardenolides that the butterflies sequester from plants (Fink and Brower 1981). The same scenario was also shown with big-eyed bugs, which prey equally on nicotine-sequestering *Manduca sexta* larvae that had been fed on either nicotine-containing or nicotine-free plants (Kumar et al. 2014). Furthermore, development of the specialist parasitoid *Cotesia melitaearum* was unaffected by plant- sequestered iridoid glycosides sequestered by its host *Melitaea cinxia* (Reudler et al. 2011) . Yet, the underpinnings of genomic variation underlying these adaptations remain unclear (Ode 2006).

A notable example of specialized herbivores that can tolerate, sequester, and use plant specialized metabolites is the western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCR) (Robert et al. 2017). The WCR is one of the most economically significant agricultural pests worldwide, responsible for over $2 billion in annual losses due to yield reduction and pest management costs in the United States alone (Gray et al. 2009). Having spread across European regions, the WCR poses a global threat to maize production, a crop that provides 5% of global caloric intake and is an important source of animal feed as well as in biofuel production (Ranum et al. 2014; Erenstein et al. 2022). WCR larvae indeed feed on maize roots and, unlike other insects, can tolerate, stabilize, and sequester maize benzoxazinoids (BXs) (Robert et al. 2012;

Robert et al. 2017). BXs are indole-derived compounds, stored as glucosides in the plant vacuole, and being deglucosylated by a glucosidase upon tissue disruption (Robert and Mateo 2022). HDMBOA-Glc and DIMBOA-Glc are two BXs known to be involved in maize defense against herbivores (Meihls et al. 2013; Robert and Mateo 2022). Upon attack, the unstable aglucones HDMBOA and DIMBOA are formed and are spontaneously converted to MBOA. While these BXs have shown to be toxic to several herbivores such as aphids (Zhang et al. 2021; Shavit et al. 2022; Tzin et al. 2015) or caterpillars such as *Ostrinia* and *Spodoptera* (Campos et al. 1988; Glauser et al. 2011; Maag et al. 2016), WCR larvae tolerate these compounds, sequester HDMBOA-Glc, and glucosylate MBOA for sequestration as MBOA-Glc (Robert et al. 2017).

Despite sequestration of toxic BXs, WCR larvae can be attacked by entomopathogenic nematodes (EPNs) such as *Heterothabditis* spp. that are obligate parasites (Dillman and Sternberg 2012)). EPN infective juveniles (IJs) are free-living in soil and enter an insect host through natural openings or by penetrating the cuticle (Zhang et al. 2021; Dillman and Sternberg 2012). Once inside the host, EPNs release their symbiotic bacteria, which act in concert with the nematode's venom in killing the insect host (Lu et al. 2017; Dillman and Sternberg 2012; Chang et al. 2019), providing a nutrient-rich environment for the EPNs to reproduce (Dillman et al. 2012). Upon resource depletion, new IJs emerge from the host cadaver.

EPN infectivity can be drastically reduced when in BX-sequestering larvae, being as low as half the infectivity in larvae that are free of BXs (Robert et al. 2017; Zhang et al. 2019). First, WCR larvae release MBOA-Glc through their skin, acting as a repellent to EPNs (Robert et al. 2017). Second, EPNs that enter the insect host encounter elevated level of HDMBOA-Glc, itself toxic for EPNs (Robert et al. 2017). Finally, HDMBOA-Glc is locally deglucosylated, leading to the rapid formation of MBOA, toxic for both EPNs and their endosymbiotic bacteria (Robert et al. 2017). Although BXs offer a robust, multilayered defense for the WCR, EPN isolates that have co-evolved with WCR (e.g. in the US) have developed the ability to overcome this defense and exhibit higher infectivity than isolates that have not evolved alongside the BX-sequestering insect (Zhang et al. 2019). Experimental selection of *Heterorhabditis bacteriophora* in BX-sequestering WCR led to higher infectivity rates compared to EPNs selected in a non-sequestering *Diabrotica* insect (Zhang et al. 2019), indicating that swift adaptation to the sequestered plant metabolites is possible, potentially through (epi)genetic changes or shifts in microbial associations.

Studies investigating EPN resistance evolution to plant specialized metabolites from their hosts have primarily focused on microbial factors, such as the role of bacterial symbionts, rather than exploring the genetic responses of the nematodes themselves. Experimental evolution of

Photorhabdus, the bacterial endosymbiont of *Heterorhabditis* EPNs, improved the ability of EPNs that carried the selected isolates to kill WCR larvae (Machado et al. 2020). Endosymbionts carrying a mutation in the aquaporin-like channel gene *aqpZ* indeed enhanced EPN effectiveness in killing BX-sequestering WCR larvae, although such endosymbiont resistance to BXs was also associated with substantial costs as four out of the five selected isolates resulted in EPN growth impairment (Machado et al. 2020). These findings indicate that bacterial symbiont modifications alone may not fully account for the observed EPN adaptation to BXs, suggesting that genetic or epigenetic changes within the nematodes themselves are also crucial for overcoming the plant's defenses (Ogi et al. in review). Recently developed genomic resources in *Heterorhabditis* (Ogi et al, in prep) now provides the essential tools to investigate the genetics of adaptation to host-sequestered BXs in EPNs. Understanding the genomic changes that modulate the infectivity success of *Heterorhabditis* EPNs toward pests provides insights into genetic variants and pathways that enhance EPN resilience to plant specialized metabolites and could lead to more effective biocontrol strategies.

In this study, we investigate the genomic basis of EPN adaptation to BXs sequestered by their insect host through a guided evolution experiment. First, we chose four resistant and four susceptible EPN isolates and confirmed their tolerance to BXs in infectivity tests. Next, we adapted all isolates to BX containing or BX lacking WCR larvae over five host cycles (F_0 to F_5). We then examined changes in infectivity and conducting DNA sequencing of F_0 and F_5 allowed us to perform genome structure and F_{ST} analyses, identifying putative genes mediating EPN resistance to BXs.

Methods

Biological resources

Maize seeds of both the benzoxazinoid mutant bx1::W22 (Tzin et al. 2015), which is a loss of function mutant in the benzoxazinoid pathway and the wild type inbred line W22 were kindly provided by Georg Jander (Boyce Thompson Institute). Western corn rootworm (WCR, *Diabrotica virgifera virgifera*) eggs were obtained from USDA-ARS-NCARL (North Central Agricultural Research Laboratory, US Department of Agriculture, Agriculture Research Service, Brookings, SD, USA). WCR eggs were incubated at room temperature and hatching larvae were raised on either bx1::W22 ("bx^-") or W22 (BX^+) seedlings. WCR larvae fed on bx1::W22 are hereafter referred to as bx^--fed WCR, whereas WCR larvae fed on W22 are hereafter referred to as BX^+-fed WCR. Second and third instar larvae were used for infectivity assays. Entomopathogenic nematodes (EPNs) were originally provided by Prof. Raquel Campos Herrera (Institute of Grapevine and Wine

Sciences, Spain) and Prof. Ralf Udo Ehlers (e-nema GmbH, Germany) and maintained in an internal collection. The detailed description of 8 individual EPN isolates used in this study can be found in Table S1. All these isolates benefit from whole genome sequencing data and were previously assessed regarding susceptibility to benzoxazinoids (Ogi et al. in review) as to select four BX resistant isolates (HU2, IT6, VM1 and DE2) and four BX susceptible isolates (De6, 0943,MG618b and EN01). EPNs were amplified in *Galleria mellonella* larvae bought from a local fish store (Fischereibedarf N. Wenger AG, Kasernenstrasse 11, 3013 Bern). Emerging EPNs were collected using white traps (White 1927), filtered through 25 µm sieves, and kept in tap water in cell culture flasks (Thermo Fisher scientific, Switzerland). All EPNs were stored in 8°C fridges until use. Infective juveniles (IJs) were used in all experiments unless specified otherwise.

Experimental selection

Each of the eight chosen EPN isolates were divided into four subpopulations. The individual EPN subpopulations were then selected in either BX^+-fed or bx^--fed WCR larvae, resulting in 64 replicates within subpopulations (8 isolates x 4 subpopulations x 2 host diet). EPNs were multiplied by adding 50 EPNs to solo cups (30 mL; Frontier Scientific Services, Inc.) containing 3.5g of moist, autoclaved sand (Selmaterra, Bigler Samen AG) and 5 WCR larvae over five host cycles (called F_0 to F_5, n= 5 per subpopulation). Each host cycle represents approximately 2-3 EPN generations depending on host size (Dillman and Sternberg 2012; Trejo-Meléndez et al. 2024; Kaya and Gaugler 1993). EPN susceptibility to BXs, BXs preference, and infectivity were tested at F_0, F_3, and F_5.

Infectivity assays

The infectivity of the selected subpopulations was tested in WCR larvae fed on either BX^+ or bx- mutant plants. Briefly, 5 WCR larvae were placed into solo cups (30 mL; Frontier Scientific Services, Inc., Newark, USA) containing 3.5 g of moist, autoclaved, sand (Selmaterra, Bigler Samen AG, Steffisburg, Switzerland). Approximately 100 EPNs suspended in 700 µl tap water were added into the solo cups and incubated at 25 ± 0.5 °C for 7 days. As infected larvae turned red within 3-5 days, the infectivity rate was recorded visually 3-7 days post exposure (n=5 per each of the 64 subpopulations).

Genomic analyses

Of the 64 subpopulations produced by the experimental evolution experiment, 46 could be reared to a large enough quantity in generation F_5 to enable DNA extraction and sequencing along with the 8 original F_0 isolates (Table S2). DNA extraction was performed using the QIAamp® Micro

kit (Qiagen), following the manufacturer's instructions with the deviations that (i) 100 µl EPNs at a concentration of >50K EPNs/ml were added to 180 µl buffer ATL, (ii) homogenization was performed by adding eight 1.4 mm zirconium oxide beads and shook in a bead beater at 30 X for 3 minutes, and 20 µl proteinase K were only added after. Elution was done using 70 µl elution buffer after a five-minute incubation time. Samples were stored at -20°C until further processing at the Next Generation Sequencing (NGS) facility of the University of Bern. A PCR-free low input library was prepared and 150 bp paired end illumina sequencing was done on half a flow cell (Illumina, California, USA).

Sequence read processing and variant calling were conducted through a custom Snakemake pipeline, SnakeGATK4_v2 (https://github.com/parisodlab/snakeGATK4_v2). Accordingly, sequencing reads were aligned to the reference genome (produced for the isolate m13e) (Ogi et al. in review) using BWA-MEM (v0.7.18; (Li 2013)), and duplicate reads were marked using Picard's MarkDuplicates tool (v3.2; Broad Institute). Variant calling was performed using GATK4 (v4.1.3; (Poplin et al. 2017)). Individual genomic variant calls (gVCF) were generated for each sample using the HaplotypeCaller function with the *Heterorhabditis bacteriophora* genome set as reference (Ogi et al. in review)). These gVCFs were subsequently merged and imported into a genomic database using the GenomicsDBImport function. Joint genotyping of the gVCF files was performed using GenotypeGVCFs. Variant filtering was conducted using stringent criteria: sites were filtered based on quality metrics, including QualByDepth (QD) < 20, StrandOddsRatio (SOR) > 5.0, variant quality score (QUAL) < 30.0, and FisherStrand (FS) > 100.0, utilizing the VariantFiltration function in GATK4. Additionally, we applied minimum depth per sample (min_depth < 5), genotype quality (GQ < 15), and reference genotype quality (RGQ < 15) filters to remove low-confidence variant and invariant sites. We excluded sites with >95% missing genotype data to ensure high-quality variant calls using bcftools (v1.20; (Danecek et al. 2011)).

To confirm the correctness of the sequenced samples to the isolate assigned to them, a concordance analysis was conducted using previous sequencing results as a baseline (Ogi et al. in review). The concordance analysis was done on vcf files using the bcftools function gtcheck (Li 2011) (Figure S1).

Population genomic analyses:

A principal component analysis was conducted on all samples, using the SNPRelate package (Zheng et al. 2017) in R on the filtered vcf file to establish both clear differentiation between the isolates used here and a lack of differentiation based on treatment for the F_5 samples. A PCA analysis was also conducted on all F_0 samples to confirm if susceptibility is not the major dividing

factor between isolates (Ogi et al. in review). PCA analyses on individual isolates was performed for all samples of the isolates to check for the level of differentiation between F_0 and F_5 samples, and on F_5 samples of all originally susceptible strains, to investigate if the adaptation to BX^+ hosts is a major dividing force. A phylogenetic tree was built using RAxML (Stamatakis 2014), using the GTRGAMMA model, selecting the best tree out of 20 and building a bootstrap support out of 100 runs. Visualization was done in R using the ape package (Paradis and Schliep 2019). A genetic structure analysis was conducted using the program STRUCTURE, using the admixture model at 10,000 burnin and 100,000 repetitions, 10 replicates for each K (K1-K8). The best K was determined using Structure harvester and different runs were combined using clumpp.

Comparative genomic analyses

Comparisons of different treatments (F_0, F_5 BX^+, F_5 bx^-, each in susceptible and resistant isolates) were conducted to get a baseline for what regions of the genome could be important for the susceptibility to BXs, but this of course also highlights differences that accumulated for other reasons, as the different isolates are not extremely closely related and these comparisons may also disguise the relevant mutations, as different isolates may adapt differently to the presence of BXs. Since there are more differences in treatment between F_0 and F_5 than between F_5 BX^+ and F_5 bx^-, as the changed environment from previously being reared in *G. mellonella* larvae to *Diabrotica virgifera* larvae may have a larger impact than the two treatments where the only difference is the level of BXs contained in the food of the host larvae, this comparison was not delved into deeply. The main comparison presented here are within susceptible isolates, comparing F_5 BX^+ and F_5 bx^- samples to get the closest idea about which loci are actually involved in the response to BXs.

The F_{ST} analysis was conducted on filtered vcf files using the R package PopGenome (Pfeifer et al. 2014). All comparisons are window based with non-overlapping windows of 1000 bp, and the F_{ST} was calculated using the F_ST.stats function. Outlier F_{ST}s were defined as windows with an F_{ST} that is larger than the third quartile plus 1.5 times the interquartile range. All windows with higher F_{ST} values were considered for functional annotation and GO term enrichment analysis, and from these regions, SNPs were extracted directly, genes that either end or start in these windows were extracted using the annotation form (Ogi et al. in review) and the sequence of these genes was extracted from the reference fasta file that was used to align the illumina sequences above.

Further investigation into the genes was done using GO term enrichment analysis, using the GO term annotation from *Caenorhabditis elegans* (Carbon and Mungall 2024). This approach

inherently lacks some depth into the specific organisms, as the relative phylogenetic distance between *H. bacteriophora* and *C. elegans* means that there are significant genomic differences, and therefore only a somewhat biased subset of genes will be considered in the GO term analysis. For non-described genes, other methods were used, including blast searches and hmmer searches. Shiny-GO was used to create a network of important GO terms, using a translation of *H. bacteriophora* genes into C. elegans genes using an adapted basic blast alignment (Ge et al. 2020).

Statistical analyses

For statistical analyses, infectivity assays statistical comparisons were done using a Two-Way Repeated Measures ANOVA (One Factor Repetition), after data passed both Normality Test (Shapiro-Wilk) and Equal Variance Test (Brown-Forsythe) in SigmaPlot. For significant different groups, all pairwise comparisons were done using Bonferroni t-test. One subpopulation was considered as one replicate, all data shown here are the average of all subpopulations, which in turn are the average of all technical replicates within that subpopulation.

Results

Susceptible EPN isolates evolved resistance to benzoxazinoids within three growth cycles in BX-fed WCR larvae

In average, F_0 resistant isolates showed a similar infectivity rate of BX^+-fed and bx^--fed WCR larvae (Figure S2), even though individual differences were noted during the assay (Figure S3). In particular, HU2 was more successful in infecting BX^+-fed WCR larvae, DE2 and VM1 in infecting bx^--fed larvae, and IT6 showed no difference (Figure S3). However, no difference could be noted between infectivity rates of BX^+ and bx^--fed WCR larvae within any of the recording days (Figure S3). As expected, F_0 susceptible isolates displayed a higher infectivity in bx^--fed WCR larvae than in BX^+-fed ones at all timepoints (Figure S2), a pattern that was consistently observed for each of the four isolates. These observations confirmed the categorization of HU2, IT6, VM1, and DE2 as resistant isolates and of DE6, 0943,MG618b, and EN01 as BX susceptible isolates.

After three host cycles (F_3) in BX^+-fed WCR larvae, BX-resistant and BX-susceptible isolates did show non-significant differences in infecting BX^+ or bx^--fed WCR larvae (Figure 1), a behaviour that was consistently observed through all individual isolates except for 09_43 (Figure S4) suggesting the adaption has already been at least partly successful. As expected, after five host cycles (F_5), BX-resistant and BX-susceptible isolates similarly infected BX^+ or bx^--fed WCR larvae

(Figure 1), a behaviour that was consistently observed through all individual isolates (Figure S5). Overall, three host cycles in BX^+-fed WCR larvae were sufficient to clear the initial differences observed between BX-resistant and BX-susceptible isolates (F_0) (Figure 1).

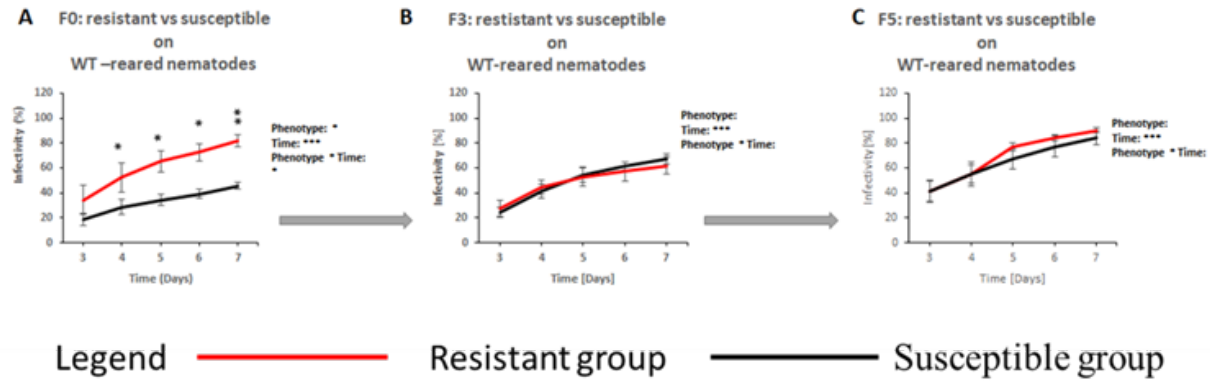


Figure 1: Comparing the infectivity of susceptible and resistant isolates in benzoxazinoid-containing larvae (BX^+-fed) over several generations (F_0s (A), F_3s(B) and F_5s (C)) Asterisk (*) indicate significant difference in infectivity between resistant and susceptible nematode isolates at a time point between day 3 and 7 post exposure ($P<0.05$). The legend on the right of each Figure shows the significance of the contribution the factors phenotype and time have in determining these differences. ,**:$0.001<p<0.01$,*: $0.01<p<0.05$, Error Bars: MSe.

The results showed that generation F_0 started with significant infectivity differences ($P<0.05$) between susceptible (black line) and resistant isolates (red line, Figure 1A). However, at F_3 as well as F_5 (Figures 1B and 1C), infectivity differences between resistant and susceptible isolates disappeared for all data points ($P>0.05$). Thus, the difference between susceptible and resistant isolates in benzoxazinoid tolerance disappeared after continuous exposure to benzoxazinoids, suggesting that adaptation to BXs has taken place within few generations.

Genomics

Population genomics

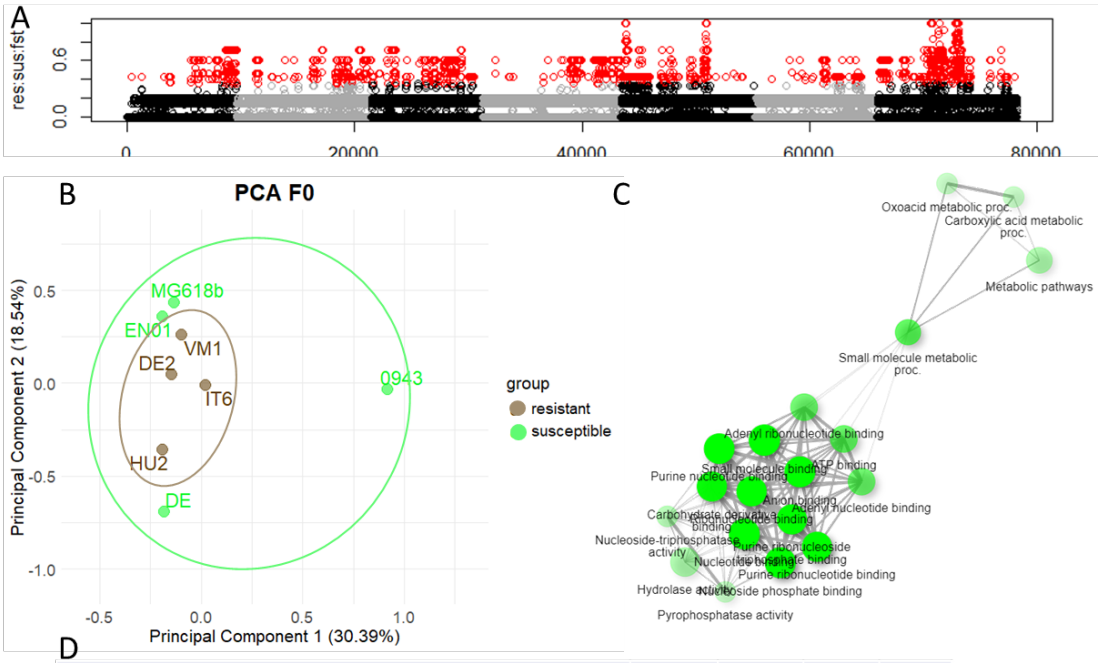
The information gathered from the PCA, the STRUCTURE output and the Phylogeny (Figure S6) all showed a much higher divergence between the individual isolates than between the different treatments, as expected due to the much longer divergence time between the different strains than within the experimental evolution, thus confirming that the experiment provoked evolution rather than large scale genetic drift. PCA analyses of individual isolates show in most cases a stronger distinction between the F_0 sample and the post evolution samples, rather than between the BX^+ and bx^- adapted post-evolution samples (separated on PC1 in 4 cases, PC2 and PC4 in 1 case each, Figures S8-S13). The STRUCTURE analysis showed a lack of differentiation large

enough to show a genome wide effect within isolates, only distinguishing between the isolates rather than the treatments, with some isolates not even having a unique structure group assigned. The phylogenetic tree, rooted using a isolate of the species *Heterorhabditis beicherriana*, showed a similar picture, with all isolates forming monophyletic groups, but no clear differentiation between treatments appearing, as was expected due to the selection experiment having been intended to act on few loci, rather than producing genome-wide divergence. DE2, which only had two sequenced samples, that F_0 and one F_5 does not conform to the monophyletic sorting, which was why they were excluded from further analyses presented here.

Comparative genomics

The pairwise F_{ST} landscape analysis of the different combinations of treatments (susceptible F_0, susceptible F_5 on BX^+, susceptible F_5 on bx^-, resistant F_0, resistant F_5 on BX^+, resistant F_5 on bx^-) showed a much higher level of divergence in some of the comparisons, particularly in ones comparing initially susceptible to initially resistant isolates (e.g. resistant F_5 adapted to BX^+ compared to susceptible F_5 adapted to bx^-) (Figure S7), which shows that there is higher differentiation between resistant and susceptible isolates than within each of the two phenotypes. Both the high level of differentiation between the different susceptibilities and the very low level of differentiation within the same susceptibility suggest that the susceptibility is a major diverging factor between the groups. The low divergence within groups allows for much more precise specification of areas of heightened differentiation between the different treatments, such as between susceptible F_5 BX^+ and susceptible F_5 bx^-, which is the comparison

where the most interesting mutations are expected to happen. However, including all isolates in one comparison may veil adaptations that are different in all isolates.



D

-- bp_peaks_genesres_sus.txt --

	GO.ID	Term	Annotated	Significant	Expected	P_value
1	GO:0040032	post-embryonic body morphogenesis	9	4	0.62	0.00034
6	GO:0007049	cell cycle	116	6	7.99	0.0038
7	GO:0008340	determination of adult lifespan	75	9	5.16	0.00405
8	GO:0006260	DNA replication	17	5	1.17	0.00506
9	GO:0010628	positive regulation of gene expression	28	5	1.93	0.00584
11	GO:0000727	double-strand break repair via break-induced replication	6	2	0.41	0.02428
12	GO:0042761	very long-chain fatty acid biosynthetic process	6	2	0.41	0.02428
13	GO:0055088	lipid homeostasis	7	2	0.48	0.03305
14	GO:0006270	DNA replication initiation	7	2	0.48	0.03305
15	GO:0016310	phosphorylation	128	10	8.81	0.03486
65	GO:0006457	protein folding	57	7	3.93	0.04286
66	GO:0032502	developmental process	430	18	29.61	0.04336

-- mf_peaks_genesres_sus.txt --

	GO.ID	Term	Annotated	Significant	Expected	P_value
1	GO:0005524	ATP binding	249	29	17.84	4.70E-07
2	GO:0051082	unfolded protein binding	28	6	2.01	0.0011
9	GO:0016887	ATP hydrolysis activity	71	9	5.09	0.0036
10	GO:0140662	ATP-dependent protein folding chaperone	17	4	1.22	0.0056
14	GO:0046872	metal ion binding	328	24	23.5	0.0155
15	GO:0003688	DNA replication origin binding	6	2	0.43	0.0261
16	GO:0004653	polypeptide N-acetylgalactosaminyltransferase activity	6	2	0.43	0.0261
17	GO:0017116	single-stranded DNA helicase activity	6	2	0.43	0.0261
18	GO:0043138	3'-5' DNA helicase activity	6	2	0.43	0.0261
19	GO:0032266	phosphatidylinositol-3-phosphate binding	7	2	0.5	0.0355
20	GO:0042803	protein homodimerization activity	7	2	0.5	0.0355
21	GO:0140359	ABC-type transporter activity	17	3	1.22	0.0368
22	GO:0003924	GTPase activity	53	6	3.8	0.0423
23	GO:0019001	guanyl nucleotide binding	71	7	5.09	0.0432
45	GO:0031625	ubiquitin protein ligase binding	8	2	0.57	0.046
46	GO:0005504	fatty acid binding	8	2	0.57	0.046

Figure 2: Population genomic analysis between susceptible and resistant F_0, including an F_{ST} landscape, a PCA, a GO term network analysis and a list of enriched GO terms. A) F_{ST} landscape comparing susceptible and resistant isolates, with red points denoting windows (1000 bp) that is higher than the 3rd quartile plus 1.5 times the interquartile range. B) a PCA showing PC 1 and 2 of the susceptible (light green) and resistant (brown) isolates. The ellipses hold no statistical significance and only show the range of both groups. C) A network analysis of gene in high F_{ST} windows, translated to C. elegans genes using a blast alignment, performed in ShinyGO. D) GO terms enriched in high F_{ST} windows, calculated using the R package topGO.

The direct comparison between non-adapted F_0 susceptible and resistant isolates showed a large amount of significantly high F_{ST} windows (Figure 2A), the highest of which were concentrated on chromosomes 5 and 7. A GO term enrichment analysis of the windows with high F_{ST} showed a selection of enriched genes, including terms involved in translation (e.g. DNA replication, double-strand break repair via break-induced replication), protein synthesis (protein

318 folding, unfolded protein binding), fatty acid metabolism (fatty acid binding, very long-chain fatty
319 acid biosynthetic process), transport processes (ABC-type transporter activity, lipid
320 homeostasis) and growth and development (developmental process). (Figure 2D). A Network
321 analysis of the same genes using a translation to C. elegans genes showed an accumulation of
322 metabolic pathways enrichment as well as many binding proteins, particularly ones involved in
323 energy storage and DNA maintenance (Figure 2C).

324 To get a clearer idea about factors that influence exclusively the adaptation to BXs, a comparison
325 between susceptible isolates after adaptation to BX^+ over 5 generations to samples of the same
326 isolate after adaptation to BX^- was conducted. The reason for the comparison of two groups post-
327 adaption rather than F_0 and F_5 despite the presence of a low concentration of BXs in the control
328 treatment was that the isolates had previously been exclusively amplified on *G. mellonella* larvae
329 since collection, and the adaptation to the different host (*D. virgifera*) would have introduced a
330 factor of differentiation that could have obfuscated the effect of the BXs. The pairwise F_{ST}
331 landscape analysis, for the three isolates that had more than one F_5 sample highlighted a very
332 restricted number of significantly high- F_{ST} windows for each of the isolates, 1 window for DE6, 7
333 windows for MG618b and 12 windows for 0943 (Figure 3 B, D, F). Further analysis, including
334 identifying the annotated genes that were at least partially inside the high- F_{ST} windows revealed
335 some genes that are of a similar family to the genes found in the comparison of the susceptible
336 and resistant F_0 samples.

337 In MG618b, the ATP-binding cassette was found in a high- F_{ST} window, which is expected to play
338 a role in the provision of energy that would be needed to provide translocation across
339 membranes. In 09_43, one high- F_{ST} window was the same as one that was identified in the
340 comparison between the two F_0 phenotypes, however, no genes were present in that window
341 which therefore makes further analysis difficult. However, in the other windows, a few genes
342 were identified. The sarcoplasmic calcium binding protein was identified through a blast search.
343 An adhesion G-coupled receptor was also identified, which may be involved in the G-protein-
344 coupled receptor signalling pathway. There were also several uncharacterized genes that are
345 also found in the human hookworm, *Necator americanus*, where they were matched with a
346 partial mRNA.

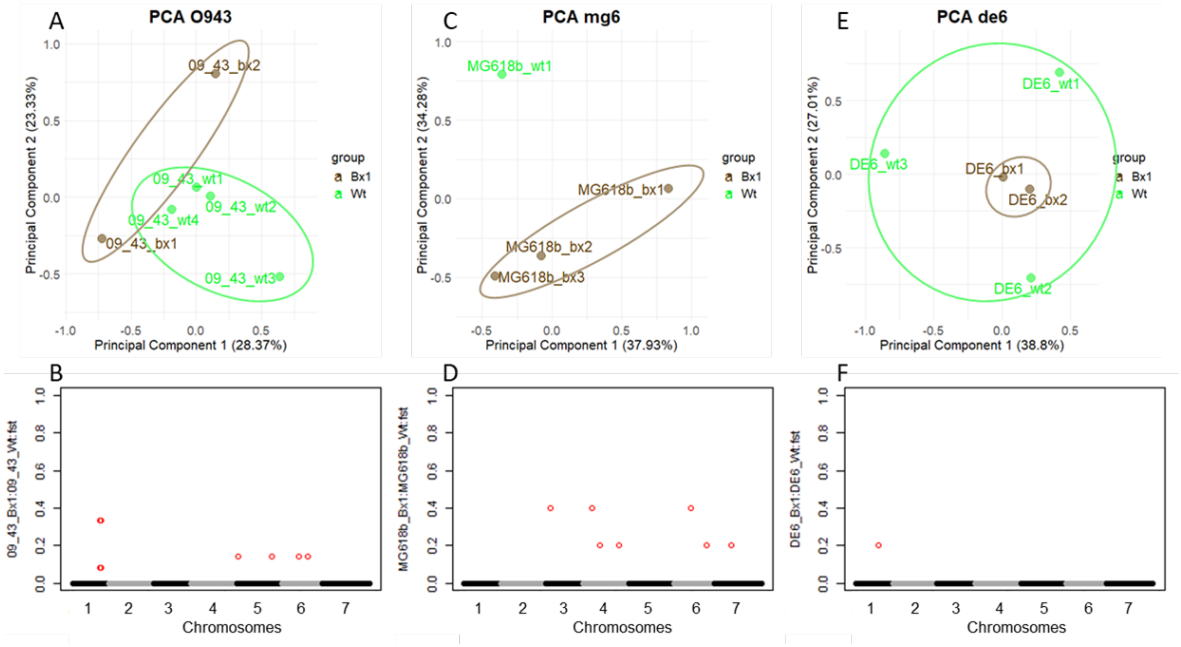


Figure 3: Population genomic analysis withing susceptible isolates, comparing samples reared on BX⁺ and bx⁻. Shown here are PCA analyses (, the ellipses hold no statistical significance) and F_{ST} landscapes (red points denoting windows with an F_{ST} value that is higher than the 3rd quartile plus 1.5 times the interquartile range). A) PCA analysis of the isolate 0943, B) F_{ST} landscape of the isolate 0943; C) PCA analysis of the isolate MG618b, D) F_{ST} landscape of the isolate MG618b; E) PCA analysis of the isolate DE6, F) F_{ST} landscape of the isolate DE6

Table 2: Enriched GO terms in regions of F_{ST} value that is higher than the 3rd quartile plus 1.5 times the interquartile range. Significant: number of genes associated to OG term that are considered significant, Expected: number of genes from dataset that would be expected to be associated to the GO term given random chance.

-- biological processes fstpeaks --						
	GO.ID	Term	Annotated	Significant	Expected	P_value
1	GO:0006412	translation	104	26	15.19	5.40E-05
2	GO:0008610	lipid biosynthetic process	71	15	10.37	6.90E-05
3	GO:0040018	positive regulation of multicellular organism growth	46	12	6.72	0.00073
5	GO:0008340	determination of adult lifespan	75	16	10.95	0.00113
6	GO:0008150	biological_process	1613	247	235.57	0.00194
7	GO:0007165	signal transduction	292	61	42.65	0.00256
8	GO:0036498	IRE1-mediated unfolded protein response	41	10	5.99	0.00349
9	GO:0055085	transmembrane transport	196	31	28.63	0.00509
10	GO:0000003	reproduction	192	27	28.04	0.00584
11	GO:0007041	lysosomal transport	8	4	1.17	0.00694
12	GO:0000027	ribosomal large subunit assembly	5	3	0.73	0.00699
16	GO:0030199	collagen fibril organization	6	3	0.88	0.01303
17	GO:0006535	cysteine biosynthetic process from serine	6	3	0.88	0.01303
18	GO:0009792	embryo development ending in birth or egg hatching	103	17	15.04	0.01309
19	GO:0018991	egg-laying behavior	42	7	6.13	0.01666
20	GO:0007229	integrin-mediated signaling pathway	9	4	1.31	0.02105
24	GO:0006099	tricarboxylic acid cycle	13	4	1.9	0.02703
25	GO:0009653	anatomical structure morphogenesis	164	29	23.95	0.0277

26	GO:0035264	multicellular organism growth	64	18	9.35	0.02839
27	GO:0006629	lipid metabolic process	140	30	20.45	0.0405
28	GO:0006631	fatty acid metabolic process	57	13	8.32	0.04161
29	GO:0007186	G protein-coupled receptor signaling pathway	33	8	4.82	0.04318
30	GO:0008361	regulation of cell size	17	4	2.48	0.04418
31	GO:0042026	protein refolding	15	4	2.19	0.04451
32	GO:0006096	glycolytic process	15	4	2.19	0.04451
33	GO:0072583	clathrin-dependent endocytosis	15	4	2.19	0.04451
40	GO:0010171	body morphogenesis	39	9	5.7	0.0463

-- molecular functions fstpeaks --

	GO.ID	Term	Annotated	Significant	Expected	P_value
1	GO:0005524	ATP binding	249	44	37.58	2.30E-05
2	GO:0003735	structural constituent of ribosome	46	13	6.94	0.00025
4	GO:0050661	NADP binding	5	3	0.75	0.00766
8	GO:0003779	actin binding	13	4	1.96	0.01418
9	GO:0050017	L-3-cyanoalanine synthase activity	6	3	0.91	0.01423
10	GO:0004124	cysteine synthase activity	6	3	0.91	0.01423
11	GO:0003756	protein disulfide isomerase activity	7	3	1.06	0.02315
18	GO:0046872	metal ion binding	328	42	49.5	0.03416
19	GO:0005544	calcium-dependent phospholipid binding	8	3	1.21	0.03444
20	GO:0005509	calcium ion binding	21	5	3.17	0.04505
21	GO:0050660	flavin adenine dinucleotide binding	21	5	3.17	0.04505

Discussion

This study provides a remarkable demonstration of the rapid adaptation of EPNs to a class of plant defense compounds sequestered by their insect host. The significant differences in infection rates between susceptible and resistant EPN isolates, which were evident at the start of the experiment, disappeared after only three generations of exposure to BX-sequestering WCR larvae. The genomic analyses highlight key loci involved in DNA repair, protein synthesis, and metabolism, providing new insights into the genetic mechanisms driving this adaptation and offering potential targets for enhancing biocontrol strategies.

The rapid adaptation to BXs highlights the remarkable plasticity and adaptability of EPNs. Such rapid adaptation to host-sequestered plant defense compounds suggests strong selective pressures in BX^+-fed WCR larvae, allowing for swift genetic shifts. This is consistent with previous findings (Zhang et al. 2019), where BX susceptibility was overcome in a short evolutionary timeframe. While the lower infectivity of resistant F_5 isolates compared to F_0 might initially seem puzzling, it is likely explained by differences in environmental factors at the time of the assay. Variations in conditions such as temperature, humidity, UV, could have influenced the outcome. This hypothesis will be further tested by performing infectivity assays with F_0 and F_5 isolates simultaneously, ensuring that environmental factors remain consistent and allowing for a more

accurate comparison of their infectivity levels. The fact that BX^+-selected susceptible and resistant isolates performed similarly emphasizes the potential for natural enemies, like EPNs, to quickly adjust to specialized herbivores. Yet, the underlying genetic mechanisms remained unexplored.

Population genomics revealed that metabolic and developmental processes might explain differences in infectivity of BX-resistant and BX-susceptible EPN isolates. The high-F_{ST} regions in the comparison between F_0 susceptible and F_0 resistant isolates highlighted a wide range of enriched GO terms which may be involved in the ability of the EPN to handle BXs. The heightened activity in translation could be linked to the BX's potential to intercalate into DNA, which could disrupt normal transcription and translation processes (Du Fall and Solomon 2011). In response, *H. bacteriophora* may be ramping up ribosomal activity to ensure that protein synthesis remains efficient, compensating for any BX-induced transcriptional hindrance. This could reflect a cellular strategy to overcome BX's interference by ensuring a steady output of necessary proteins, crucial for survival and infectivity. The regulation of genes involved in protein synthesis, particularly translation and ribosome-associated processes could be important given that BX compounds are known to inhibit enzymes, especially those containing cysteine residues. This increased demand for protein production may represent a compensatory mechanism. The organism might be overproducing proteins to counteract the inhibitory effects of BX on key enzymes, such as those involved in digestion, ensuring that essential functions are maintained even under BX stress. Fatty acid metabolism, another enriched process, plays a significant role in the context of BX exposure. BXs could impair membrane integrity or lipid biosynthesis, key components for maintaining cellular structure and energy storage. The organism's response appears to involve upregulation of lipid biosynthesis and fatty acid metabolism, which may be linked to maintaining membrane stability and adapting energy resources to support survival in the presence of BX. Since lipids are crucial for infectivity, particularly in processes like membrane fusion during host invasion, this metabolic adjustment might also help maintain the nematode's infective potential despite BX stress. Such gene families are already known to be important in the ability of other EPN species to infect their hosts (Dillman et al. 2015) and have been shown to potentially play a role in BX resistance (Ogi et al. in review). Transport processes, particularly transmembrane transport, could be pivotal in managing BX exposure. The enrichment in transmembrane transport pathways suggests that *H. bacteriophora* might be employing transporter proteins to exclude BX from entering its cells, thus mitigating the toxic effects (as in Machado et al. (2020)). Regulating the movement of BX across membranes could potentially limit its accumulation and minimizing its harmful impact on cellular processes.

Finally, the enrichment of pathways related to growth and development, such as the regulation of multicellular organism growth and anatomical structure morphogenesis, indicates that *H. bacteriophora* is balancing stress adaptation with maintaining developmental processes. Despite the toxic environment, the organism appears to prioritize growth, possibly to ensure its ability to develop and remain infective. This suggests a robust adaptive mechanism that integrates stress response with essential developmental pathways, allowing the nematode to maintain its life cycle and infectivity even under BX exposure. Some of the GO terms, particularly polypeptide N-acetylgalactosaminyltransferase activity has already been found to be in areas of divergence in previous studies on the aspect of plant toxin tolerance in EPNs (Ogi et al. in review)

The population genomic approaches showed a stronger divergence between F_0 and F_5 isolates. The single isolate PCAs separating the F_0 samples more strongly from the F_5 samples in most cases could be due to the fact that the nematode isolates had been exclusively reared on *G. mellonella* larvae between their entry into the lab rearing and the start of the experiment presented here, so the adaptation for five generations on a different insect larvae (D. virgifera) who is not only much smaller than *G. mellonella* but also has a massively different diet, even disregarding the presence of BXs. The greater wax moth larva is a parasitic organism that prays on honeybees, which would on one hand include much more animal protein in their diet and also make them an organism that may not be part of the natural rang of hosts for *H. bacteriophora*, which are aquatic nematodes exclusively found underground.

One gene family that was found in single isolate comparisons between BX^+ and bx^- F_5 adapted samples, particularly the one involved in ATP binding has already previously been found to show difference between susceptible and resistant nematode isolates (Ogi et al. in review). This particular apparent importance of energy conservation could be a change that is relevant for all the different potential aspects listed above, as the basis of energy conservation is instrumental in the execution of the other processes. The sarcoplasmic calcium binding protein that was identified in the isolate 09_43 can exclusively be found in invertebrates, particularly in neuronal and muscle tissue (Hermann and Cox 1995). They are characterized as EF-hand calcium buffering proteins and hypothesised to play a role in invertebrate muscle relaxation. Calcium has been shown to play an important role int both neural functioning an aging/neurodegeneration in *C. elegans* (Tanimoto et al. 2017; Alvarez et al. 2020), which could mean that a calcium binding protein would be important in the ability of *H. bacteriophora* to ensure the damage acquired from the BXs could be evaded, such as through rapid development followed by refocusing much of the energy present into reproduction, to heighten chances of survival for the following generations.

The adhesion G-coupled receptor is known to play a role in the immune system, where it is shown to be involved in the response to systemic inflammation (Yona et al. 2008). Since G-protein coupled receptors are very diverse sensory receptors, they also have a wide variety of application in an organism, particularly as a signalling receptors in immune response. Though most of the knowledge of their role in immune response is gathered from the response of C. elegans to pathogens and damage associated molecular patterns (Gupta and Singh 2017), a similar importance could be assigned to them in response to other types of damage, such as the presence of benzoxazinoids. Though BXs interact with cells and cell functioning in different ways to pathogens, the importance of actually transmitting information regarding any type of attack can not be underestimated, and in such a generalist organism as H. bacteriophora, the need to use any possible mechanism for the lack of specific responses may be present.

In conclusion, in this study we demonstrate the remarkable ability of *Heterorhabditis bacteriophora* to rapidly adapt to the host-sequestered plant defense compounds benzoxazinoids within as few as three generations of selection. The genomic analyses revealed key loci involved in DNA repair, protein synthesis, and metabolic processes that likely contribute to this swift adaptation. These findings highlight potential targets for enhancing the efficacy of EPNs in biocontrol strategies and provide insights into the broader mechanisms by which organisms can overcome defense mechanisms. These genes and loci will have to be confirmed by conducting additional and complementary experiments, such as identifying selective sweeps in addition to the F_{ST} analyses, as well as performing RNAi silencing on the identified genes to establish if the ascribed function is truly their effect.

Acknowledgements

We would like to thank the Next Generation Sequencing Platform of the University of Bern for performing the high-throughput sequencing experiments. The work of VO and DM was supported by the Swiss National Science Foundation [310030_192564] to ME. The work of CAMR was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme [ERC-2019-STG949595] and the Swiss National Science Foundation [310030_189071].

Tables

Table 3: Enriched GO terms in regions of F_{ST} value that is higher than the 3rd quartile plus 1.5 times the interquartile range

Figure legend

Figure 1: Comparing the infectivity of susceptible and resistant isolates in benzoxazinoid-containing larvae (BX$^+$-fed) over several generations (F_0s (A), F_3s(B) and F_5s (C)) Asterisk (*) indicate significant difference in infectivity between resistant and susceptible nematode isolates at a time point between day 3 and 7 post exposure ($P<0.05$). The legend on the right of each figure shows the significance of the contribution the factors phenotype and time have in determining these differences. ,**:$0.001<p<0.01$,*: $0.01<p<0.05$, Error Bars: MSe.

Figure 2: Population genomic analysis between susceptible and resistant F_0, including an F_{ST} landscape, a PCA, a GO term network analysis and a list of enriched GO terms. A) F_{ST} landscape comparing susceptible and resistant isolates, with red points denoting windows (1000 bp) that is higher than the 3rd quartile plus 1.5 times the interquartile range. B) a PCA showing PC 1 and 2 of the susceptible (light green) and resistant (brown) isolates. The ellipses hold no statistical significance and only show the range of both groups. C) A network analysis of gene in high F_{ST} windows, translated to C. elegans genes using a blast alignment, performed in ShinyGO. D) GO terms enriched in high F_{ST} windows, calculated using the R package topGO.

Figure 3: Population genomic analysis withing susceptible isolates, comparing samples reared on BX$^+$ and bx$^-$. Shown here are PCA analyses (, the ellipses hold no statistical significance) and F_{ST} landscapes (red points denoting windows with an F_{ST} value that is higher than the 3rd quartile plus 1.5 times the interquartile range). A) PCA analysis of the isolate 0943, B) F_{ST} landscape of the isolate 0943; C) PCA analysis of the isolate MG618b, D) F_{ST} landscape of the isolate MG618b; E) PCA analysis of the isolate DE6, F) F_{ST} landscape of the isolate DE6

Supplementary Figures

Figure S1: Concordance analysis of all samples compared to previously sequenced samples of the eight isolates represented here

Figure S2: Benzoxazinoid (BX)-susceptible entomopathogenic nematodes (EPNs) can be selected to be as infective as BX-resistant EPNs within three parasitism cycles in BX^+-fed Western Corn Rootworm larvae (WCR).

Figure S3: F_0 infectivity rates of individual entomopathogenic nematode (EPN) isolates in Western Corn Rootworm larvae (WCR) fed on wild type plants containing benzoxazinoids (BX^+) and bx mutant plants (bx).

Figure S4: F_3 infectivity rates of individual entomopathogenic nematode (EPN) isolates (F_0) in Western Corn Rootworm larvae (WCR) fed on wild type plants containing benzoxazinoids (BX^+) and bx mutant plants (bx)

Figure S5: F_5 infectivity rates of individual entomopathogenic nematode (EPN) isolates (F_0) in Western Corn Rootworm larvae (WCR) fed on wild type plants containing benzoxazinoids (BX^+) and bx mutant plants (bx)

Figure S6: Population genetic comparison between all the samples created and used in this study

Figure S7: F_{ST} landscape of all comparisons between different treatments (susceptible isolates F_0, resistant isolates F_0, both in F_5 adapted to BX^+ and both in F_5 adapted to bx^-

Figure S8: PCA showing principal components (PC) 1 to 5 for the isolate VM1, a resistant isolate

Figure S9: PCA showing principal components (PC) 1 to 5 for the isolate HU2, a resistant isolate

Figure S10: PCA showing principal components (PC) 1 to 5 for the isolate IT6, a resistant isolate

Figure S11: PCA showing principal components (PC) 1 to 5 for the isolate MG618b, a susceptible isolate

Figure S12: PCA showing principal components (PC) 1 to 5 for the isolate DE6, a susceptible isolate

Figure S13: PCA showing principal components (PC) 1 to 5 for the isolate 0943, a susceptible isolate

Figure S1

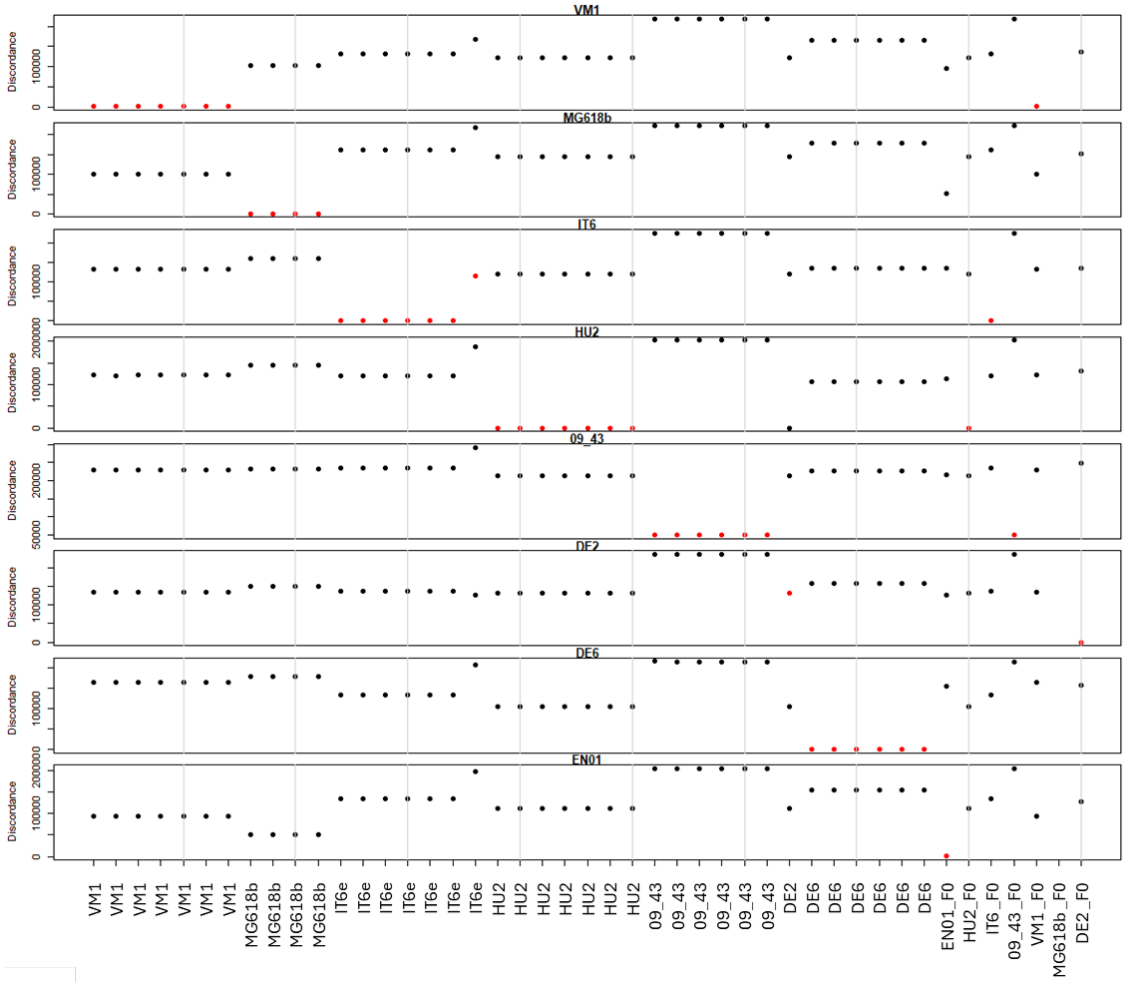


Figure S1: Concordance analysis of all samples compared to previously sequenced samples of the eight isolates represented here. Each plot represents one previous sample compared to all current samples, points in red mean that this samples is supposed to be the same as the old sample. Lower values show lower discordance, i.e. higher likelihood of the samples being of the same isolate.

Figure S2

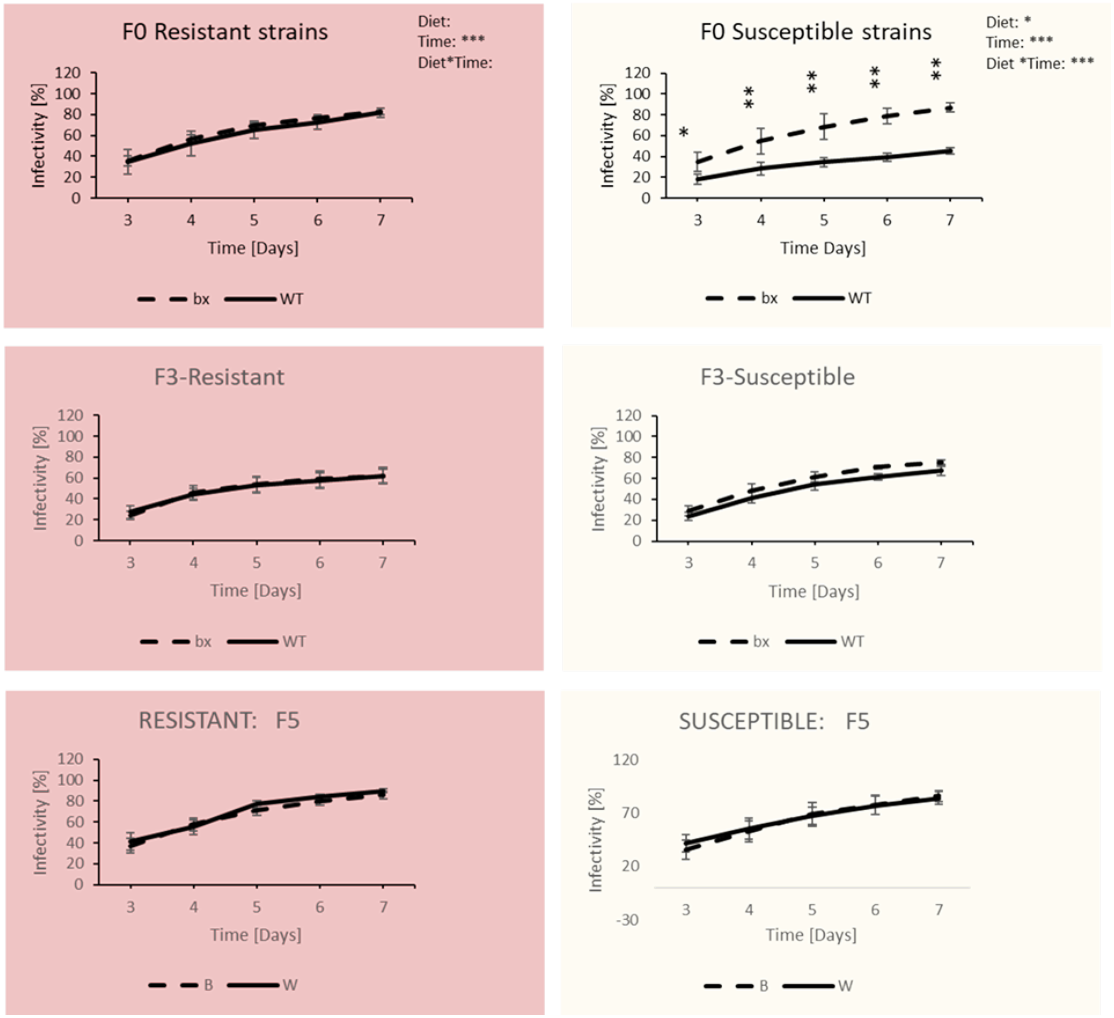


Figure S2: Benzoxazinoid (BX)-susceptible entomopathogenic nematodes (EPNs) can be selected to be as infective as BX-resistant EPNs within three parasitism cycles in BX$^+$-fed Western Corn Rootworm larvae (WCR). EPNs were selected by rearing them in BX$^+$-fed WCR larvae for 5 parasitism cycles (F_0 to F_5) in BX$^+$-fed WCR larvae (n=4 subpopulation per isolate and WCR diet). EPN infectivity (Mean ± SEM) was recorded by adding 50 EPNs to 5 WCR larvae and visually inspecting the larvae for colour change daily between day 3 and day 7 after EPN addition. Means of the subpopulation averages are shown. Red panel (left): BX-resistant EPN isolates. Yellow panel (right): BX susceptible EPN isolates. Solid line: EPN infectivity in BX$^+$-fed WCR. Dotted line: EPN infectivity in bx$^-$-fed WCR. Diet: Diet of the WCR larvae used for infectivity. Two-Way ANOVA on repeated measured were conducted. Stars indicate significant differences between EPN infectivity on BX$^+$ and bx$^-$-fed WCR larvae within the day of observation. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Figure S3

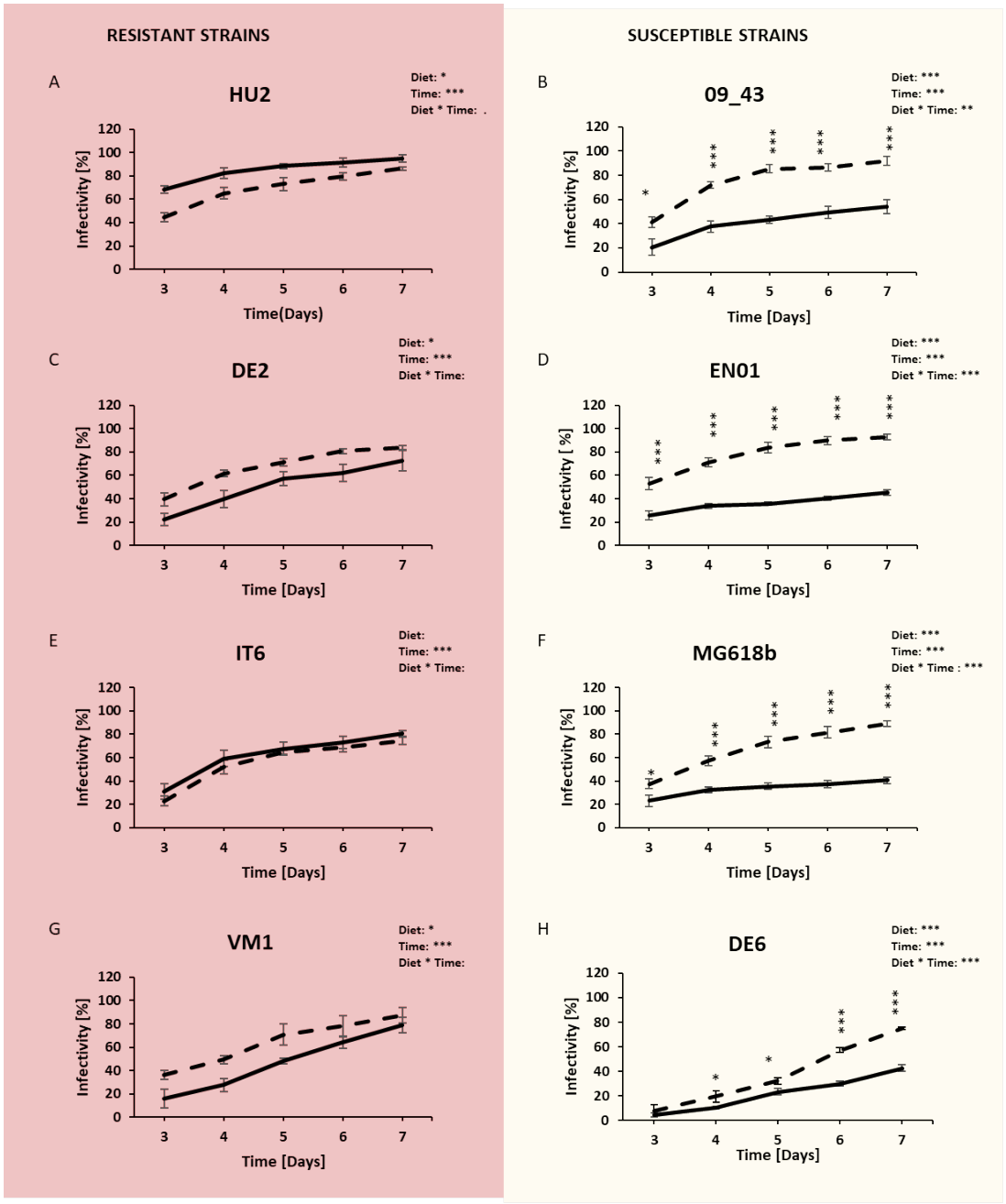


Figure S3: F_0 infectivity rates of individual entomopathogenic nematode (EPN) isolates (F_0) in Western Corn Rootworm larvae (WCR) fed on wild type plants containing benzoxazinoids (BX^+) and bx mutant plants (bx). EPN infectivity rate (Mean ± SEM) was recorded by adding 50 EPNs to 5 WCR larvae and visually inspecting the larvae for colour change daily between day 3 and day 7 after EPN addition (n=5). Red panel (left): BX-resistant EPN isolates. Yellow panel (right): BX susceptible EPN isolates. Solid line: EPN infectivity in BX^+-fed WCR. Dotted line: EPN infectivity in bx^--fed WCR. Diet: Diet of the WCR larvae used for infectivity. Two-Way ANOVA on repeated measured were conducted. Stars indicate significant differences between EPN infectivity on BX^+ and bx^--fed WCR larvae within the day of observation. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Figure S4

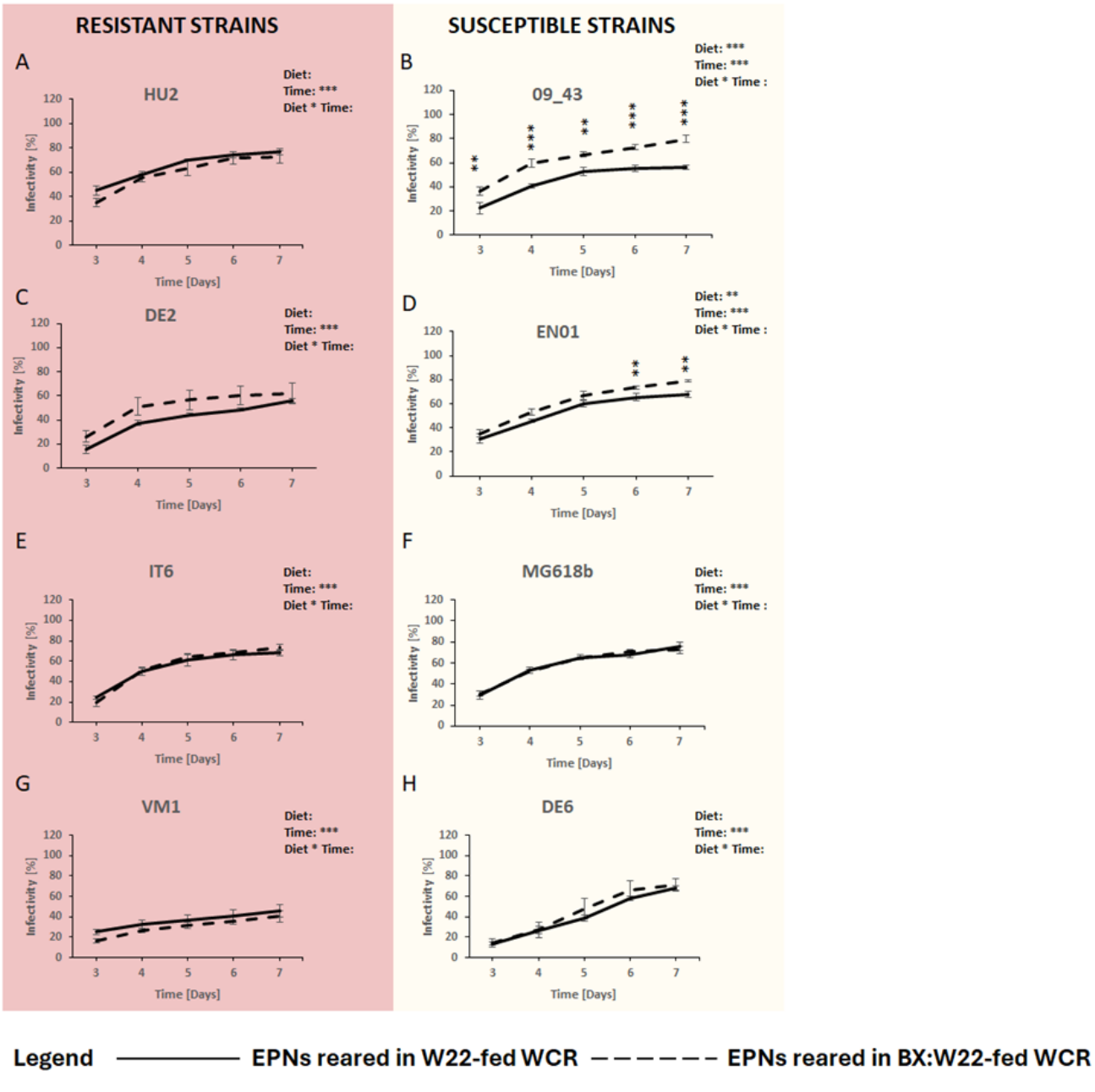


Figure S4: F_3 infectivity rates of individual entomopathogenic nematode (EPN) isolates (F_0) in Western Corn Rootworm larvae (WCR) fed on wild type plants containing benzoxazinoids (BX^+) and bx mutant plants (bx). EPN infectivity rate (Mean ± SEM) was recorded by adding 50 EPNs to 5 WCR larvae and visually inspecting the larvae for colour change daily between day 3 and day 7 after EPN addition (n=5). Red panel (left): BX-resistant EPN isolates. Yellow panel (right): BX susceptible EPN isolates. Solid line: EPN infectivity in BX^+-fed WCR. Dotted line: EPN infectivity in bx^--fed WCR. Diet: Diet of the WCR larvae used for infectivity. Two-Way ANOVA on repeated measured were conducted. Stars indicate significant differences between EPN infectivity on BX^+ and bx^--fed WCR larvae within the day of observation. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Figure S5

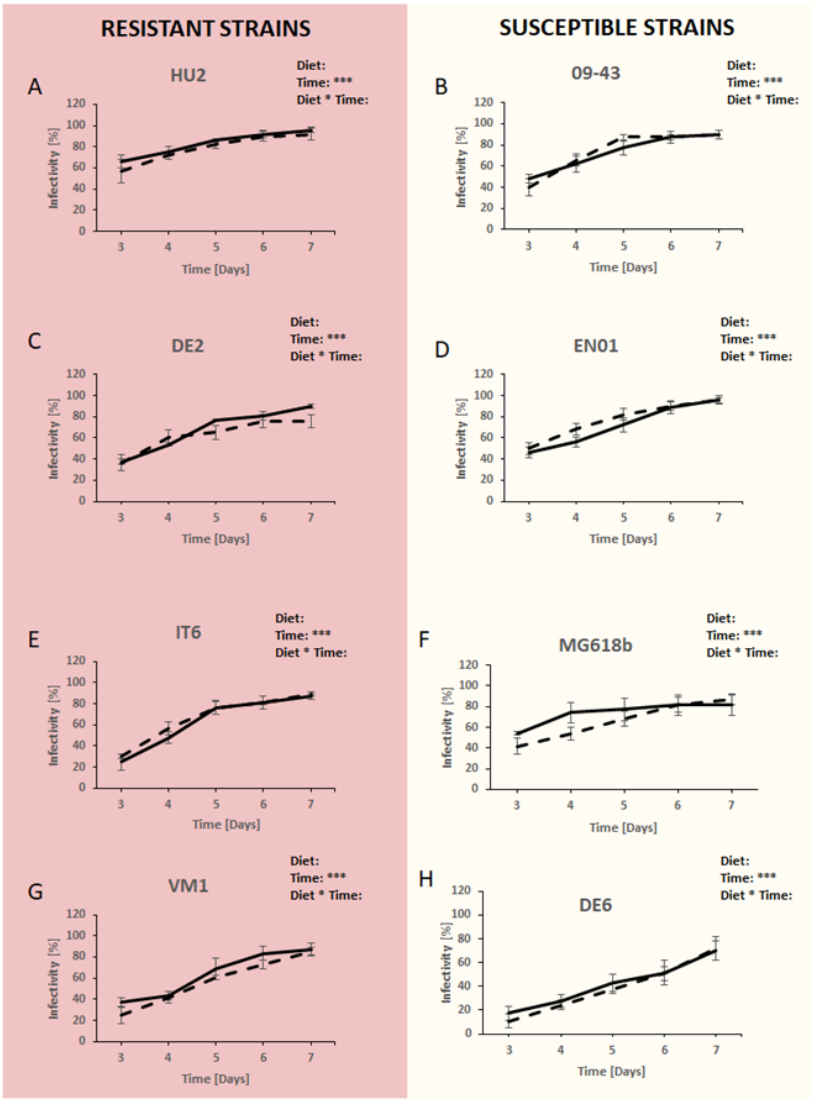
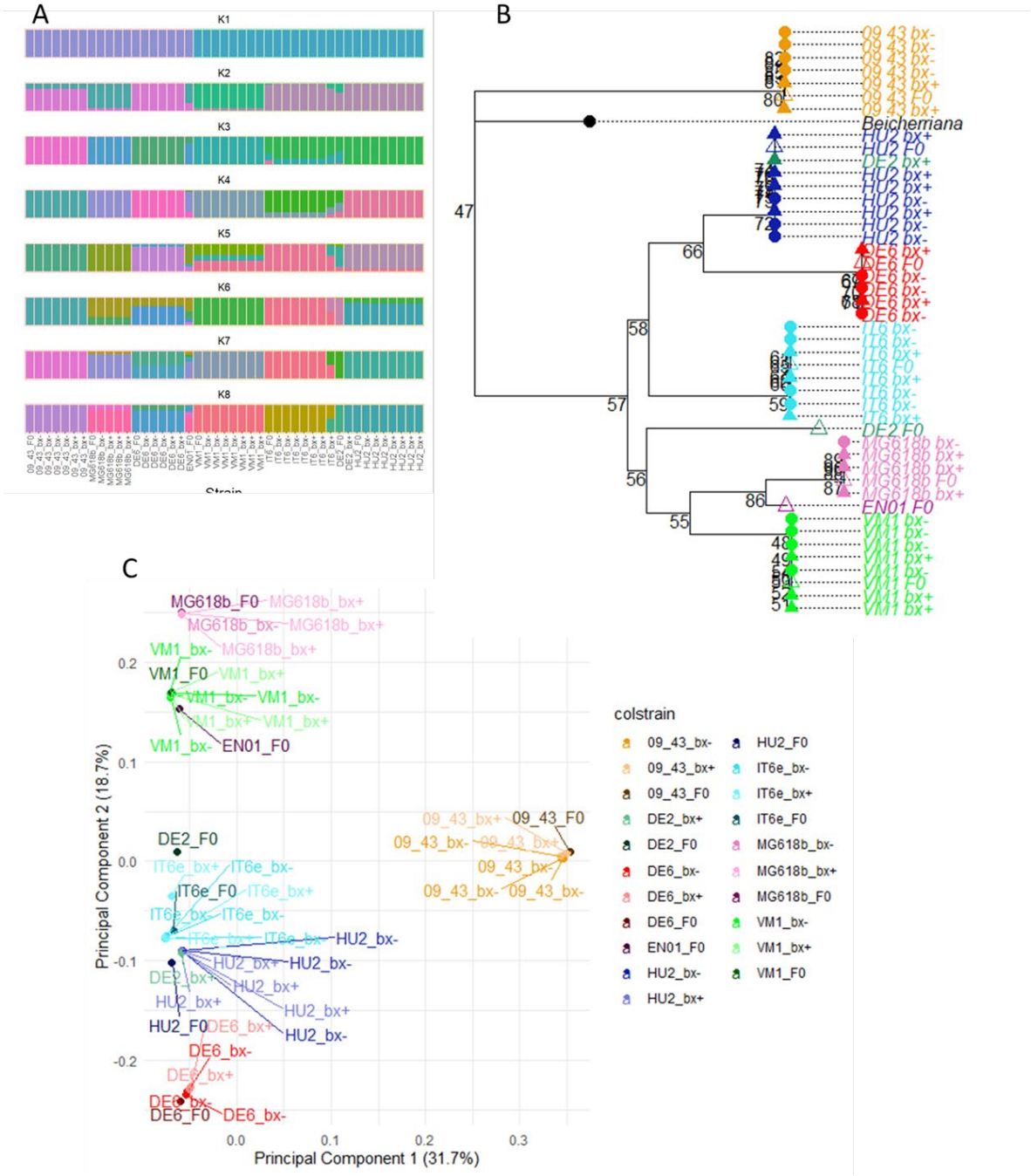


Figure S5: F_5 infectivity rates of individual entomopathogenic nematode (EPN) isolates (F_0) in Western Corn Rootworm larvae (WCR) fed on wild type plants containing benzoxazinoids (BX^+) and bx mutant plants (bx). EPN infectivity rate (Mean ± SEM) was recorded by adding 50 EPNs to 5 WCR larvae and visually inspecting the larvae for colour change daily between day 3 and day 7 after EPN addition (n=5). Red panel (left): BX-resistant EPN isolates. Yellow panel (right): BX susceptible EPN isolates. Solid line: EPN infectivity in BX^+-fed WCR. Dotted line: EPN infectivity in bx^--fed WCR. Diet: Diet of the WCR larvae used for infectivity. Two-Way ANOVA on repeated measured were conducted. Stars indicate significant differences between EPN infectivity on BX^+ and bx^--fed WCR larvae within the day of observation. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

582 Figure S6



583

584 **Figure S6: Population genetic comparison between all the samples created and used in this study**. A)
585 STRUCTURE analysis of all samples, with K=1-K=8. Samples are ordered by isolates, and withing isolates by
586 treatment, first F_0, then F_5 control (adapted to bx^-) and then F_5 treatment (adapted to BX^+) The colors used in this plot
587 have no specific implications and only represent the difference between STRUCTURE groups. B) Phylogenetic tree of
588 all samples, the colors represent the species, with cold colors representing resistant isolates and warm colors
589 representing susceptible isolates. C) A PCA analysis of all samples. The colors are the same ones as in the
590 Phylogeny, the different shades of one color represent the different treatments, with lighter colors showing F_5
591 samples adapted to BX^+, and dark colors showing F_0. The median color is samples adapted to bx^-.

Figure S7

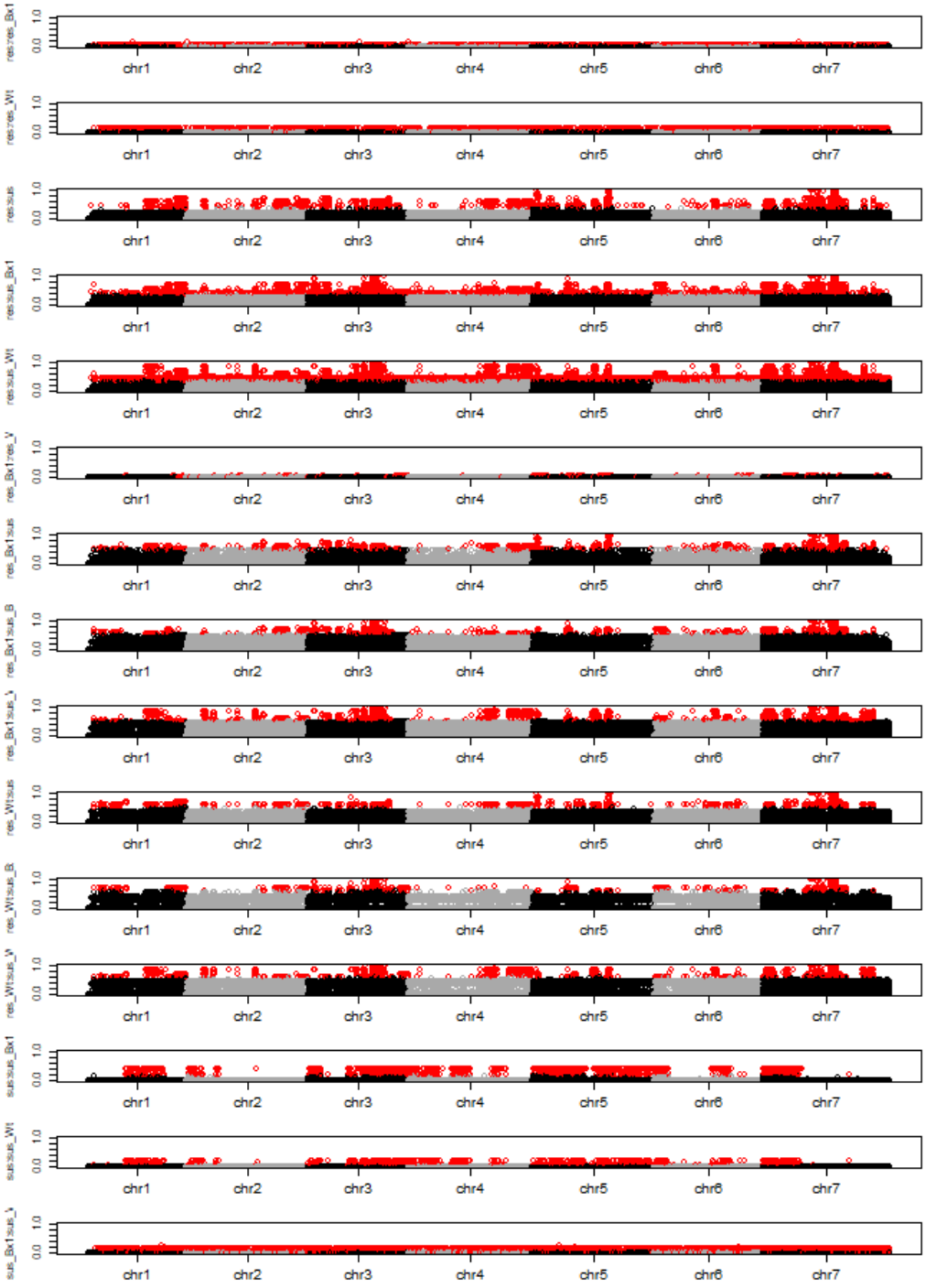
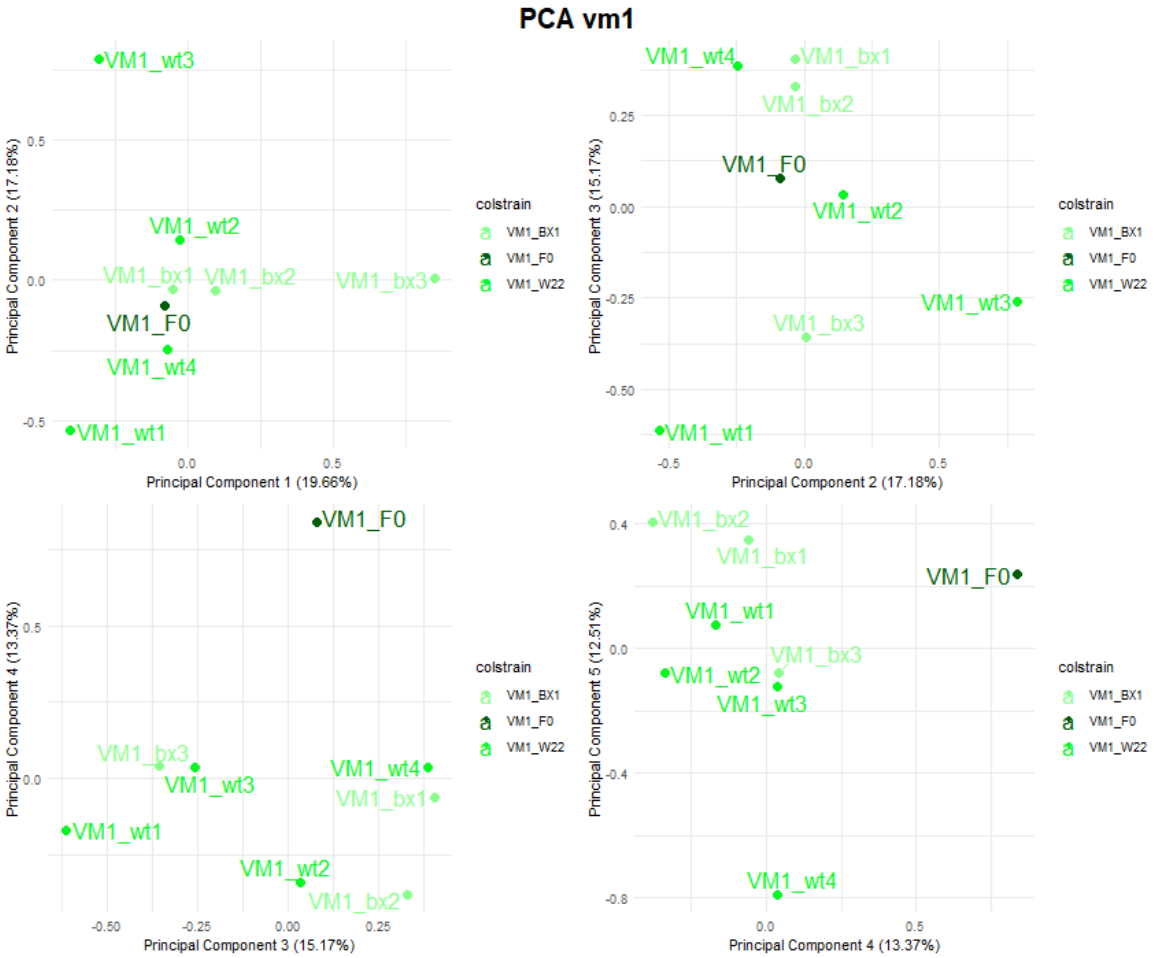


Figure S7: F_{ST} landscape of all comparisons between different treatments (susceptible isolates F_0, resistant isolates F_0, both in F_5 adapted to BX^+ and both in F_5 adapted to bx^-. F_{ST} landscapes of all comparisons present in this study, the red dots represent windows with an F_{ST} value that is higher than the 3rd quartile plus 1.5 times the interquartile range. From top to bottom: resistant F_0 to resistant F_5 bx^-; resistant F_0 to resistant F_5 BX^+; resistant $F_0$to susceptible F_0; resistant $F_0$to susceptible F_5 bx^-; resistant $F_0$to susceptible F_5 BX^+; resistant F_5 bx^- to resistant F_5 BX^+; resistant F_5 bx^- to susceptible F_0; resistant F_5 bx^- to susceptible F_5 bx^-; resistant F_5 bx^- to susceptible F_5 BX^+; resistant F_5 BX^+ to susceptible F_0; resistant F_5 BX^+ to susceptible F_5 bx^- ;resistant F_5 BX^+ to susceptible F_5 BX^+; susceptible F_0 to susceptible F_5 bx^-; susceptible F_0 to susceptible F_5 BX^+; susceptible F_5 bx^- to susceptible F_5 BX^+.

604 Figure S8

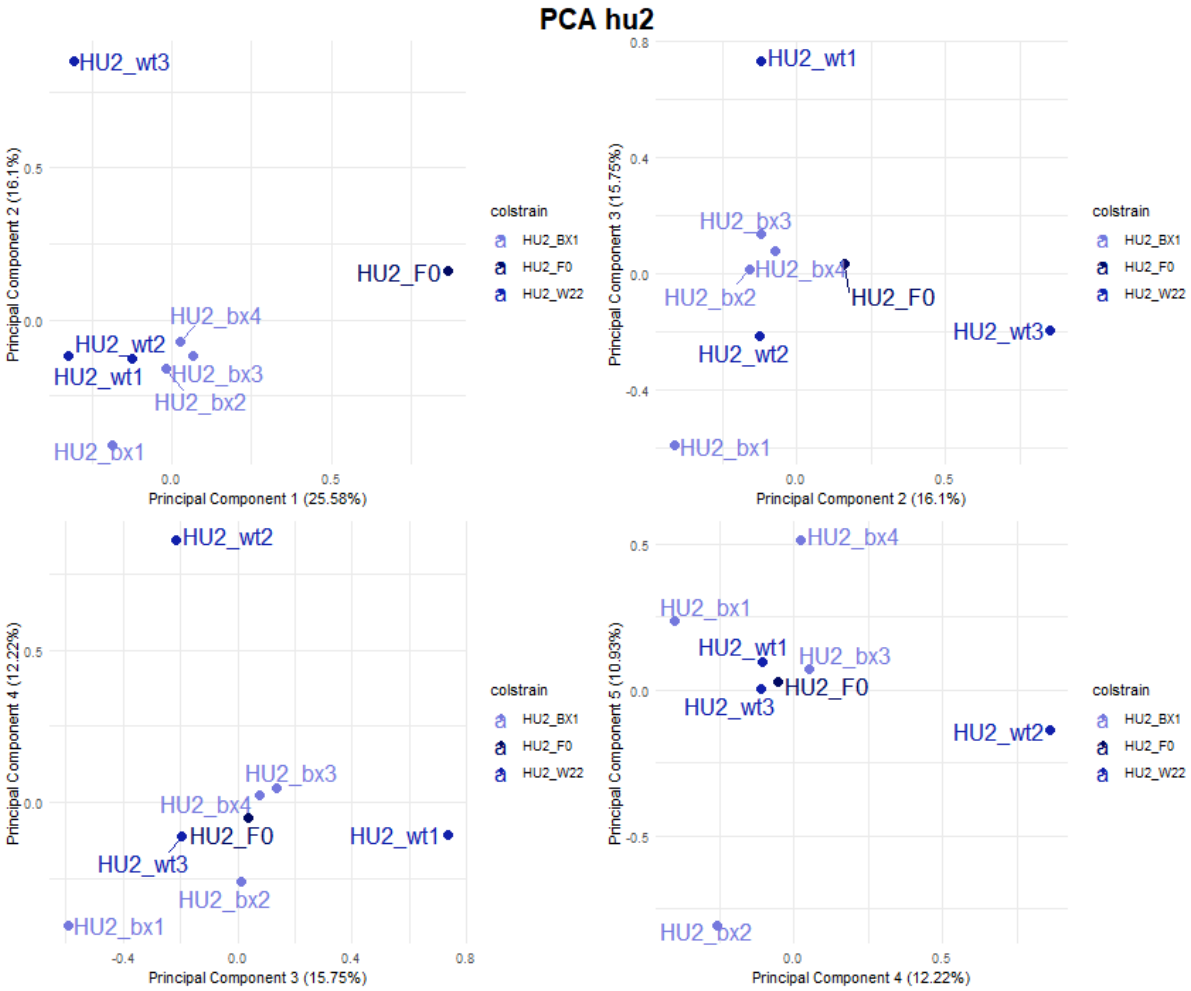


605

606 **Figure S8: PCA showing principal components (PC) 1 to 5 for the isolate VM1, a resistant isolate**. Each of the four
607 plots represents 2 PCs, with the lower PC always appearing on the x-axis. The lighter color represents F_5 bx^-, the
608 medium color F_5 BX^+ and the dark color F_0.

609

610 Figure S9



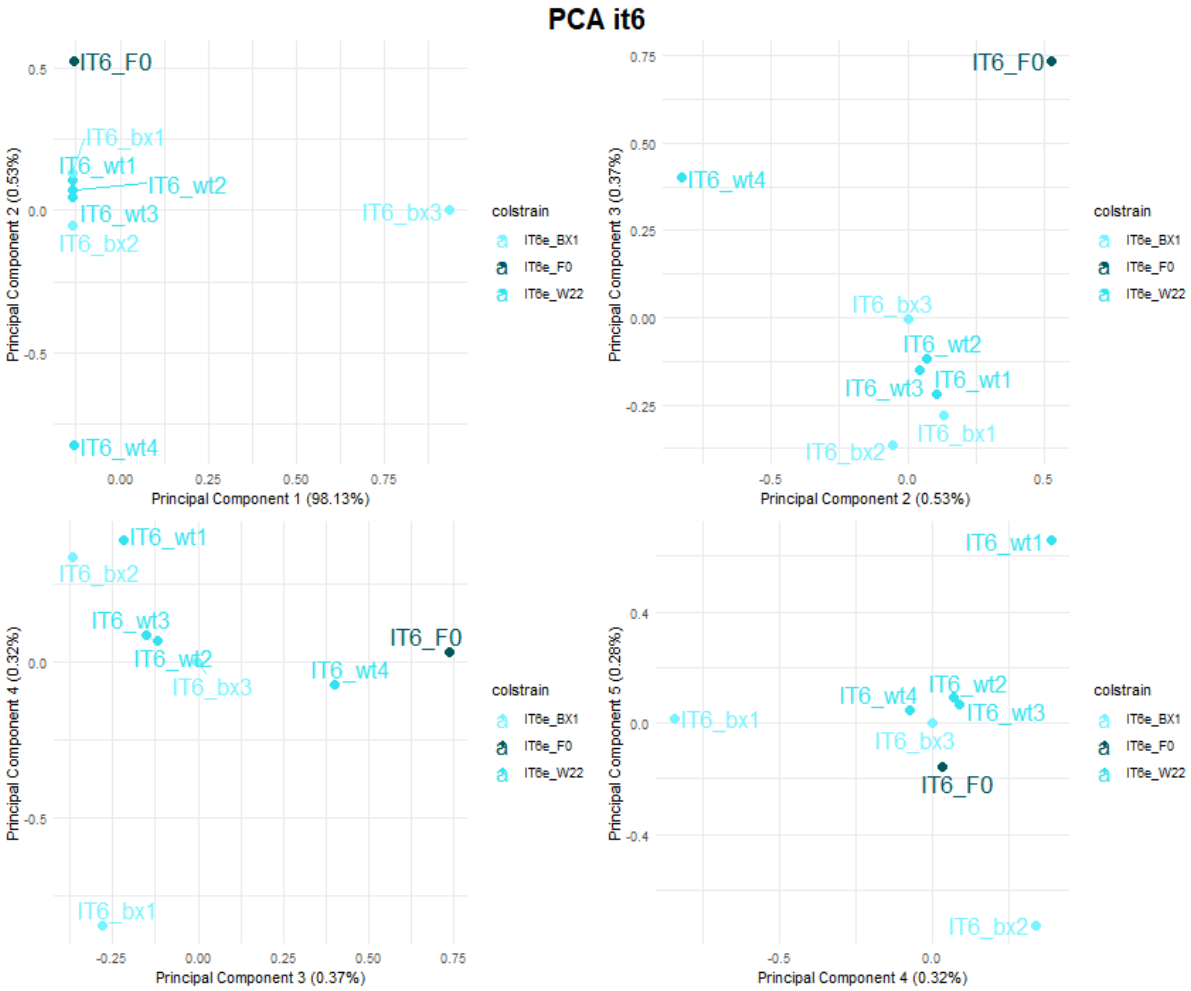
611

612 **Figure S9: PCA showing principal components (PC) 1 to 5 for the isolate HU2, a resistant isolate**. Each of the four
613 plots represents 2 PCs, with the lower PC always appearing on the x-axis. The lighter color represents F_5 bx^-, the
614 medium color F_5 BX^+ and the dark color F_0.

615

616

617 Figure S10



618

619 **Figure S10: PCA showing principal components (PC) 1 to 5 for the isolate IT6, a resistant isolate.** Each of the four
620 plots represents 2 PCs, with the lower PC always appearing on the x-axis. The lighter color represents F_5 bx^-, the
621 medium color F_5 BX^+ and the dark color F_0.

622

623

Figure S11

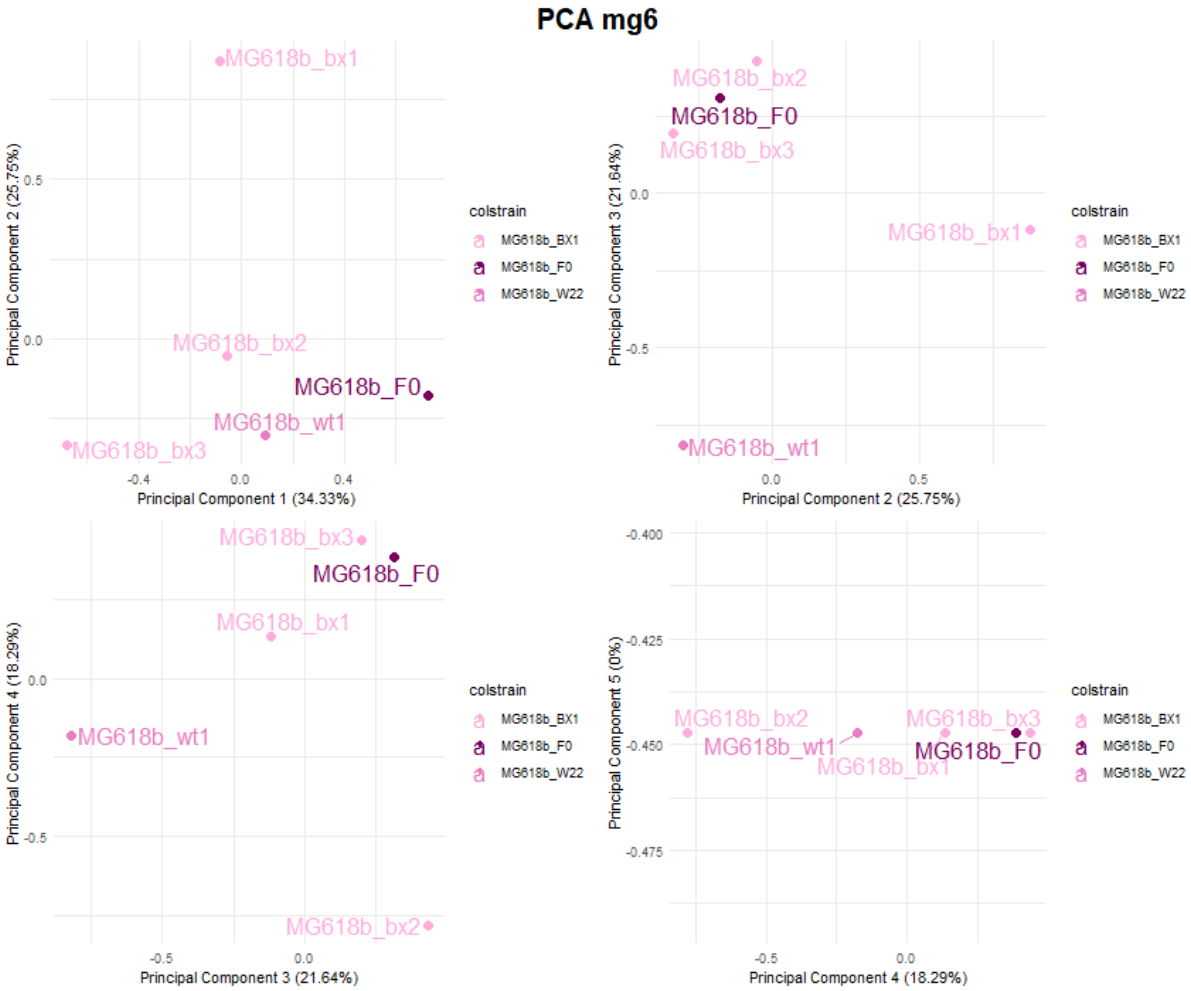
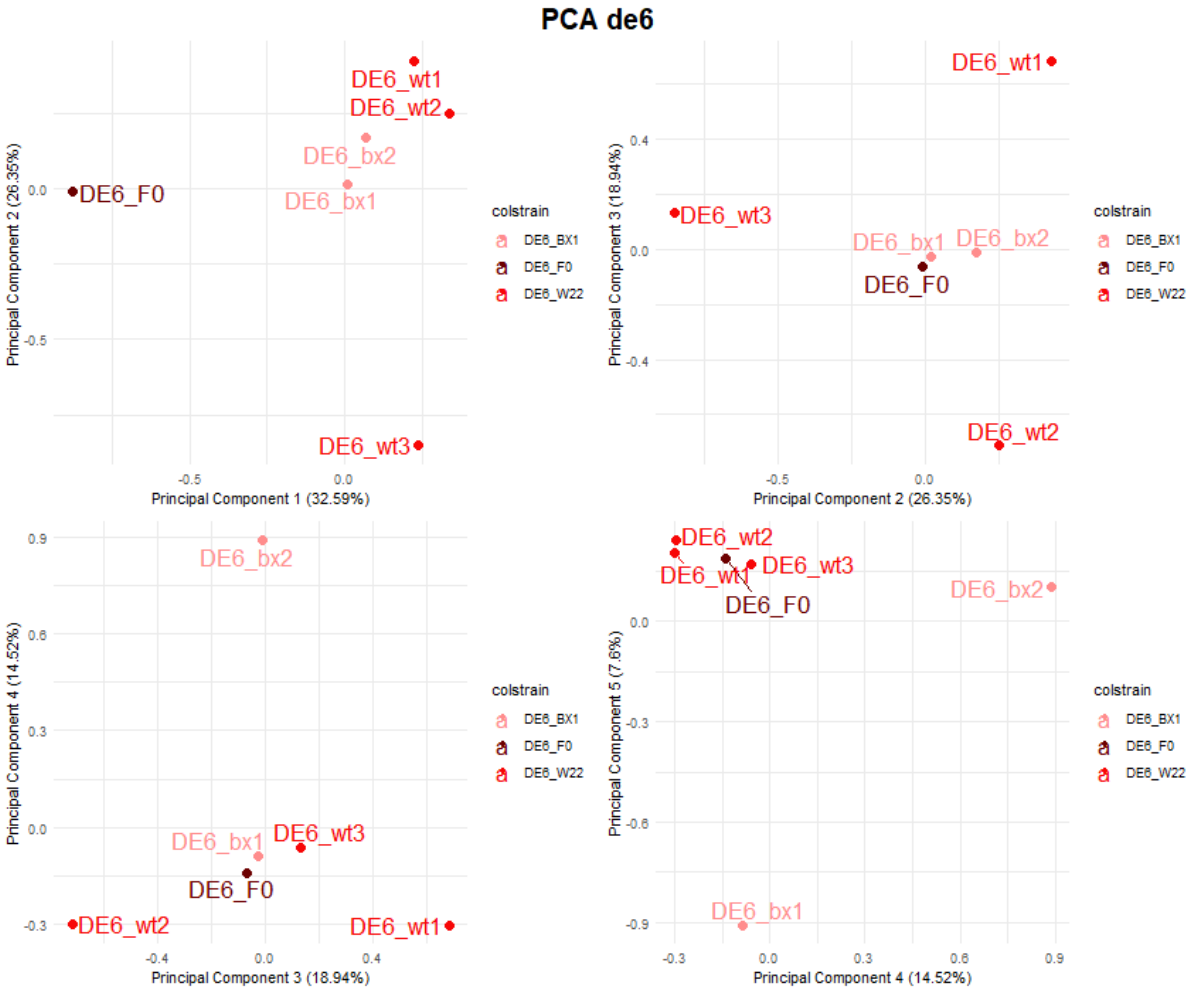


Figure S11: PCA showing principal components (PC) 1 to 5 for the isolate MG618b, a susceptible isolate. Each of the four plots represents 2 PCs, with the lower PC always appearing on the x-axis. The lighter color represents F_5 bx^-, the medium color F_5 BX^+ and the dark color F_0.

632 Figure S12



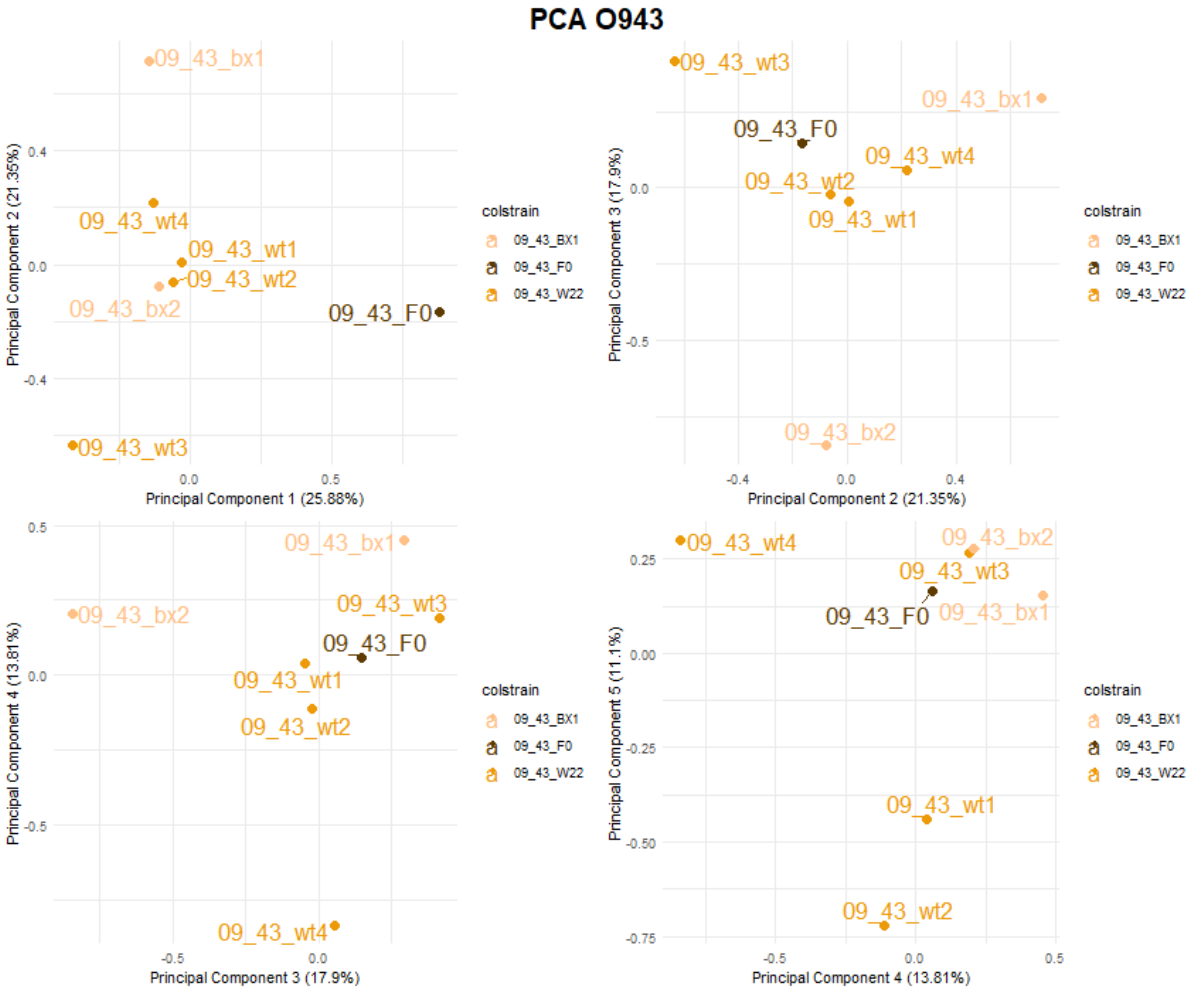
633

634 **Figure S12: PCA showing principal components (PC) 1 to 5 for the isolate DE6, a susceptible isolate.** Each of the
635 four plots represents 2 PCs, with the lower PC always appearing on the x-axis. The lighter color represents F_5 bx^-, the
636 medium color F_5 BX^+ and the dark color F_0.

637

638

639 Figure S13



640

641 **Figure S13: PCA showing principal components (PC) 1 to 5 for the isolate 0943, a susceptible isolate**. Each of the
642 four plots represents 2 PCs, with the lower PC always appearing on the x-axis. The lighter color represents F_5 bx^-, the
643 medium color F_5 BX^+ and the dark color F_0.

644

645

Supplementary Tables

Table S1

Table S1: List of entomopathogenic nematode (EPN) isolates, response to benzoxazinoids, and origin.

Isolate	Response to benzoxazinoids	Origin	susceptibility
MG618b	Susceptible	Switzerland	Susceptible
09-43	Susceptible	Turkey	Susceptible
DE6	Susceptible	Germany	Susceptible
EN01	Susceptible	Germany	Susceptible
IT6	Resistant	Italy	Resistant
DE2	Resistant	Germany	Resistant
HU2	Resistant	Hungary	Resistant
VM1	Resistant	Spain	Resistant

653 Table S2

654 **Table S2:** List of samples of entomopathogenic nematode (EPN) isolates, that were reared to
655 large enough quantities for DNA extraction and sequencing.

Isolate	Diet	Sus_res	Subgroup
09_43	F_0	susceptible	F_0
VM1	Wt	resistant	B
VM1	Wt	resistant	C
VM1	Wt	resistant	D
VM1	Bx1	resistant	A
VM1	Bx1	resistant	B
VM1	Bx1	resistant	C
MG618B	Wt	susceptible	D
MG618B	Bx1	susceptible	A
MG618B	Bx1	susceptible	B
MG618B	Bx1	susceptible	C
IT6E	Wt	resistant	A
IT6E	Wt	resistant	B
IT6E	Wt	resistant	C
IT6E	Wt	resistant	D
IT6E	Bx1	resistant	A
IT6E	Bx1	resistant	B
IT6E	Bx1	resistant	D
HU2	Wt	resistant	A
HU2	Wt	resistant	B
HU2	Wt	resistant	D
HU2	Bx1	resistant	A
HU2	Bx1	resistant	B
HU2	Bx1	resistant	C
HU2	Bx1	resistant	D
09_43	Wt	susceptible	A
09_43	Wt	susceptible	B
09_43	Wt	susceptible	C
09_43	Wt	susceptible	D
09_43	Bx1	susceptible	A
09_43	Bx1	susceptible	C
DE2	Bx1	resistant	C
DE6	Wt	susceptible	A
DE6	Wt	susceptible	B
DE6	Wt	susceptible	D
DE6	Bx1	susceptible	C
DE6	Bx1	susceptible	D
VM1	Wt	resistant	A
DE2	F_0	resistant	F_0
DE6	F_0	susceptible	F_0
EN01	F_0	susceptible	F_0
HU2	F_0	resistant	F_0
IT6E	F_0	resistant	F_0

MG618B	F_0	susceptible	F_0
VM1	F_0	res	F_0

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General discussion

In this thesis, I investigated the mechanisms and biological consequences of benzoxazinoid resistance in natural enemies of herbivores. We present a de-novo chromosome scale genome assembly of the entomopathogenic nematode (EPN) *Heterorhabditis bacteriophora* as well as a comparative genomic analysis looking at genetic features that are unique to EPNs. We disentangled the relative importance of the symbiotic bacterium *Photorhabdus* in the evolution of different *Heterorhabditis* species and identified candidate genes for the difference in susceptibility to plant toxins in *H. bacteriophora* isolates that share evolutionary history with benzoxazinoids compared to those that do not. We also conducted a large-scale guided evolution experiment on a sample set of both resistant and susceptible isolates to distinguish the genes most likely involved in the actual susceptibility to benzoxazinoids without accounting for genetic differences accumulated over prolonged evolutionary distance. We also confirmed the chromosomal structure of *H. bacteriophora* using immunofluorescence imaging. Below, I discuss the results of the chapters in my thesis, I particularly give a more general overview over EPN specific genes and what is important in plant toxin tolerance, explain what will still have to be done, and I discuss how these results impact the field.

What are features that are unique to EPNs?

EPNs are a monophyletic clade of nematodes that are identified by their life cycle involving a period of time existing in the haemolymph of an insect or mollusc host, while feeding on symbiotic bacteria that had been ejected into the host by the EPN itself (Ciche and Ensign 2003; Poinar 1966; Martens et al. 2004; Ciche et al. 2006). As only two genera of EPNs are undisputedly known (Heterorhabditis and Steinernema) and until recently not many high-quality genetic resources have been available, the genetic basis of this unique life strategy has not been well studied (Maushe et al. 2023). We use a gene-based approach to investigate the differences between EPNs and nematodes with other life strategies, including mammalian parasites, plant parasites and free-living nematodes (Chapter 1). Many of these nematodes share certain aspects of what defines the life strategy of the EPNs, for example mammalian parasites are also exposed to an immune system within their host, or the free-living nematodes such as *C. elegans*, which is also a bacterivore nematode. However other aspects of the EPN lifestyle are more restricted, such as the (obligate) inclusion of a symbiotic bacterium (Sajnaga and Kazimierczak 2020; Machado et al. 2018). While both genera can survive and kill host insects without the symbiotic bacteria, Heterorhabditis relies on the symbiont for successful infection and reproduction inside

the insect host (Ruby 2008). Steinernema can infect their hosts without the symbiotic bacterium (*Xenorhabdus*) but their mutualistic interaction aides the infection process greatly (Han and Ehlers 2000).

The gene families that were found to potentially be involved in the EPN lifestyle include ones that could reasonably be supposed to play a role in response to the insect immune system (Chapter 1). Particularly striatin domain-containing proteins, which could be involved in pathways relating to signalling proteins and could therefore be involved in the response to the host immune system (Tanti et al. 2023) as well as Heparan-alpha-glucosaminide N-acetyltransferase which is involved in the lysosomal degradation of heparin sulfate and as a transferase playing a role degradation could also be expected to interact with proteins other compounds produced by the host insect as an immune response (Schiff et al. 2020). Glyco_trans_2-like domain-containing proteins, however, while also being different in EPNs compared to other nematodes, could be more likely expected to be involved in adaptation to the food source due to its role in glycosyltransferase during the biosynthesis of sugars (Sobhanifar et al. 2016). Due to the special case of a mostly bacterivorous nematode living in an insect host, the processing of nutrients could be expected to differ somewhat from many of the other nematodes presented here. Even though there are other bacterivorous nematodes represented in the study, such as *C. elegans*, the difference in their environment could lead to different needs for processing and digestion of the food. Other genes have previously been identified as being putatively involved in the parasitism of EPNs, particularly in *Steinernema*, where proteases and protease inhibitors are postulated to be important in the parasitic life strategy, which are known to be involved in digestion, development and other processes as well as immune responses (Dillman et al. 2015). The other family of genes supported in that study are fatty acid- and retinol-binding proteins, which are inhibiting eicosanoid biosynthesis in insect hosts, which is another way to circumvent the immune system (Dillman et al. 2015). The gene families identified in this study differ from the ones described above, but they can be considered to work towards the same goal, i.e. the evasion of the host immune system in any way possible. Since this work includes both genera of EPNs, the identification would off course be different than in (Dillman et al. 2015), as the two genera of EPNs are phylogenetically relatively distantly related, since they are a paraphyletic group (Trejo-Meléndez et al. 2024).

How does *H. bacteriophora* contend with plant toxins in host haemolymph?

We present a list of candidate gene families that show differences between susceptible and resistant isolates of *H. bacteriophora* (Chapter 2) and ones that show differences between susceptible isolates that have been adapted to BX^+ host insects and bx^- host insects (Chapter 3). Among these candidate genes are several that are potentially relevant in the ability of an organism to cope with toxic compounds, particularly benzoxazinoids. BXs are known to have quite a widespread effect on the organism they are affecting, as such they can interrupt the digestive process, particularly affecting the breakdown of proteins, thereby reducing the energy efficiency of an organism (Wouters et al. 2016). The genes that may help with that effect are particularly ones involved in the regulation of protein synthesis, as increasing the production of digestive enzymes may reduce the negative effect achieved by the BXs and allow for a smaller loss of energy. Other candidate genes that may be involved in the response to this aspect of BX toxicity are the ones affecting growth and development, since the lowered efficacy of the digestive system likely leads to a necessary reallocation of energy reserves, and allocating a large part of the resources to rapid growth and therefore quick reproduction would be a way to on one hand ensure reproductive success and on the other hand allow more chances for offspring to escape the presence of BXs, for example in their infective juvenile form, where they are protected by a double cuticle and do not need to eat (Timper and Kaya 1989; Johnigk and Ehlers 1999), thereby lowering the exposure to BXs.

BXs are also known to damage DNA by intercalating into them, which can lead to issues with downstream mechanisms including replication and translation which can lead to the production of dysfunctional proteins which in turn can lead to cellular issues and ultimately cell death (Du Fall and Solomon 2011). This is why it might be important for process involved in translation processes to adapt, particularly to ensure continued production of proteins such as the enzymes mentioned above that are essential to keep a productive digestive system.

Another effect BXs are known to have is the increase of oxidative stress through the production of reactive oxygen species (ROS) which are unstable molecules which easily and detrimentally interact with various structures within a cell, such as lipids, proteins and DNA (Bayir 2005). Two of these and their relative importance as well as potential ways for organisms to react to them were already mentioned above, but the effect of ROS on cell functioning are widespread, and since oxidative stress can detrimentally interact with membrane integrity (Stanley and Kim 2018), the candidate gene related to the fatty acid metabolism pathway that was identified and that plays a major role in the response to ROS can be essential in membrane integrity and therefore in the maintenance of the cell structure and energy storage. Fatty acid metabolism has already

previously been identified to be important in EPNs in general (Dillman et al. 2015) where it was found to be important in the ability of Steinernema to infect insects. It may be that the importance in that context, which could be in accordance with the response to insect immune systems would be exacerbated in a case where not only the autonomous immune system of the insect is involved but also a defense compound, which could be considered to be part of the plant immune system and has been hijacked by the insect itself, thereby increasing both the defense of the insect as well as the need of the EPN to defend itself against it.

Outlook and further perspectives

This study provides, apart from a lot of resources for further analysis, a list of candidate genes for EPN life strategy and for BX tolerance in *H. bacteriophora*. However, many questions still remain to be answered and experiments to be conducted. Further remaining questions are:

- Are there differences between bacterivorous and non-bacterivorous nematodes, as well as between parasitic and non-parasitic nematodes, based on their environment?
- How do the genetic changes actually interact with the plant toxins?
- What are the molecular changes achieved by the genetic differences between susceptible and resistant isolates?
- Are the identified genetic changes in response to BX adaptation needed and sufficient to affect the changes observed?

To get a better understanding of the actual genes underlying the differences between susceptible and resistant isolates, further experiments are needed (Chapter 3). Particularly a similar experiment with more controlled conditions, so external, non-controllable effect would be less pronounced. If these effects could not be completely ruled out, a method to allow comparison of different generation at the same time would have to be established, be that by allowing an intermediate generation in *Galleria mellonella* larvae to get the different generations to be of the same age at the time of the experiment or by establishing a method to stagnate the infective juveniles at a certain age without the strong decline in both living nematodes and infectivity that is observed when keeping infective juveniles for a prolonged period of time using the current methods. One of the issues that would be encountered with the first of these options is that in a *G. mellonella* larvae, which is many times bigger than a *Diabrotica virgifera* larva, many more generation could happen, which in turn may reduce the effect of the guided evolution experiment strongly. Of course, given that all analyses presented here were conducted on nematodes that had at least on generation in *G. mellonella* larvae before DNA extraction and sequencing, doing the final infectivity assay on nematodes that had been subjected to the exact same treatment

may give even stronger results regarding the actual correlation of the genetic changes and the changes in infectivity. However, getting the F_0 generation to a good, highly infective state with only one intermediary generation in *G. mellonella* may not be very easy, as different isolates of *H. bacteriophora* lose infectivity at different rates, and therefore some of the isolates may have a much different infection rate after the have been re-amplified after multiple months in stasis. The alternative method of storage is at the moment only a hypothetical option, as such a method would first have to established and well tested to ensure that the storage would not have any major effects on the infection rate in the stored nematodes and to ensure no other aspects of the nematode behaviour and biology would change.

A lager samples size, both for the number of isolates used for the experiment as well as for the number of replicates within samples could also be useful in the future. This is naturally difficult to accomplish, as the scope of the experiment with such an increase would be difficult to carry out in a reasonable amount of time while ensuring similarity of treatment between different replicates. The larger number of isolates, particularly of susceptible isolates would allow for more statistical significance, both in the comparison of F_0 samples and in the comparison of the comparisons of the different treatments in F_5 of the isolates. Having more replicates, both for samples adapted to BX^+ and bx^- would allow for a much stronger F_{ST} analysis and would make any signatures less likely to be due to errors either in sequencing or other steps of the process. The way the experiment presented in this thesis was conducted lead to a relatively small number of F_5 samples that were adapted to either BX^+ or bx^- hosts. In some cases, this leads to a very low-confidence F_{ST} analysis, particularly in the case of MG618b, where only a single sample adapted to bx^- was sequenced, which makes the F_{ST} analysis, a population-based differentiation analysis, less strong than would be preferred. Given the still high cost of sequencing many high-quality whole genome sequences, it may be possible to lay a stronger focus on the sequencing of the susceptible isolates, as a reduced number of resistant isolates especially in the F_5 generation would still allow for a relevant and significant control.

The types of analyses conducted here were limited, partly due to time constraints and partly due to sample size issues. There are further analyses that would support the ones conduced here and hopefully uncover more interesting aspects of the adaptation of EPNs to benzoxazinoids. Particularly analysis identifying selective sweeps would be very interesting, as selective sweeps can be more informative regarding recent positive selection, as would be expected from such a relatively short evolution experiment. Selective sweeps are especially indicative of selection, being considered direct evidence thereof due to a rapid increase in frequency of a beneficial mutation, which leads to a decrease of genetic variation in the regions surrounding this mutation

(Smith and Haigh 1974), whereas a high F_{ST} value must not necessarily be due to positive selection and can be an artifact, for example due to genetic drift. Selective sweeps also allow a more in-depth insight into the temporal dynamics of adaptation, as they have properties that leave discernible patterns in linkage disequilibrium and haplotype structures, which is not the case for F_{ST} analysis, which in turn merely show the presence or absence of mutations.

To establish the importance of the candidate genes identified in this study and in any additional analysis that may be conducted in the future, targeted testing will have to be conducted. One way to do this is to use a process called RNAi, which exploits a cell's innate defences to silence specific genes. The way this is done is by artificially creating a piece of double stranded RNA according to the gene that is supposed to be silenced and exposing the cell to it. This dsRNA is then recognized by the cell and cleaved into small interfering RNA by the enzyme Dicer, which are then incorporated into the RNA induced silencing Complex where they are guided to complementary mRNA sequences (the ones produced by the cell itself) which leads to the cleavage and degradation of those mRNAs, thereby silencing the effect of the genes without any genome editing (Kim and Rossi 2008). One of the main issues with this technique and therefore one of the main reasons this has not been done yet in this project, is the difficulty of conducting RNAi experiments in *Heterorhabditis bacteriophora*. While it is a widely used and generally well-established practice, the main stumbling block for its application is the way the dsRNA gets to the cells. While this has been tried and tested in other nematode species, such as C. elegans, where the nematode is exposed to the dsRNA through one of three methods, feeding (by altering their food source, *Escherichia coli*, to produce the dsRNA (Timmons and Fire 1998)), soaking (which incubates the nematode in a solution containing the dsRNA (Tabara et al. 1998)) or microinjection (which uses extremely thin glass needles to precisely puncture the nematode and administer the dsRNA solution (Fire et al. 1998)), none of those methods have been used to great success in our lab in *H. bacteriophora*. The feeding method is of course much more difficult given that the preferred method of reproduction and therefore feeding for *H. bacteriophora* is in an insect host. It is possible to grow the nematodes outside of insects, be that on agar plates containing the symbiotic bacterium *Photorhabdus* or in bioreactors, but the dsRNA would need to be somehow introduced into the bacteria or produced by the bacteria themselves, which is much more difficult to accomplish in a bacterium that is much less well studied than *E. coli*. Both of the other methods mentioned above, soaking (Moshayov et al. 2013; Ciche and Sternberg 2007) and microinjection (Ratnappan et al. 2016) have been used successfully in *H. bacteriophora*, however, as neither of these protocols are established in the lab, we have not had the chance to test the importance of the identified candidate genes yet. However, to confidently

identify the genes that are at the basis of this phenotype, such a next step is necessary and will have to be conducted.

Impact of the presented work

This work provides the field with a new, high-quality chromosome scale genome assembly of the EPN species *H. bacteriophora*, which will make more studies into the genetics of EPNs in general and *H. bacteriophora* in particular feasible. It will allow for a clearer understanding of how this species can be used in agriculture and which regions of the genome are responsible for certain responses to different biotic and abiotic conditions (Chapter 1). While this study focuses solely on the interaction of *H. bacteriophora* with the western corn rootworm *Diabrotica virgifera* and the maize toxin benzoxazinoid sequestered therein, it still gives a basis to be able to potentially extrapolate from the data collected here and get a better overview of how the nematode handles challenges that it may not be used to, as the environment and challenges faced by the nematodes are constantly changing and evolving, thus forcing the nematode itself to also change in an evolutionary arms race. As this type of interaction is very common and extends far beyond the constraints of both the study system and application, it can provide valuable insights into the process in general.

Apart from insights into the interaction between EPNs and their host, the data presented here can also be helpful to further the knowledge regarding specifics of the parasitic lifestyle (Chapter 1). The genes highlighted during this work are a starting point to establish more knowledge about the genes and mutations therein that would be important for such a widespread and still unique lifestyle. Even though this work only focuses on nematode parasites, even more specifically nematodes that are entomopathogenic, it provides knowledge to aspects of the parasitic lifestyle, and the genome that is presented can be used further to compare with other types of parasites in all genera that have parasitic representatives, in order to find similarities that are special to parasites. It would of course not be expected that the same genes would be involved in parasitism in all organisms but knowing what mutations all the different types of parasites have could be used to more easily find parasitism genes in new, non-model organisms, and could therefore be used to protect against harmful parasites while providing knowledge about how to support 'beneficial' parasites.

The commercial use of this nematode is already happening, but from our data it can be seen that the most widely commercially distributed isolate of *H. bacteriophora*, EN01, is actually one of the few isolates that do not have the ability to cope with the presence of BXs in their host haemolymph (Chapters 2&3). Therefore, it may be helpful to identify the specific genes

responsible for this adaptation to facilitate easier crossing of a resistant isolate with already established isolates, which are high performing in other important aspects, such as desiccation or heat tolerance, to increase the resistance of such a generally available isolate, and therefore increasing the area of applicability for this product. The knowledge that can be gathered from this new genomic resource can of course also be applied to other aspects of nematode biology, ecology and physiology, helping to identify mutations and adaptations to other types of stressors, be they biotic, such as fungi or viruses; or abiotic, such as UV stress or desiccation, which are important factors in the success and applicability of EPNs (Maushe et al. 2023). While EPNs with high tolerance to these various stressors have been successfully produced through conventional methods of crossbreeding of various isolates with beneficial mutations, knowing the specific loci responsible for these differences would be helpful to create more consistent and widely successful isolates more easily.

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Annex I

Chapter 4 – Chromosome structure of *Heterorhabditis bacteriophora*

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Abstract

While the genome of *Heterorhabditis bacteriophora*, an entomopathogenic nematode, has been sequenced, significant gaps remain in our understanding of its chromosomal structure. This study aims to characterize the chromosomal organization of *H. bacteriophora* and comparing it with *Caenorhabditis elegans*. Our findings revealed a unique chromosomal architecture in *H. bacteriophora*, where TEs are predominantly concentrated in the central regions of chromosomes, while genes are less biased toward the center and, in some cases, more prevalent at the chromosomal ends. In contrast, *C. elegans* showed the expected pattern of gene-rich central regions and TE accumulation at the chromosomal ends. Further comparative analysis across *Heterorhabditis* species identified patterns of reduced F_{ST} and d_{XY} in the central regions, suggesting evolutionary conservation. Yet, immunofluorescence staining confirmed that *H. bacteriophora* exhibits holocentric chromosomes, with centromeric activity distributed along the entire chromosome length. These results suggest that, despite central TE accumulation, these regions do not function as conventional centromeres. This study advances our understanding of *H. bacteriophora* genome architecture and raises important questions about the role of TEs in holocentric chromosomes, warranting further investigation into their evolutionary and functional implications.

Introduction

The genome of *Heterorhabditis bacteriophora*, an entomopathogenic nematode, provides a valuable model for understanding the genetics of parasitism and symbiosis with bacterial partners. With a genome size of approximately 85 megabases (Mb) and around 17,787 predicted protein-coding genes (Ogi et al. in review), *H. bacteriophora* displays a compact genome, similar in some respects to that of *Caenorhabditis elegans*, the most well-characterized nematode model (C. elegans Sequencing Consortium 1998). *C. elegans* has long served as a reference for

studying genome structure, organization, and chromosomal dynamics due to its well-resolved six chromosomes (C. elegans Sequencing Consortium 1998). Despite the similarities in overall genome size and coding potential between *H. bacteriophora* and *C. elegans*, much remains to be uncovered about the detailed chromosomal structure of *H. bacteriophora*, particularly with regard to the spatial organization of its genome (Ogi et al. in review). Our previous work provided a chromosome-scale assembly of the *H. bacteriophora* genome (Ogi et al. in review), key chromosomal features, such as the location of centromeres and specific patterns of recombination, are still underexplored.

The chromosomal structure of *H. bacteriophora*, consisting of five autosomes and one sex chromosome, remains less defined in terms of the precise positioning of essential genomic elements like the centromeres. Centromeres play a crucial role in chromosome segregation during cell division, and understanding their positioning and function is central to deciphering genome dynamics and stability (Black and Cleveland 2011). In *C. elegans*, centromeres are holocentric, meaning that spindle attachment occurs along the entire length of the chromosome (Albertson and Thomson 1993). Whether *H. bacteriophora* follows a similar pattern or exhibits distinct centromeric regions is currently unknown.

Comparative studies of chromosomal dynamics in different *Heterorhabditis* isolates, being susceptible or resistant to plant benzoxazinoids, revealed reduced genetic divergence (lowered F_{ST} and d_{XY}) in the central regions of chromosomes, suggesting that these areas may play a critical role in maintaining genetic integrity or adapting to environmental pressures (Ogi et al. in review). Additionally, an accumulation of Mutator Retrotransposons in the centre of the *H. bacteriophora* chromosomes as previously reported (Ogi et al. in review), potentially indicating that these transposons are either tolerated in or drawn to centromeric regions, possibly due to the reduced recombination and the structural organization of centromeres. Such patterns raise important questions about the structure and function of these central chromosomal regions in *H. bacteriophora* as these regions may be associated with centromeric activity or lower recombination rates. Understanding the chromosomal dynamics within *H. bacteriophora* in the context of these patterns will provide important insights into the evolutionary history of this species.

This study aims to address these gaps by characterizing the centromeric regions of *H. bacteriophora* through a combination of genomic and cytogenetic approaches. By comparing gene and TE densities, and chromosomal divergence between *H. bacteriophora* and *C. elegans,* we aimed at better understanding the relationship between chromosomal structure and

function, and at identifying potential centromeric regions based on patterns of genetic diversity. Finally, we provide a direct visualization of centromeric markers by immunofluorescence to elucidate the positioning of these critical regions in *H. bacteriophora*. Together, these approaches will enhance our understanding of the genome structure of this important nematode species but also contribute to broader insights into chromosomal evolution and dynamics in parasitic nematodes.

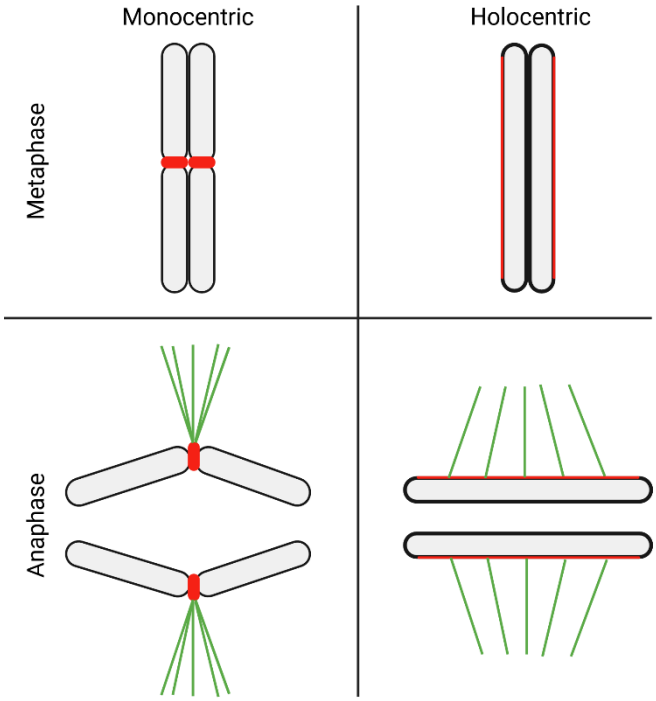

Figure 5: Difference between monocentric (left) and holocentric (right) Chromosomes during metaphase (top) and anaphase (bottom). Centromeric regions are highlighted in red, microtubules in green

Materials and Methods

Genomic aspects

For genomic analyses, the *H. bacteriophora* reference genome, annotation and WGS data created in (Ogi et al. in review). The distribution of TEs (annotated using EDTA) and genes (annotated using maker for *H. bacteriophora*, downloaded from ncbi for *C. elegans*) along the chromosomes for both *H. bacteriophora* and *C. elegans* was calculated in windows of 1 Mbp, and visualization of the data was done in R (Team RDC 2010). The analysis of the F_{ST}, d_{XY} and π was also done in R, using the PopGenome package (Pfeifer et al. 2014). The comparisons conducted here were done between different species of *Heterorhabditis*, namely *H. bacteriophora*, *H. beicherriana*, *H. zacatecana*, *H. georgiana* and *H. ruandica*. All F_{ST} and d_{XY}

comparisons were done between *H. bacteriophora* and each of the other species, π was calculated for all species.

Immunofluorescence

The chromosome structure of *H. bacteriophora* embryos was investigated using an immunofluorescence methanol fixation protocol originally adapted for *C. elegans* from a combination of a protocol by Pierre Gönczy and (Lee et al. 2016). In detail, 10 nematodes washed in salt water were applies to a microscopy slide coated in poly-L-lysine (1-1.5 mg/ml) and covered with a cover slip. The slide was then placed on a metal block that had been pre-cooled on dry ice and left for at least five minutes. The coverslip was quickly removed by flipping it with a razorblade, upon which the slide was put into -20°C methanol for 30 minutes. Rehydration was done in PBS for 5 minutes, followed by blocking in 200 µl AbDil (4% BSA and 0.1% Triton X-100 in PBS) at room temperature in a humid chamber for 20 minutes. The slide was then incubated with primary antibodies (MAB2609 (1:500) and HCP-3 (1:200)) at 4C overnight in a humid chamber, followed by washing in PBS twice for 5 minutes, a secondary incubation in the secondary antibody (1:1000 goat anti-mouse/Alexa 488 and 1:1000 goat anti rabbit/Cy3 in PBS) for 45 min and again washing twice in PBS. The slide was then carefully dried, 6 µl of mounting medium was added to the coverslip which was sealed to the slide using nail polish. Pictures of the resulting sample were taken on a fluorescence microscope to evaluate the chromosomal structure of the nematode embryos.

Results

Gene and TE density

The comparison of Gene and TE density of *H. bacteriophora* and *C. elegans* showed a very different distribution (Figure 2). While in *C. elegans* the majority of TEs was accumulated along the ends of chromosomes, whereas the genes tended to be slightly more common in the centre of the chromosomes. On contrast, in *H. bacteriophora*, TEs appeared to be more concentrated in the centres of the chromosomes, whereas the distribution of the genes was less clearly biased toward the middle of the chromosome, in some cases even being more at the ends of the chromosomes.

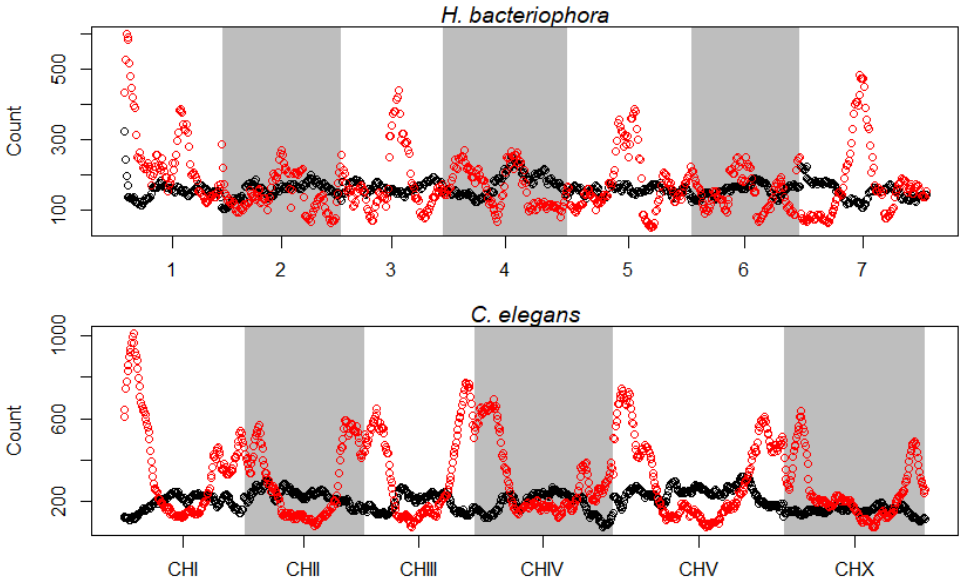

Figure 2: Distribution of TEs and genes along the chromosomes of *H. bacteriophora* and *C. elegans*. A) The distribution along *H. bacteriophora* chromosomes. TEs are shown in red, Genes in black, the change in background denotes a switch in chromosomes. Sliding windows of size 1,000,000bp with a 100,000bp jump. B) the same analysis for *C. elegans*. *These data were obtained from (Ogi et al. in review).*

F_{ST}, d_{XY} and π

The comparisons of the different Heterorhabditis species showed a distinct pattern of lowered F_{ST} and d_{XY} in the centre of chromosomes (Figure 3). This, along with an increase in π in these same regions, is a pattern that is not very well described. This is the result that led to the confirmation of the pattern using immunofluorescence staining.

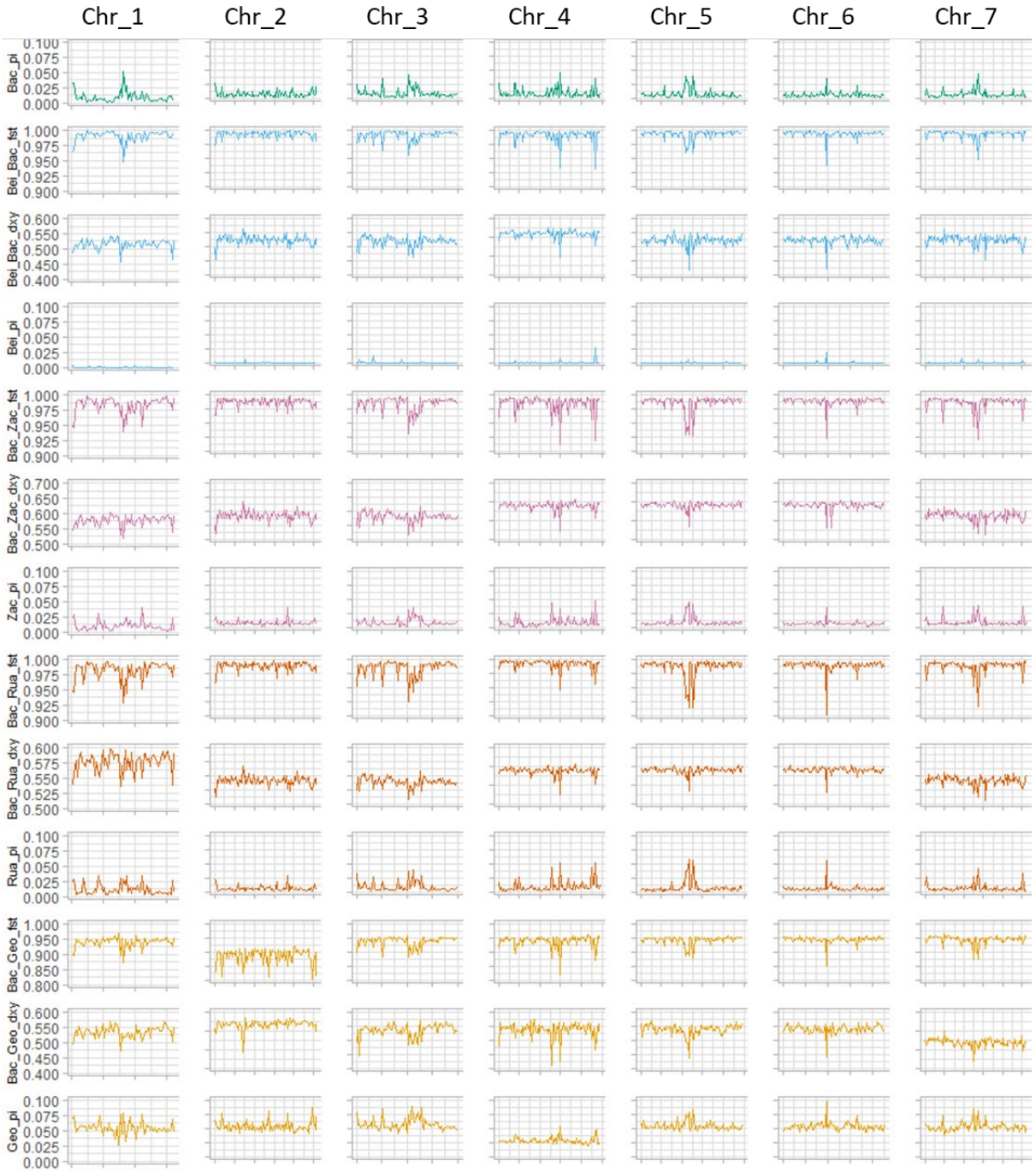

Figure 3: F_{ST}, d_{XY} and π of different comparisons of other Heterorhabditis strains to *H. bacteriophora*. From top to bottom, π of *H. bacteriophora*; and then always the F_{ST}, then d_{XY} and finally π of *H. beicherriana*, *H. zacatecana*, *H. ruandica* and *H. georgiana* in order, with the F_{ST} and d_{XY} comparisons always being to *H. bacteriophora* and the π being just the species mentioned. *These data were obtained from (Ogi et al. in review).*

Immunofluorescence staining

The resulting pictures were assessed optically, and when comparing the DAPI staining which highlights the AT rich regions of the chromosome and therefore shows where the chromosomes are located, to the CempA staining which highlights centromeric regions, is could be seen that

the CempA stained along the entirety of the chromosome, suggesting that there is evidence for monocentric chromosomes (Figure 4).

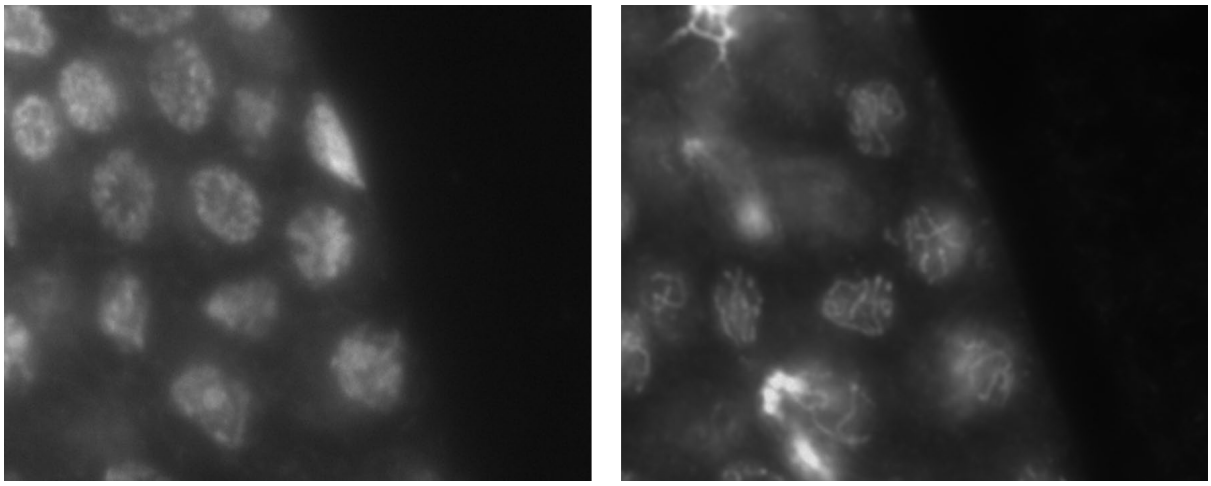

Figure 4: Microscope picture of germline cells of H. bacteriophora. A) Picture of the DAPI staining of the germline cells, highlighting the AT regions of the DNA, therefore showing where the chromosomes are located. B) Picture of the same region with a cempA staining which highlights the centromeric activity.

Discussion

The primary genomic results suggested that the chromosome structure would be different from the structure found in *C. elegans*, especially the strikingly different distributions of TEs and genes along the chromosomes. This result in combination with the surprising dip in F_{ST} and d_{XY} in the centres of the chromosomes led to conducting the visual assessment of the actual structure present in the nuclei. The results showed that the supposition that many nematodes are monocentric is in fact true.

The comparison of gene and TE density between *H. bacteriophora* and *C. elegans* revealed striking differences in chromosomal organization. In *C. elegans*, the majority of TEs are concentrated towards the chromosomal ends, while genes are more densely packed in the central regions, a pattern that is well-established in nematode genomics (Laricchia et al. 2017). This distribution likely reflects a balance between functional gene-rich regions and the accumulation of repetitive elements in areas with lower selective pressure, such as the telomeric and subtelomeric regions. In contrast, *H. bacteriophora* displays a distinct organization, with TEs predominantly concentrated in the center of the chromosomes, while the distribution of genes shows no clear central bias, and in some cases, they are even more frequent toward the chromosomal ends (Ogi et al. in review).

This unique pattern in *H. bacteriophora* raises interesting questions about the forces driving genome architecture in this species. The central accumulation of TEs may indicate that these regions are less active in terms of recombination and selective pressure, allowing for a greater tolerance of TE insertions. This contrasts with *C. elegans*, where central chromosomal regions are more gene-dense and under stronger selective constraint. The tendency of genes in *H. bacteriophora* to be distributed more evenly, or even towards the chromosomal ends, suggests that different evolutionary pressures or structural constraints may govern chromosomal organization in this species. It is possible that *H. bacteriophora* chromosomes have evolved distinct strategies to maintain gene expression integrity while tolerating the central accumulation of TEs.

The comparative analysis across different *Heterorhabditis* species and isolates further supports the idea that the central regions of chromosomes in *H. bacteriophora* are subject to unique evolutionary dynamics. The lowered F_{ST} and d_{XY} values in the centers of chromosomes, combined with an increase in nucleotide diversity, suggest that these regions are highly conserved and possibly functionally important. This pattern, which has not been widely reported in other nematodes, indicates that the central chromosomal regions may play a crucial role in maintaining genetic integrity, potentially by harboring essential genes or regulatory elements that are preserved across species.

To explore whether these conserved central regions could correspond to centromeres, we employed immunofluorescence staining using CempA, a known centromere marker. The results showed CempA staining along the entirety of the chromosomes, suggesting the presence of monocentric chromosomes. Contrary to our hypothesis that the centromeres would be concentrated in the central regions of the chromosomes, the staining revealed a holocentric chromosome structure. This holocentric organization is consistent with what has been observed in other nematodes, such as *C. elegans*, where spindle attachment during cell division occurs along the entire chromosome. The discovery of holocentricity in *H. bacteriophora* aligns with previous findings in nematodes but adds an interesting contrast given the accumulation of transposable elements in the center of the chromosomes, which may indicate other structural or functional roles for these regions beyond typical centromeric activity. Understanding the implications of this holocentric organization in combination with the central TE accumulation will require further investigation, particularly in terms of how it influences chromosomal stability and segregation during meiosis and mitosis.

In summary, the divergent chromosomal organization in *H. bacteriophora*, characterized by central TE accumulation and a less defined gene distribution, reveals unique evolutionary and structural dynamics in this species. The holocentric nature of its chromosomes contrasts with the central TE accumulation and the absence of a clear pattern of gene enrichment in the middle of the chromosomes, raising interesting questions about the functional role of these central regions. The concentration of TEs in these regions could play a role in maintaining chromosomal stability or influencing chromatin structure, possibly contributing to the genome's evolutionary adaptability. Further investigation into the interplay between TE accumulation, chromosomal structure, and holocentric activity will be crucial for understanding the evolutionary pressures shaping *H. bacteriophora*'s genome architecture, as well as its broader implications for nematode chromosome biology.

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Declaration of consent

on the basis of Article 18 of the PromR Phil.-nat. 19

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Study program: Ecology and Evolution

Bachelor ☐ Master ☐ Dissertation ☑

Title of the thesis: Population Genomics of Benzoxazinoid Resistance in Entomopathogenic Nematodes

Supervisor: Prof. Dr. Matthias Erb
Prof. Dr. Christelle Robert
Prof. Dr. Christian Parisod

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