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Basic laboratory and field manual for conducting research with the entomopathogenic nematodes, *Steinernema* and *Heterorhabditis*, and their bacterial symbionts

Selçuk HAZIR\(^1\)\(^*\), Harry K. KAYA\(^2\), Mustapha TOURAY\(^1\), Harun ÇİMEN\(^1\), David SHAPIRO-ILAN\(^3\)

\(^1\)Department of Biology, Faculty of Arts and Sciences, Aydın Adnan Menderes University, Türkiye
\(^2\)Department of Entomology and Nematology, University of California, Davis, USA
\(^3\)USDA-ARS, Southeastern Fruit and Tree Nut Research Station, Byron, USA

Abstract: Broad spectrum chemical pesticides are harmful to humans and other nontarget organisms. Biological control, which entails the use of natural enemies, is a viable alternative. Isolation, identification, and application of biocontrol agents such as the entomopathogenic nematodes (EPNs), *Steinernema* and *Heterorhabditis* and their symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* have increased substantially over the last four decades, and the trend continues with advancement of molecular techniques. Yet, there is a need for a simple hands-on guide for their proper identification, classification, and handling, especially for researchers and users who are not totally familiar with these biocontrol agents. Thus, this manual is intended to provide a practical guide for students and researchers interested in or wanting to focus on these organisms. The manual describes the general biology and bionomics of these nematode/bacterium complexes and explains various basic standard protocols and methods frequently used in research and field laboratories ranging from isolation to application methods. Methods for rearing the insects, *Galleria mellonella* and *Tenebrio molitor*, which are routinely used in bioassays and recovery of EPNs, are also included.

Key words: Biological control, insect-parasitic nematodes, *Steinernema*, *Heterorhabditis*, basic methods, *Photorhabdus*, *Xenorhabdus*, standard protocols

1. Introduction

Pest management strategies include methods to control destructive organisms that directly or indirectly cause harm to humans, crops, and livestock (Birch et al., 2011). These strategies are projected to assist in feeding more than 9.8 billion people by 2050 (UN, 2019). Pest management tactics can be grouped into chemical, biological, genetically modified plants, or resistant varieties, and cultural or mechanical methods. Among these, chemical control generally delivers fast and effective results, but many of these pesticides are toxic to humans, livestock, plants, and other nontarget organisms. Moreover, broad spectrum chemical pesticides have negative effects on the environment, result in pesticide resistance, and can induce resurgence of secondary pests. Therefore, chemical insecticides must be used judiciously to minimize negative effects. Accordingly, safer alternatives are being sought to replace these toxic chemical pesticides (WHO, 1990; Debach and Rosen, 1991; Nauen, 2007). One of the safest and environmentally friendliest alternative approaches is to use biological control agents.

Biological control uses natural enemies or their byproducts to control populations of noxious organisms. Humans basically rely on the natural feeding hierarchy and intervene by using one or more living beneficial organisms or their byproducts to control or suppress the target pests. Some of the most destructive pests in food production are invertebrates that include arthropods (e.g., insects and mites), mollusks, and plant-parasitic nematodes. Arthropod pests have their own natural enemies such as parasitoids (e.g., braconid, ichneumonid and chalcid wasps and tachinid flies), predators (lacewings, ladybugs, and mites) and pathogens (viruses, bacteria, fungi, and nematodes) (Debach and Rosen, 1991; Lacey and Georgis, 2012; Lacey et al., 2015). Our emphasis is on entomopathogenic nematodes which can be excellent biological control agents of pest insects and other invertebrates that spend all or some portion of their life cycle in soil (Shapiro-Ilan et al., 2017, 2018; Koppenhöfer et al., 2020).

Nematodes are commonly called roundworms with most life stages being microscopic, and they are...
the most numerous animal group on earth (Stock and Goodrich-Blair, 2012, Van Den Hoogen et al., 2019). They belong to the phylum Nematoda which has 2 classes — Adenophorea and Secernentea — and over 180 families. At least 30 of these families are associated with insects and invertebrates, but only 7 families have the potential to be biocontrol agents. These families are Allantonematidae, Mermithidae, Sphaerulariidae, Rhabditidae, Neotylenchidae, Heterorhabditidae and Steinernematidae (Stock and Goodrich-Blair, 2012). Nematode general characteristics include a long, elongated, and tubular body with a complete nervous and digestive system within the body cavity (pseudocoelom). The nematode body is simple and unsegmented, and tapered at both ends, and its epidermis is made up of cuticle which it molts up to four times as it grows. Nematodes lack circulatory, respiratory, and endocrine systems (Pechenik, 2005; Poinar, 2005). Usually, nematodes have separate sexes; males have a copulatory spicule and are smaller than their female counterparts. Nematodes, unlike other worm-like animals, have no circular muscles; they move wavyly by the action of longitudinal muscles found on the dorsal and ventral parts of their body and their hydrostatic skeleton. The muscles are controlled by two nerves that also run along the same parts of the nematode's body (Kiontke and Fitch, 2013). Nematodes are believed to have originated in the marine environment (Blaxter and Koutsouvolos, 2014) and have adapted to a terrestrial habitat where they live as free-living organisms or parasites of plants and animals (Poinar, 2005).

There are three nematode families that infect and kill their invertebrate hosts in association with bacteria: Rhabditidae, Steinernematidae, and Heterorhabditidae. Within the Rhabditidae, there are two genera, Oscheius and Caenorhabditis, which are known to use pathogenic bacteria to parasite and kill their insect hosts (Zhang et al., 2009; Torres-Barragan et al., 2011; Dillman et al., 2012). Oscheius and Caenorhabditis nematodes (like C. briggsae) reportedly are associated with different bacteria such as Serratia nematophila, S. marcescens, Enterococcus sp., Pseudomonas sp., Bacillus cereus, etc., (Abebe et al., 2011; Fu and Liu, 2019). Another rhabditid is Phasmarhabditis hermaphroditica, which infects pestiferous mollusks, is produced commercially using the bacterium Moraxella osloensis, and is available in some countries as a commercial product for mollusks biocontrol (Wilson et al., 2000; De Ley et al., 2016).

In this manual, our focus is on the families Steinernematidae and Heterorhabditidae because they are the only nematodes that have been mass-produced and applied commercially as biocontrol agents to control insects. We provide introductory information on these entomopathogenic (i.e. insect-killing) nematodes (EPNs) and their symbiotic bacteria as well as various methods and techniques needed to conduct laboratory and field research with the EPN/bacterium complex. These include (1) the classification and bionomics of EPNs and their symbiotic bacteria, (2) explanation of basic standard protocols/techniques on how to collect and extract EPNs from soil, (3) identification of EPNs and their symbiotic bacteria using both morphometric and molecular techniques, (4) determination of virulence bioassays for EPNs and methods of rearing these organisms, (5) stain improvement and stabilization techniques, (6) application methods for EPNs in the field, and (7) useful methods for rearing the EPN host insects (Galleria mellonella and Tenebrio molitor). We realize that there are already several useful resources that describe techniques for conducting research with EPNs (e.g., Woodring and Kaya, 1988; Kaya and Stock, 1997; Stock and Goodrich-Blair, 2012; Glazer and Lewis, 2000), but since these references were published new information and/or techniques have been added to the literature. In addition, the previous sources are in books or special publications and not readily available to students and researchers in many countries. Therefore, in this open access article, we present an updated overview of techniques for studying EPNs and their symbiotic bacteria.

1.1. Steinernematidae and Heterorhabditidae – biology and ecology

The classification of the families Steinernematidae and Heterorhabditidae is presented in Table 1. Steinernematidae has two genera, Neosteinernema and Steinernema, whereas Heterorhabditidae is monogeneric represented by the genus Heterorhabditis. As of this writing, Steinernema has 102 species (Table 2), and Neosteinernema has only one species (N. longicurvicauda) which kills termites (Nguyen and Smart, 1994). Heterorhabditis has 22 species (Table 3) (Gulcu et al., 2017; Shapiro-Ilan et al., 2018; Bhat et al., 2020). Some of the described species have wide distributions occurring in temperate and tropical regions or at least 3 continents (such as S. feltiae, H. bacteriophora, S. carpocapsae, S. glaseri) (Addis et al., 2011; Akyazi et al., 2012; Chaubey and Garg, 2019), whereas others have more limited distributions. For example, thus far S. riobrave has only been discovered in the Rio Grande valley of the US and Mexico (Stuart et al., 2004). However, more surveys need to be conducted to elucidate their distribution (Gulcu et al., 2017). EPNs have been isolated from soil on all continents, except Antarctica, and many islands by using insects as bait (Dillman et al., 2012). Their presence across the globe, however, is patchy which is affected by the availability of hosts, soil type and vegetation (Stock, 2015).

Steinernema and Heterorhabditis are soil-dwelling nematodes and are mutualistically associated with bacteria in the genus Xenorhabdus and Photorhabdus, respectively.
Table 1. List of *Steinernema* nematode species and associated *Xenorhabdus* bacterial symbiont species associated with the nematode species. Many bacterial symbionts of *Steinernema* species have not been characterized or described. Also note that the same *Xenorhabdus* species has been isolated from different *Steinernema* species.

<table>
<thead>
<tr>
<th>Nematode species</th>
<th>Bacterial symbiont</th>
<th>Country first isolated from</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. abbasi</td>
<td>X. indica</td>
<td>Sultanate of Oman</td>
<td>Elawad et al., 1997</td>
</tr>
<tr>
<td>S. aciari</td>
<td>X. ishibashii</td>
<td>China</td>
<td>Qiu et al., 2005a</td>
</tr>
<tr>
<td>S. affine</td>
<td>X. bovienii</td>
<td>Denmark</td>
<td>Wouts et al., 1982</td>
</tr>
<tr>
<td>S. akhursti</td>
<td>Undescribed</td>
<td>China</td>
<td>Qiu et al., 2005b</td>
</tr>
<tr>
<td>S. anatoliense</td>
<td>Undescribed</td>
<td>Turkey</td>
<td>Hazir et al., 2003c</td>
</tr>
<tr>
<td>S. apuliae</td>
<td>Undescribed</td>
<td>Italy</td>
<td>Triggiani et al., 2004</td>
</tr>
<tr>
<td>S. arasbaranense</td>
<td>Undescribed</td>
<td>Iran</td>
<td>Nikdel et al., 2011</td>
</tr>
<tr>
<td>S. arenarium</td>
<td>X. kozodoii</td>
<td>Central Russia</td>
<td>(Artyukhovsky et al., 1997) Wouts et al. 1982</td>
</tr>
<tr>
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<td>Undescribed</td>
<td>Japan</td>
<td>Phan et al., 2006b</td>
</tr>
<tr>
<td>S. asiaticum</td>
<td>Undescribed</td>
<td>Pakistan</td>
<td>Anis et al., 2002</td>
</tr>
<tr>
<td>S. australae</td>
<td>X. magdalenensis</td>
<td>Chile</td>
<td>Edgington et al., 2009b</td>
</tr>
<tr>
<td>S. backanense</td>
<td>Undescribed</td>
<td>Vietnam</td>
<td>Phan et al., 2006a</td>
</tr>
<tr>
<td>S. batuswanae</td>
<td>Undescribed</td>
<td>South Africa</td>
<td>Didiza et al., 2021</td>
</tr>
<tr>
<td>S. beddingi</td>
<td>Undescribed</td>
<td>South Africa</td>
<td>Katumanyane et al., 2020</td>
</tr>
<tr>
<td>S. bertusi</td>
<td>Undescribed</td>
<td>South Africa</td>
<td>Cimen et al., 2016a</td>
</tr>
<tr>
<td>S. bicornutum</td>
<td>X. budapestensis</td>
<td>Strazilovo, Serbia</td>
<td>Tallosi et al., 1995</td>
</tr>
<tr>
<td>S. biddulphi</td>
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<td>Cimen et al., 2016b</td>
</tr>
<tr>
<td>S. boemarei</td>
<td>Undescribed</td>
<td>France</td>
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<td>S. borjomiense</td>
<td>Undescribed</td>
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<td>Gorgadze et al., 2018</td>
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<td>Undescribed</td>
<td>Brazil</td>
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<tr>
<td>S. carpocapsae</td>
<td>X. nematophila</td>
<td>Czechoslovakia</td>
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<tr>
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</tr>
<tr>
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<td>X. budapestensis</td>
<td>China</td>
<td>Jian et al., 1997</td>
</tr>
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<td>S. changbaiense</td>
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</tr>
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<td>China</td>
<td>Nguyen et al., 2008a</td>
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<td>S. citrae</td>
<td>Undescribed</td>
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<td>Stokwe et al., 2011</td>
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<td>Lopez-Nunez et al., 2008</td>
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<td>Undescribed</td>
<td>Costa Rica</td>
<td>Uribe-Lorío et al., 2007</td>
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<tr>
<td>S. cubanum</td>
<td>X. poinarii</td>
<td>Western Cuba</td>
<td>Mracek et al., 1994</td>
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<td>S. cumgarense</td>
<td>Undescribed</td>
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<td>Phan et al., 2006a</td>
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<td>S. diaprepesi</td>
<td>X. doucetiae</td>
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<td>Nguyen and Duncan, 2002</td>
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<td>X. eapokensis</td>
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<td>Phan et al., 2006a; Kampfer et al., 2017</td>
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<td>Ethiopia</td>
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<td>S. fabii</td>
<td>Undescribed</td>
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<td>Abate et al., 2016</td>
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<td>S. feltiae</td>
<td>X. bovienii</td>
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<td>S. glaseri</td>
<td>X. poinarii</td>
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<td>(Steiner, 1929) Wouts et al., 1982</td>
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<td>Venezuela</td>
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<td>83</td>
<td>S. sangi</td>
<td>X. thuongxuanensis, X. vietnamensis</td>
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(Gaugler and Kaya, 1990; Adams and Nguyen, 2002; Shapiro-Ilan et al., 2020). With *Neosteinernema*, a bacterium was isolated from this nematode but was not identified (Nguyen and Smart, 1994), and the role of the bacterium has not been determined. In the case of mutualistic relationship between *Steinernema* and its *Xenorhabdus* symbiont, and *Heterorhabditis* and its *Photorhabdus* symbiont, the nonfeeding infective juveniles (IJJs) of the nematodes have specific roles. The role of the IJJs is to house and protect the bacterium against external environmental conditions within the nematode's intestine, to serve as a vector for the bacterial cells to get into an insect host, and to preemptively suppress the host's antibacterial immune response before bacterial release. Once released by the IJJs into the insect's hemocoel, the role of the bacterium is to multiply and produce secondary metabolites, enzymes, toxins, and other compounds that further suppress the host immune system, quickly kill the insect host as well as to create a suitable monoxenic environment for nematode development and bioconvert the host tissues into a food source for the developing nematodes (Akhurst and Boemare, 1990; Forst and Clarke, 2002; Hazir et al., 2003a; Stock and Goodrich-Blair, 2008). Based on this potent combination, the nematode/bacterium complex infects the insect host and can kill it within 48 h.

Many EPN/bacterium complexes can be mass-produced in vivo or in vitro and have been sold commercially for biological control or IPM programs. They are of economic importance and much research effort has been expended to optimize their production and application (Abate et al., 2017). Moreover, the mutualistic bacteria produce antibiotics and other secondary metabolites that have the potential to be used in medical, veterinary, and agricultural fields (Webster et al., 2002; Böszörményi et al., 2009; Pidot et al., 2014; Kim et al., 2017; Kajla et al., 2019).

### 1.2. Symbiotic bacteria

The symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, belong to the family Morganellaceae (Adeolu et al., 2016) and can be considered to be entomopathogenic because they can kill their insect host, but they require the IJJs to get into the insect’s hemocoel. They are gram-negative, enteric symbiont bacteria linked to *Steinernema* for *Xenorhabdus* and to *Heterorhabditis* for *Photorhabdus*, *Xenorhabdus* cells are sequestered in a special anterior pouch of the intestine of steinernematid IJJs, whereas *Photorhabdus* cells are found in the lumen of the intestine of heterorhabditid IJJs. This pairing of the nematode and bacterium is quite specific (Boemare and Akhurst, 2006; Ferreira and Malan, 2014; Adeolu et al., 2016). Aside from *P. asymbiotica*, which has been isolated from human soft tissue infections and soil from where these infections originated in the United States and Australia (Gerrard et al., 2006), these bacteria have only been found in association with EPNs and have yet to be isolated from soil (Dillman et al., 2012).

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**Table 1.** (Continued).

<table>
<thead>
<tr>
<th>No</th>
<th>Species</th>
<th>Symbiont</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
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<td>Undescribed</td>
<td>Vietnam</td>
<td>Phan et al., 2006a</td>
</tr>
<tr>
<td>85</td>
<td>S. scapterisci</td>
<td>X. innexi</td>
<td>Uruguay</td>
<td>Nguyen and Smart Jr, 1990</td>
</tr>
<tr>
<td>86</td>
<td>S. scarabaei</td>
<td>X. koppenhoeferi</td>
<td>USA</td>
<td>Stock and Koppenhöfer, 2003</td>
</tr>
<tr>
<td>87</td>
<td>S. schleemannii</td>
<td>Undescribed</td>
<td>Germany</td>
<td>Spiridonov et al., 2010</td>
</tr>
<tr>
<td>88</td>
<td>S. siamkayai</td>
<td>X. stockae</td>
<td>Thailand</td>
<td>Stock et al., 1998</td>
</tr>
<tr>
<td>89</td>
<td>S. sichuanense</td>
<td>X. bovienii</td>
<td>China</td>
<td>Mracek et al., 2006</td>
</tr>
<tr>
<td>90</td>
<td>S. silvaticum</td>
<td>X. bovienii</td>
<td>Germany</td>
<td>Sturhan et al., 2005</td>
</tr>
<tr>
<td>91</td>
<td>S. surkhetense</td>
<td>Undescribed</td>
<td>Nepal</td>
<td>Khatri-Chhetri et al., 2011b</td>
</tr>
<tr>
<td>92</td>
<td>S. taiwanensis</td>
<td>Undescribed</td>
<td>Taiwan</td>
<td>Tseng et al., 2018</td>
</tr>
<tr>
<td>93</td>
<td>S. texanum</td>
<td>Undescribed</td>
<td>Texas, USA</td>
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<td>95</td>
<td>S. thanhi</td>
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<td>Phan et al., 2001b</td>
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<tr>
<td>96</td>
<td>S. tielingense</td>
<td>X. bovienii</td>
<td>China</td>
<td>Ma et al., 2012c; Mamiya et al., 2021</td>
</tr>
<tr>
<td>97</td>
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<td>South Africa</td>
<td>Cimen et al., 2014</td>
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<tr>
<td>98</td>
<td>S. unicornum</td>
<td>Undescribed</td>
<td>Chile</td>
<td>Edington et al., 2009a</td>
</tr>
<tr>
<td>99</td>
<td>S. xinbinense</td>
<td>Undescribed</td>
<td>China</td>
<td>Ma et al., 2012b</td>
</tr>
<tr>
<td>100</td>
<td>S. xueshanense</td>
<td>Undescribed</td>
<td>China</td>
<td>Mracek et al., 2009</td>
</tr>
<tr>
<td>101</td>
<td>S. weiseri</td>
<td>X. bovienii</td>
<td>Europe</td>
<td>Mracek et al., 2003</td>
</tr>
<tr>
<td>102</td>
<td>S. yirgalemense</td>
<td>Undescribed</td>
<td>Ethiopia</td>
<td>Nguyen et al., 2004b</td>
</tr>
</tbody>
</table>
Photorhabdus and Xenorhabdus and bacteria in the genera Arsenophonus, Moellerella, Morganella, Proteus, and Providencia have a monophyletic origin (Adeolu et al., 2016).

Currently, Xenorhabdus consists of 27 bacterial species with *X. thuongxuanensis, X. eapokensis* and *X. lircayensis* being the most recently described species (Kampfer et al., 2017; Sajnaga and Kazimierczak, 2020; Castaneda-
Table 3. Comparative morphometrics (in μm) of infective juveniles of *Steinernema* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>L</th>
<th>W</th>
<th>EP</th>
<th>NR</th>
<th>ES</th>
<th>TL</th>
<th>D%</th>
<th>E%</th>
<th>Reference</th>
</tr>
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<tr>
<td><em>S. carpocapsae</em></td>
<td>558</td>
<td>25</td>
<td>38</td>
<td>85</td>
<td>120</td>
<td>53</td>
<td>26</td>
<td>60</td>
<td>Poinar, 1990</td>
</tr>
<tr>
<td><em>S. scapterisci</em></td>
<td>572</td>
<td>24</td>
<td>39</td>
<td>97</td>
<td>127</td>
<td>54</td>
<td>31</td>
<td>73</td>
<td>Nguyen and Smart, 1990</td>
</tr>
<tr>
<td><em>S. siamkayai</em></td>
<td>446</td>
<td>21</td>
<td>35</td>
<td>72</td>
<td>94</td>
<td>36</td>
<td>37</td>
<td>96</td>
<td>Stock et al., 1998</td>
</tr>
<tr>
<td><em>S. affine</em></td>
<td>693</td>
<td>30</td>
<td>62</td>
<td>95</td>
<td>126</td>
<td>66</td>
<td>49</td>
<td>94</td>
<td>Poinar, 1988</td>
</tr>
<tr>
<td><em>S. beddigi</em></td>
<td>743</td>
<td>70</td>
<td>96</td>
<td>125</td>
<td>77</td>
<td>57</td>
<td>92</td>
<td>Qiu et al., 2005c</td>
<td></td>
</tr>
<tr>
<td>(700–790)</td>
<td>(NA)</td>
<td>(64–75)</td>
<td>(80–113)</td>
<td>(113–130)</td>
<td>(72–83)</td>
<td>(52–64)</td>
<td>(84–103)</td>
<td></td>
<td></td>
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<tr>
<td><em>S. intermedium</em></td>
<td>680</td>
<td>28</td>
<td>65</td>
<td>92</td>
<td>121</td>
<td>64</td>
<td>51</td>
<td>96</td>
<td>Poinar, 1985</td>
</tr>
<tr>
<td><em>S. abbreviata</em></td>
<td>541</td>
<td>29</td>
<td>48</td>
<td>68</td>
<td>89</td>
<td>56</td>
<td>53</td>
<td>86</td>
<td>Elawad et al., 1997</td>
</tr>
<tr>
<td><em>S. bicornutum</em></td>
<td>769</td>
<td>29</td>
<td>61</td>
<td>92</td>
<td>124</td>
<td>72</td>
<td>50</td>
<td>84</td>
<td>Tallosi et al., 1995</td>
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<tr>
<td><em>S. riobreave</em></td>
<td>622</td>
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<td>56</td>
<td>87</td>
<td>114</td>
<td>54</td>
<td>49</td>
<td>105</td>
<td>Cabanillas et al., 1994</td>
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<tr>
<td><em>S. akhurstii</em></td>
<td>812</td>
<td>33</td>
<td>59</td>
<td>90</td>
<td>119</td>
<td>73</td>
<td>47</td>
<td>77</td>
<td>Qiu et al., 2005b</td>
</tr>
<tr>
<td><em>S. feltiae</em></td>
<td>849</td>
<td>29</td>
<td>63</td>
<td>113</td>
<td>136</td>
<td>86</td>
<td>46</td>
<td>74</td>
<td>Nguyen, 2007</td>
</tr>
<tr>
<td><em>S. litorale</em></td>
<td>909</td>
<td>31</td>
<td>61</td>
<td>96</td>
<td>125</td>
<td>83</td>
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<td><em>S. arenarium</em></td>
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<td>109</td>
<td>138</td>
<td>75</td>
<td>55</td>
<td>119</td>
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<tr>
<td><em>S. diaprepsi</em></td>
<td>1022</td>
<td>34</td>
<td>74</td>
<td>102</td>
<td>138</td>
<td>83</td>
<td>54</td>
<td>90</td>
<td>Nguyen and Duncan, 2002</td>
</tr>
<tr>
<td><em>S. glaseri</em></td>
<td>1130</td>
<td>43</td>
<td>102</td>
<td>120</td>
<td>162</td>
<td>78</td>
<td>65</td>
<td>131</td>
<td>Wouts et al., 1982</td>
</tr>
</tbody>
</table>

L = length; W = greatest body diam.; EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = pharynx length; T = tail length; D% = EP/ES × 100; E% = EP/T × 100; NA = not available.

Alvarez et al., 2021) (Table 2). *Photorhabdus* has 21 species since whole genome sequencing by Machado et al. (2018) elevated 15 subspecies to the species level, and the latest species descriptions are *P. aegyptia* and *P. hindustanensis* by Machado et al. (2021a, 2021b, 2021c). *Photorhabdus luminescens* has *P. luminescens mexicana* as a subspecies; *Photorhabdus laumondii* is divided into *P. laumondii* subsp. *laumondii* and *P. laumondii* subsp. *clarkei*; *P. akhurstii* into *P. akhurstii* subsp. *akhurstii* and *P. akhurstii* subsp. *bharatensis*; *P. khanii* into *P. khanii* subsp. *guanajuatensis*; and *P. heterorhabditis* into *P. heterorhabditis* subsp. *aluminescens* and *P. heterorhabditis* subsp. *heterorhabditis*; *P. australis* into *P. australis* subsp. *thailandensis* and *P. australis* subsp. *australis* (Machado et al., 2018, 2019, 2021a, 2021b, 2021c) (Table 3). *Xenorhabdus* and *Photorhabdus* exist as primary or secondary phases (i.e. variants), which can be monitored by their ability to absorb dye and produce antibiotic compounds. Dye absorption and antibiotic production are observed in Phase-I, which is also the natural state of...
the bacteria. Phase-II, on the other hand, spontaneously occurs under unfavorable conditions and is believed to be due to reversible DNA imbalance (Leclerc and Boemare, 1991; Volgyi et al., 1998; Boemare, 2002). Both these bacterial phases are motile and facultative anaerobes. Their optimum growth temperature ranges between 25 °C and 30 °C depending on species and strain. Catalase activity, bioluminescence and red pigment production are characters that differentiate *Phototobdus* from *Xenorhabdus*; *Phototobdus* is positive for these characteristics, whereas *Xenorhabdus* is negative (Boemare, 2002). These bacteria are well-known for the plethora of secondary metabolites they produce.

### 1.3. Life cycle of entomopathogenic nematode/bacterium complex

Like all nematodes, EPNs have four larval stages. They have a specialized larval stage, the IJ that is resistant to unfavorable conditions (Kiontke and Fitch, 2013). These IJs or dauers, the only free-living stage, roam freely or lie in wait for passing insects in soil. The IJs infect their host through natural openings such as the mouth and anus and then move from the intestine into the hemocoel, or they infect via the spiracle and move directly into the hemocoel. With *Heterorhabditis* and some *Steinernema* species, the IJs can also penetrate through thin areas of the insect’s integument and enter directly into the insect’s hemocoel (Bedding and Molyneux, 1982; Peter and Ehlers, 1994). The alimentary tract of the IJs is closed, and hence they are nonfeeding but once they enter the insect’s hemocoel, they open their alimentary tract and release the symbiont bacteria which are pathogenic to insects. In the case of *Heterorhabditis*, the IJs regurgitate the bacterial symbionts, whereas with *Steinernema*, the IJs defecate the bacterial cells into the insect’s hemocoel (Ciché et al., 2006). The bacteria produce compounds that suppress the insect immune system, and host death subsequently occurs due to toxemia or septicemia (Dutky, 1959; Forst et al., 1997; Dowd and Peters, 2002). *Xenorhabdus* and *Phototobdus* also produce antimicrobials to prevent saprophytic organisms such as other bacteria, protozoa, and fungi from invasion as well as compounds that deter other organisms from scavenging on the insect cadaver (Baur et al., 1998; Zhou et al., 2002; Foltan and Puza, 2009; Gulcu et al., 2012). IJs initiate their development and feed on the symbiotic bacteria and host tissues, molt to the 4th stage and then to the adult stage (Hazir et al., 2003a) (Figure 1).

Adult steinernematids are dioecious except for *Steinernema hermaphroditum* (Stock et al., 2004). In heterorhabditids, the first-generation adults resulting from IJs are hermaphrodites, but progenies of these hermaphrodites have males, females, and hermaphrodites in the subsequent generations (Koltai et al., 1995). Unlike steinernematids where male and female IJs are needed to infect an insect host, a single IJ of heterorhabditids can infect and reproduce in an insect host. Copulation occurs when adult steinernematid males coil around females or heterorhabditid males lie parallel to female genital region and inserting their copulatory spicules into the females’ vulva. Also, adult steinernematid males battle rival males by coiling around and constricting their opponent; the rival male is paralyzed and killed using the copulatory spicules (O’Callaghan et al., 2014; Zenner et al., 2014). In both EPN genera, some fertilized eggs hatch within the mother to produce offspring that consumes her, a phenomenon known as *endotokia matricida* (Figure 2). Generally, a few days after death, heterorhabditid-infected insect hosts (cadavers) turn red, but some species/strains can also cause the cadaver to turn orange, purple, brown,
yellow, or green. Steinernematids cause the infected host to become brown, tan, gray, dark gray, or ochre after death (Figure 3). The color of the insect cadavers is due to the pigment associated with the bacteria — red or other colors for *Photorhabdus* and brown, tan, gray or dark gray, ochre for *Xenorhabdus* (Devi, 2018) (Figure 3). Depending on the size of their insect host and availability of nutrients, there may be one or more generations of the EPNs in each host. Upon depletion of resources, IJs are formed and exit the cadaver to seek new hosts (Poinar, 1990; Kaya and Gaugler, 1993; Griffin, 2012).

1.4. Ecology of entomopathogenic nematode/bacterium complex

As previously stated, IJs are the only form found in soil. Their survival and distribution are affected by various abiotic factors such as vegetation, soil texture, temperature, moisture, etc. Biotic factors also affect their presence and persistence in soil, but the availability of a suitable insect host plays the most crucial role (Stuart et al., 2015).

1.4.1. Abiotic factors
1.4.1.1. Moisture

Nematodes require moisture for movement and respiration, making it a vital and critical necessity. An adequate and flowing water film between soil particles is required for movement from one place to another. Also, due to a lack of a circulatory and respiratory system, nematodes respire through their moist body walls. Consequently, nematodes are less active or survive less in overly dry or overly wet soil due to oxygen deprivation (Koppenhöfer et al., 1995; Yadav and Lalramliana, 2011; Stuart et al., 2015).

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**Figure 2.** Endotokia matricida stage of an entomopathogenic nematode.

**Figure 3.** White trap system with entomopathogenic nematode-infected *Galleria mellonella* and *Tenebrio molitor* cadavers. Color change of infected cadavers is observed a few days after death. Heterorhabditud-infected cadaver generally turns red (A and C); steinernematids-infected are brown, tan or even black (C and D).
1.4.1.2. Temperature

EPN species have been isolated from nearly all climates, but different species have different temperature tolerances beyond which their survival is threatened. For example, some species can infect, develop, and reproduce at lower or higher temperature thresholds depending on their natural temperature habitat or innate physiology (Grewal et al., 1994a; Brown and Gaugler, 1997; Hazir et al., 2001). Prevention of ice crystal formation, or tissues unaffected by its formation, are ways nematodes isolated from cold regions like arctic and subarctic regions and high altitudes endure cold stress. *S. feltiae* is an example of an EPN that can infect below 10 °C (Grewal et al., 1994a; Hazir et al., 2001). When placed under cold temperatures, *S. feltiae* produces cyroprotectants like glycerol and trehalose (Ali and Wharton, 2015). The presence of heterorhabditids in desert and arid regions of Israel, Sri Lanka, India, and Egypt demonstrates that they can withstand hot temperatures, and it is believed that heat stress-related proteins like heat-shock proteins are useful for this purpose (Glazer, 2002; Koppenhöfer and Fuzy, 2003; Ramalingam et al., 2011; Stuart et al., 2015). Previous studies have demonstrated that similarities and differences occur among isolates of the same EPN species in their response to temperature (Hazir et al., 2001).

1.4.1.3. Soil texture

Soil texture can determine the moisture retention, water and airflow and organic matter of any given soil; hence nematode survival and persistence vary in different soil types. Sandy and loamy soils are generally most favored because these soils have moderate moisture and aeration and provide less resistance to nematode movement. The least favorable soil type is clay soil probably due to oxygen deprivation. (Kung et al., 1990a; Hazir et al., 2003b). However, there are exceptions to this rule where heavier soils have been more conducive to EPN activity (Shapiro et al., 2000).

1.4.1.4. Ultraviolet

UV radiation is another environmental stress that impacts the survival, virulence, and reproduction of EPNs. Lab studies by Shapiro-Ilan et al. (2015) assessed the UV tolerance of 21 different EPN species/strains; they reported significant variation in UV tolerance among EPNs with steinernematids usually being more tolerant than heterorhabditids. Moreover, shorter wavelengths (254 nm) were reportedly more harmful towards EPN virulence compared to longer wavelengths (366 nm).

1.4.1.5. pH

Soil pH can also affect EPN survival (Kung et al., 1990b; Fischer and Fuhrer, 1990). Khathwayo et al. (2021) screened a diverse array of EPN species and found that generally steinernematids had a higher survival ability across pH ranges than heterorhabditids. Additionally, *S. carpocapsae* and *S. riobrave* exhibited consistently higher survival in both acidic and alkaline solutions, when compared to the other steinernematids, suggesting that they may be applied in both acidic and alkaline soils. The study also indicated that pH tolerance may be correlated to geographic origin (Khathwayo et al., 2021).

1.4.2. Biotic factors

Soil environments consist of a multitude of organisms competing for limited resources using characters or behaviors that maximize their fitness. EPNs and insect cadavers are vulnerable to biotic factors in soil. Natural enemies of nematodes include nematophagous fungus, tardigrades, collembolans, mites, predaceous nematodes, etc., that prey on EPNs (Kaya, 2002; Kaya and Koppenhöfer, 1996; Deacon, 2006; Stuart et al., 2015). As a means of protecting the infecting symbiotic unit and monopolizing the nutritional resources in the insect cadaver, *Xenorhabdus* and *Photorhabdus* produce secondary metabolites to outcompete potential rival microorganisms, and these metabolites are effective deterrents designed to steer away or ward off scavengers from feeding on the insect cadavers (a phenomenon now referred to as scavenger deterrence) (Baur et al., 1998; Gulcu et al., 2012; Ulug et al., 2014; Raja et al., 2017, 2021).

1.4.2.1. Host range

Under laboratory conditions, EPNs are adept parasites of a wide range of insect species. For example, *S. carpocapsae* can infect more than 100 different insect species in the laboratory (Poinar, 1979). Under field conditions, however, host range is limited by ecological factors in soil, such as the ability of the IJs to successfully locate, penetrate and infect a host, and the host’s ability to suppress infection. Moreover, some nematodes are specialists; for instance, *S. scapterisci* is a highly virulent nematode that specializes in killing mostly Orthoptera (Nguyen and Smart, 1991; Gulcu et al., 2017). Other specialists are *S. kushidai* and *S. scarabei*, which are particularly virulent to larvae of Scarabaeidae (Koppenhöfer and Fuzy, 2003).

1.4.2.2. Dispersal

EPNs can seek out suitable hosts in soil and cryptic habitats such as in tree trunks and rhizomes. Reportedly, *S. carpocapsae* and *H. bacteriophora* can in the absence of hosts cover approximately 6.0 cm/day or surface area of 2.5 m² in 10 days; this is equivalent to dispersing a minimum of 120 times the IJ body length per day (Bal et al., 2014a). Little information is available about their host finding behavior due to difficulties in studying the different dispersal/host finding behaviors of these roundworms in the soil environment. However, based on laboratory studies EPN species are grouped accordingly as cruisers or ambushers (Campbell and Gaugler, 1993; Grewal et al.,
HAZIR et al. / Turk J Zool

2. Isolation of EPNs

2.1. Koch’s postulates and etiological role of nematode/bacterium complex in insect infection

When a new probable EPN species is isolated from the natural environment, it is necessary to confirm that the isolate suspected is indeed the causal pathogen of an observed disease. In the 19th century, Robert Koch developed four postulates that have been and are used as important guidelines for confirming that an organism is pathogenic, i.e. is an etiological agent of an infection or a disease. He theorized that a suspected infectious agent:

1. Must be present in all infected organisms and absent in healthy organisms.
2. Must be isolated and grown as a pure culture.
3. When inoculated in a healthy and susceptible experimental organism as a pure culture, it must produce the same symptoms or signs as observed in the first infection or disease.

4. Must be reisolated/recovered from an infected host (Falkow, 1988; Klassen, 2014; Madigan et al., 2015).

Koch used these criteria to easily identify infectious agents like *Bacillus anthracis* and *Mycobacterium tuberculosis* because these bacteria could be cultured in the laboratory, and there was a suitable host on which they produced pathognomonic signs of the original infection. However, this is not the case for all disease-causing organisms: some infectious agents have yet to be cultured in pure in vitro conditions and some agents cause asymptomatic or opportunistic infections. To overcome these shortcomings, these postulates have been greatly improved to include molecular techniques to Koch’s postulates which seek the presence of genes that code for virulence factors in pathogenic species/strains of an organism and whether activation or deactivation of these genes leads to disease/infection. Interestingly, however, Koch’s postulates are still used by scientists to assess pathogen-host interactions and mutualistic relationships (Falkow, 1988; Klassen, 2014; Madigan et al., 2015; Cohen, 2017), namely, the etiological role of agents like EPNs in invertebrate diseases, and also, the symbiotic association between *Xenorhabdus* and *Photorhabdus* and their respective nematode host (Kaya and Stock, 1997; Kaya and Vega, 2012).

2.2. Collection and extraction of EPNs from soil

Several different methods to extract nematodes from soil have been developed [see J. van Bezooijen (2006) http://www.nematologia.com.br/files/tematicos/5.pdf] including centrifugal flotation, sieving, and Baermann funnel. However, using these extraction methods generally results in the recovery of many different species of free-living, plant-parasitic, and animal-parasitic nematodes which require extra labor and equipment to identify them. In addition, the Baermann funnel method, for example, results in a relatively low recovery rate for quiescent IJs of EPN species like *S. feltiae* and *S. carpocapsae* (Barker, 1985; Bedding and Akhurst, 1975; Kerry and Hominick, 2002). To obtain EPNs from soil, Bedding and Akhurst (1975) developed a more specific insect-baiting method that focuses on extraction of EPN species from soil that is less laborious, is more precise, and saves time. The principle of this method is to lure the IJs to infect a susceptible insect host like *Galleria mellonella* (Lepidoptera: Pyralidae) or *Tenebrio molitor* (Coleoptera: Tenebrionidae) in freshly collected soil. However, if one is interested in isolating EPNs that are particularly virulent to a specific target pest (for example, *Steinernema scapterisci* and *S. scarabaei*) infect mole and field crickets and white grubs more readily than *G. mellonella* or *T. molitor*), it is recommended to also bait with that insect as well (Shapiro-Ilan et al., 2003a; Orozco et al., 2014).

**Procedure** (Bedding and Akhurst, 1975) (Figure 4)

Taking soil samples that are extremely dry or saturated with water (mud) should be avoided as many nematode
species cannot survive in such environments. The most ideal soil is the one that does not disperse and stick to your hand when you squeeze it in your palm. Another important point besides soil structure is the selection of agricultural areas or areas where there is an abundance of plants (lawns, pastures, orchards, forests, chaparrals, etc.). The presence of plants and insect hosts increases the possibility of finding EPNs. Until now these soil conditions have been the most favorable for EPN isolation; however, isolating EPNs from extreme environments may assist in finding superior resistant strains. Hence, collection of soil samples from different habitats should be encouraged rather than discouraged.

The following procedure outlines how to take soil samples to isolate EPN species:

1. Remove surface litter, large rocks, plants, plant debris, etc., from sampling area/spot.
2. Bore holes using a clean soil probe or auger from the surface to a depth of ca. 20 cm. Other tools like shovels or trowels can be used, but these tools collect large amounts of soil samples (Figure 5) that are not needed. Wash tools between sample collections with 70% alcohol and water to prevent cross contamination. Soil sample tools are available commercially, but they can be made by most ironsmiths.
3. Collect 8–10 subsamples from the same area and combine them to get approximately 1–1.5 kg of soil in a plastic bag. Label each plastic bag with the location, GPS, vegetation type, and soil temperature (a simple meat thermometer works well). Note: EPNs have an uneven distribution and cannot disperse over long distances in soil. If possible, multiple soil samples should be collected from each sampling area to effect higher chances of isolation. Therefore, a soil core sampler will be more suitable to collect different subsamples from the same field. Otherwise, huge amount of soil will be collected when using a shovel or trowel; however, subsamples can be taken from pooled samples. Both transects or bulk soil sampling in a grid pattern can be used to isolate different strains and species (Stuart et al., 2004).
4. Transport soil samples in cooler chests with ice or icepacks to the laboratory. Remove additional roots, rocks, and vegetation by putting the soil through a coarse sieve. Moisten soil if necessary with distilled water (optimum soils moisture would be approximately field capacity. Generally, for sandy soil 6%–10% is fine).
5. After mixing the soil subsamples are collected from the same field, dispense the soil into plastic cups (250–500 mL) with lids and add 5–10 last instar larvae of inactivated *G. mellonella* (see “Important note below”) and or whatever host is deemed appropriate. Turn plastic containers upside down so that the larvae would remain trapped below the soil. Incubate cups at ca. 24–25 °C in the dark. To isolate cold or heat tolerant species, you can alter the incubation temperatures. Check containers at various time intervals (e.g., every 2–3 days) and flip over if the larvae crawl to the surface.

Important note: Commercially sold *G. mellonella* larvae are inactivated. Such treated larvae do not produce silk or molt to the pupal stage. EPN IJs are hampered by...
the silken cocoon from infecting into the pupae. If you rear and maintain a *G. mellonella* culture, you should inactivate the larvae before adding into the soil (see details of inactivation in rearing of *G. mellonella* section).

6. Check for larval mortality in the soil every 3 days for 8–9 days; rinse the cadavers in a beaker with distilled water to remove soil particles and possible saprophytic nematodes and transfer to White traps (White, 1927) (Figure 4).

7. Check the water availability in between the smaller and bigger Petri dishes and moisture level of filter papers in White traps. If necessary, add 100–150 mL water with a pipet to the filter paper. **Caution:** Do not put too much water on filter papers (the filter paper should be moist but without standing water).

**Note:** Insects killed by EPNs would not putrefy or smell badly because the symbiotic bacteria, *Xenorhabdus* or *Photorhabdus*, inhibit the growth of saprophytic organisms by producing a variety of antimicrobial compounds.

8. Emerged nematodes (IJs) from the cadavers will migrate into water in the Petri dish. Collect the IJs by pouring the water into a beaker every day or every other day depending on how densely the nematodes are building up. Refresh the water level again in the Petri dish for the next series of IJ emergence. It is important to collect the IJs from the White traps regularly because if the population density is too high, the IJs will die due to the lack of oxygen.

9. To confirm that nematodes are responsible for death of the larvae, use Koch’s postulates by reinfecting with *G. mellonella* or other available insect host (see the subsection on Koch’s postulates above). The reason for this step is that new and/or inexperienced students or researchers can mistakenly isolate saprophytic nematodes that utilize the dead *G. mellonella* larvae or other insect hosts as a food source. In these cases, the larvae died from other causes, and the saprophytic nematodes colonized and reproduced on or in the dead larvae. Dispense 200–300 µL nematodes emerging from cadavers in the White trap to a new Petri dish lined with filter papers. Add 5 *G. mellonella* larvae and cover with lid. To avoid water loss, place the Petri dishes in plastic bags or seal with parafilm and store at room temperature in the dark. Check for mortality for 3–4 days. EPN IJs will infect the new *G. mellonella* larvae showing the same signs associated with a heterorhabditid or steinernematid infection. Place cadavers in new White traps to collect the EPN IJs for storage and further studies.

10. For long-term storage of IJs, collect and transfer the water with IJs from the White trap into a beaker and allow the nematodes to settle to the bottom of the beaker for ca. 30 min. If the water contains insect tissues and other debris, the nematodes can be washed to obtain a cleaner culture. To wash, decant the supernatant and add more water to remove the debris. Repeat this procedure several times. If needed, the live IJs can be separated from dead ones by using a 270-mesh sieve (53 µm aperture). Finally, store the washed IJs in tissue culture flasks or Tetra Pak containers (Figure 6) (Gulcu and Hazir, 2012) at 5–15 °C depending on your isolate. Keep the flasks or containers flat so that there is good aeration of the water.
Cautions: 1) Do not store IJs in beakers because IJ survival decreases if the water depth is too high. 2) IJ density is important for long-term storage. Optimum number of IJs in tissue culture flasks or Tetra-pak containers is approximately 1000-5000 IJs/mL.

3. Identification of EPNs

3.1. Morphological and morphometric analysis

Morphometric and morphological analyses gather the quantitative and qualitative data of homologous morphological characters in 2- or 3-dimensional space to formulate hypotheses on nematode systematics, phylogeny, and behavior. The objective of morphometric analyses is to compare the measurements of the length, width, their ratios, and the structural comparisons of different anatomical parts of a nematode with other nematodes of the same genus (de Man, 1876; Cobb, 1890; Baldwin and Perry, 2004) in order to trace inter- and intraspecific variation in these traits.

Procedure

1. Dissect nematode-infected G. mellonella larva in Ringer’s solution (dissolve 7.2 g NaCl, 0.17 g CaCl₂, and 0.37 g KCl in 1 L distilled water, pH adjusted to 7.3–7.4) under a stereomicroscope from 2- to 6-day and from 5- to 9-days postinfection for first generation and second generation steinernematid adults, respectively. For heterorhabditids, obtain the first-generation hermaphrodites 3- to 5-day postinfection and second-generation adults (both male and female) 7- and 9-days postinfection. IJs can be collected as they emerge.

2. Newly emerged IJs may be ensheathed with their second-stage cuticle [(heterorhabditids retain this layer longer than steinernematids (Hazir et al., 2003b)], and the presence of the second-stage cuticle affects the measurement and the quality of images. Nematodes can be desheathed by treating the nematodes with 1% sodium hypochlorite solution (NaOCl) for 5 min (Campbell and Gaugler, 1992).

3. The IJs and adult nematodes are transferred into a test tube with Ringer’s solution. Seal the test tube and incubate in a water bath at 60 °C for 2 min to instantaneously heat-kill the nematodes. This ensures that the nematodes are nicely stretched and do not coil as they are killed. A cold fixative will not instantaneously kill the nematodes, they will contract and coil to a degree that often makes them useless for detailed study.

4. Fix heat-killed nematodes using equal volume of triethanolamine–formalin (TAF) fixative (97 mL 40% formalin, 2 ml triethanolamine, and 91 mL distilled water) at 80 °C. Leave in water bath for 12–24 h (Kaya and Stock, 1997).

5. Carefully pick nematodes with a needle and transfer to watch glass with 0.5 mL anhydrous glycerol solution made with ethanol, glycerin, and distilled water in the ratio 20:1:79 (Seinhorst, 1959).

6. Dip the opened side of the test tube in the melted paraffin and transfer the paraffin on the slide in a ring shape and let stand until the paraffin hardens. Place nematode on a slide with a drop of pure glycerin inside paraffin ring. Melt paraffin support by placing slid on a hot place at 80 °C. The slide can also be sealed with nail polish or other sealants.

7. Observe and make measurements under microscope and make measurements with an image...
analysis program. Make the following measurements shown in Table 3 (Kaya and Stock, 1997; Plichta et al., 2009).

8. Compare the morphometric traits (range and mean) of each stage of measured nematode with the same stages of other nematodes of the same genus. Tables 3–6 show measurements for some of the main species described.

3.2. Scanning electron microscope

The use of scanning electron microscope (SEM) is not essential in routine identification of nematodes, although if molecular analyses show that the EPN isolate is a new species, then scanning electron microscope is necessary to show various distinctive characters on the cephalic region like amphids or cephalic and labial papillae, lateral lines for IJs or morphology of the reproductive systems (Figures

<table>
<thead>
<tr>
<th>Species</th>
<th>SP</th>
<th>GL</th>
<th>D%</th>
<th>SW%</th>
<th>GS%</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. carpocapsae</td>
<td>65</td>
<td>47</td>
<td>39</td>
<td>151</td>
<td>72</td>
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</tr>
<tr>
<td></td>
<td>(59–72)</td>
<td>(39–56)</td>
<td>(NA)</td>
<td>(NA)</td>
<td>(NA)</td>
<td></td>
</tr>
<tr>
<td>S. scapterisci</td>
<td>83</td>
<td>65</td>
<td>36</td>
<td>252</td>
<td>78</td>
<td>Nguyen and Smart, 1990</td>
</tr>
<tr>
<td></td>
<td>(72–92)</td>
<td>(59–75)</td>
<td>(32–39)</td>
<td>(204–280)</td>
<td>(69–84)</td>
<td></td>
</tr>
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<td>S. siamkayai</td>
<td>78</td>
<td>54</td>
<td>42</td>
<td>170</td>
<td>70</td>
<td>Stock et al., 1998</td>
</tr>
<tr>
<td>S. affine</td>
<td>70</td>
<td>46</td>
<td>61</td>
<td>117</td>
<td>66</td>
<td>Poinar, 1988</td>
</tr>
<tr>
<td></td>
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<td>(37–56)</td>
<td>(60–66)</td>
<td>(NA)</td>
<td>(NA)</td>
<td></td>
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<td>S. beddingi</td>
<td>71</td>
<td>43</td>
<td>58</td>
<td>108</td>
<td>61</td>
<td>Qiu et al., 2005c</td>
</tr>
<tr>
<td>S. intermedium</td>
<td>93</td>
<td>62</td>
<td>72</td>
<td>124</td>
<td>69</td>
<td>Poinar, 1985</td>
</tr>
<tr>
<td>S. abassi</td>
<td>65</td>
<td>45</td>
<td>60</td>
<td>156</td>
<td>70</td>
<td>Elawad et al., 1997</td>
</tr>
<tr>
<td>S. bicornatum</td>
<td>65</td>
<td>48</td>
<td>52</td>
<td>222</td>
<td>72</td>
<td>Tallosi et al., 1995</td>
</tr>
<tr>
<td></td>
<td>(53–70)</td>
<td>(38–50)</td>
<td>(50–60)</td>
<td>(218–226)</td>
<td>(NA)</td>
<td></td>
</tr>
<tr>
<td>S. riobrave</td>
<td>67</td>
<td>51</td>
<td>71</td>
<td>114</td>
<td>76</td>
<td>Cabanillas et al., 1994</td>
</tr>
<tr>
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<td>(48–56)</td>
<td>(60–80)</td>
<td>(NA)</td>
<td>(NA)</td>
<td></td>
</tr>
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<td>S. akhursti</td>
<td>90</td>
<td>64</td>
<td>56</td>
<td>180</td>
<td>71</td>
<td>Qiu et al., 2005b</td>
</tr>
<tr>
<td>S. feltiae</td>
<td>70</td>
<td>41</td>
<td>60</td>
<td>113</td>
<td>59</td>
<td>Nguyen, 2007</td>
</tr>
<tr>
<td></td>
<td>(65–77)</td>
<td>(34–47)</td>
<td>(51–64)</td>
<td>(99–130)</td>
<td>(52–61)</td>
<td></td>
</tr>
<tr>
<td>S. litorale</td>
<td>75</td>
<td>53</td>
<td>40</td>
<td>174</td>
<td>71</td>
<td>Yoshida, 2004</td>
</tr>
<tr>
<td>S. arenarium</td>
<td>76</td>
<td>53</td>
<td>78</td>
<td>210</td>
<td>70</td>
<td>Artyukhovsky, 1967</td>
</tr>
<tr>
<td></td>
<td>(63–93)</td>
<td>(45–63)</td>
<td>(53–96)</td>
<td>(NA)</td>
<td>(NA)</td>
<td></td>
</tr>
<tr>
<td>S. diaprepesi</td>
<td>79</td>
<td>54</td>
<td>80</td>
<td>180</td>
<td>69</td>
<td>Nguyen and Duncan, 2002</td>
</tr>
<tr>
<td>S. glaseri</td>
<td>77</td>
<td>46</td>
<td>70</td>
<td>205</td>
<td>70</td>
<td>Wouts et al., 1982</td>
</tr>
<tr>
<td></td>
<td>(64–90)</td>
<td>(44–59)</td>
<td>(60–78)</td>
<td>(164–243)</td>
<td>(64–84)</td>
<td></td>
</tr>
</tbody>
</table>

SP = spicule length; GL = gubernaculum length; D% = EP/ES × 100; SW% = SP/Anal body diameter × 100; GS% = GL/SP × 100; NA = not available.
Table 5. Comparative morphometrics (in μm) of infective juveniles of *Heterorhabditis* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>L</th>
<th>W</th>
<th>EP</th>
<th>NR</th>
<th>ES</th>
<th>TL</th>
<th>D%</th>
<th>E%</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. indica</em></td>
<td>528</td>
<td>20</td>
<td>98</td>
<td>82</td>
<td>117</td>
<td>101</td>
<td>84</td>
<td>94</td>
<td>Poinar, 1992</td>
</tr>
<tr>
<td><em>H. baujardi</em></td>
<td>551</td>
<td>20</td>
<td>97</td>
<td>81</td>
<td>115</td>
<td>90</td>
<td>84</td>
<td>108</td>
<td>Phan et al., 2003</td>
</tr>
<tr>
<td><em>H. bacteriophora</em></td>
<td>558</td>
<td>23</td>
<td>103</td>
<td>85</td>
<td>125</td>
<td>98</td>
<td>84</td>
<td>112</td>
<td>Poinar, 1990</td>
</tr>
<tr>
<td><em>H. floridensis</em></td>
<td>562</td>
<td>21</td>
<td>109</td>
<td>86</td>
<td>135</td>
<td>103</td>
<td>81</td>
<td>105</td>
<td>Nguyen et al., 2006a</td>
</tr>
<tr>
<td><em>H. mexicana</em></td>
<td>578</td>
<td>23</td>
<td>102</td>
<td>81</td>
<td>122</td>
<td>99</td>
<td>81</td>
<td>104</td>
<td>Nguyen et al., 2004a</td>
</tr>
<tr>
<td><em>H. amazonensis</em></td>
<td>589</td>
<td>23</td>
<td>107</td>
<td>85</td>
<td>121</td>
<td>107</td>
<td>88</td>
<td>100</td>
<td>Andalo et al., 2006</td>
</tr>
<tr>
<td><em>H. georgiana</em></td>
<td>598</td>
<td>22</td>
<td>104</td>
<td>85</td>
<td>127</td>
<td>98</td>
<td>82</td>
<td>107</td>
<td>Nguyen et al., 2008b</td>
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<tr>
<td><em>H. beicherriana</em></td>
<td>639</td>
<td>24</td>
<td>113</td>
<td>95</td>
<td>137</td>
<td>101</td>
<td>83</td>
<td>112</td>
<td>Li et al., 2012</td>
</tr>
</tbody>
</table>

L = length; W = greatest body diam.; EP = distance from anterior end to excretory pore; NR = distance from anterior end to nerve ring; ES = pharynx length; T = tail length with second stage cuticle; D% = EP/ES × 100; E% = EP/T × 100.

Table 6. Comparative morphometrics (in μm) of first-generation males of *Heterorhabditis* species.

<table>
<thead>
<tr>
<th>Species</th>
<th>SP</th>
<th>GL</th>
<th>D%</th>
<th>SW%</th>
<th>GS%</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. indica</em></td>
<td>43</td>
<td>21</td>
<td>122</td>
<td>187</td>
<td>50</td>
<td>Poinar, 1992</td>
</tr>
<tr>
<td></td>
<td>(35–48)</td>
<td>(18–23)</td>
<td>(NA)</td>
<td>(NA)</td>
<td>(40–60)</td>
<td></td>
</tr>
<tr>
<td><em>H. baujardi</em></td>
<td>40</td>
<td>20</td>
<td>70</td>
<td>182</td>
<td>50</td>
<td>Phan et al., 2003</td>
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<tr>
<td></td>
<td>(33–45)</td>
<td>(18–22)</td>
<td>(NA)</td>
<td>(138–208)</td>
<td>(44–61)</td>
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</tr>
<tr>
<td><em>H. bacteriophora</em></td>
<td>40</td>
<td>20</td>
<td>117</td>
<td>174</td>
<td>50</td>
<td>Poinar, 1990</td>
</tr>
<tr>
<td></td>
<td>(36–44)</td>
<td>(18–25)</td>
<td>(NA)</td>
<td>(NA)</td>
<td>(NA)</td>
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<tr>
<td><em>H. floridensis</em></td>
<td>42</td>
<td>23</td>
<td>112</td>
<td>157</td>
<td>54</td>
<td>Nguyen et al., 2006a</td>
</tr>
<tr>
<td><em>H. mexicana</em></td>
<td>41</td>
<td>23</td>
<td>129</td>
<td>167</td>
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<td>Nguyen et al., 2004a</td>
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<td><em>H. amazonensis</em></td>
<td>41</td>
<td>21</td>
<td>103</td>
<td>152</td>
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<td>Andalo et al., 2006</td>
</tr>
<tr>
<td><em>H. georgiana</em></td>
<td>44</td>
<td>25</td>
<td>110</td>
<td>172</td>
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<td>Nguyen et al., 2008b</td>
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<td>(41–49)</td>
<td>(20–28)</td>
<td>(100–122)</td>
<td>(150–200)</td>
<td>(51–64)</td>
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<tr>
<td><em>H. beicherriana</em></td>
<td>45</td>
<td>24</td>
<td>112</td>
<td>180</td>
<td>54</td>
<td>Li et al., 2012</td>
</tr>
</tbody>
</table>

SP = spicule length; GL = gubernaculum length; D% = EP/ES × 100; SW% = SP/Anal body diameter × 100; GS% = GL/SP × 100; NA = not available.
7–9) (Nyugen and Smart, 1995, 1996, 1997; Andalo et al., 2006).

Procedure
1. Kill nematodes in Ringer’s solution over a water bath at 60 °C for 2 min.
2. Fix nematodes in 4% formalin buffered with 0.1 M sodium cacodylate at pH 7.2 for 24 h from 4 °C to 6 °C.
3. Postfix in 2% osmium tetroxide (OsO₄) solution for 12 h at 25 °C, then rinse in water three times.
4. Serially transfer in ethanol (from 10% to 100%) to dehydrate samples.
5. Dry in liquid CO₂. Mount on SEM then coat with gold.
6. Examine samples with a scanning electron microscope.

3.3. Molecular analysis
At present, due to the quantity of EPN species and relatively low number of morphological differences, molecular approaches must be used in the identification of a nematode. A wide range of molecular techniques such as rapid amplified polymorphism (RAPD), restriction fragment length polymorphism (RFLP) or DNA sequence analysis have been used as diagnostic tools to identify and distinguish EPN species, but sequence analysis remains as the only viable option which further allows the researcher to trace evolutionary relationships among heterorhabditid and steinernematid nematodes. Analysis and blasting of DNA sequences of various gene regions like 18S region, 28S region and internal transcribed spacer region (ITS1, 5.8S, ITS2) sequences in databases like NCBI Genbank.
can yield the informative data for phylogenetic studies. Analysis of the ITS and 28S gene regions are the most widely used and accepted gene regions in nematode diagnosis, and NCBI Genbank has the most data for these gene regions. Mitochondrial genes can be used as well (Table 7) (Blaxter et al., 1998; Spiridonov et al., 2004b; Stock and Hunt, 2005; Adams et al., 2006).

3.3.1. DNA extraction (Vierstaete, 2009)

There are many different DNA extraction methods; commercial DNA extraction kits, phenol-chloroform extraction method, etc.

1. Crush newly obtained nematodes (a few hundred IJs or 5–10 females) in 500 µL of TE buffer with 2% cetyl trimethyl ammonium bromide (CTAB), 100 g/mL proteinase K and 1% β-mercaptoethanol with a sterile homogenizer. If you use the IJ stage for DNA extraction process, it will take a longer period because of the durable cuticle layer of the IJs.

2. Incubate tubes in a water bath at 95 °C for 10 min and then transfer to –80 °C for 10 min. Repeat cycle thrice with 15 s of vortexing in between.

3. Centrifuge at 13,000 rpm for 5 min at 4 °C. Transfer supernatant to a clean tube.


5. Add equal volume chloroform/isoamyl alcohol (24:1 v/v) to remove phenol residues. Then centrifuge at 13,000 rpm for 15 min.

6. Collect upper phase into a fresh 1.5 mL tube. Add 750 µL cold isopropanol and 1/10 volume sodium acetate.

7. Maintain tubes at –20 °C overnight. Centrifuge at 13,000 rpm for 30 min at 4 °C, decant supernatant and wash DNA pellet with 200 µL of cold 70% ethanol.

8. Air dry tubes and add 50 µL of sterile water and store at -20 °C until further use.

3.3.2. Rapid DNA extraction method (Mracek et al., 2014)

In this method, DNA is obtained from a single female.

1. The nematode is transferred to the bottom of the sterilized 0.5 mL microcentrifuge tube.

2. Crushed in 20 mL of lysis buffer (17.7 mL of ddH2O, 2 mL of 10× PCR buffer, 0.2 mL of 1% Tween 20 and 0.1 mL of 60 mg mL⁻¹ proteinase K).

3. The tube is frozen at –20 °C for 20 min, incubated at 65 °C for 60 min, followed by 95 °C incubation for 10 min.

4. Afterward, the tube is cooled on ice and centrifuged at 9000 g for 2 min.

Figure 8. Scanning electron microscopy images of *Steinernema beitlechemi* male. A, B: First generation male. A: Tail with paired genital papillae (numbered), single papilla (s) and post-deirid (arrow), dorso-lateral; B: Spicules with rounded tip, ventro-lateral. C–E: Second-generation male. C: Tail with paired genital papillae (numbered), single papilla (s) and postdeirid (arrow), lateral; D: Postdeirid, detail; E: Tail with part of paired genital papillae (numbered), single papilla (s) and mucron (m), ventro-lateral.
5. The supernatant containing DNA is ready to use for PCR amplification and can be kept at -20 °C for future use.

DNA can also be extracted from female or IJ stage of the nematodes using a commercial DNA extraction kit. Methods of the extraction can be found in the user manual of the company.

In general, some species from the steinernematid clades “feltiae” and “glaseri” may have intrapopulation and even intraindividual variability on the sequence of the ITS region (Puza et al., 2015). This fact has implications for the sequencing process and in such case, only a small part of the sequence can be obtained. In case the variability is among individuals in the population, extraction from single nematodes solves the problem. If the polymorphism is within individuals, the only way to obtain the sequence is cloning.

3.3.3. PCR amplification

1. Prepare PCR master mix by adding 7.25 mL of ddH₂O, 1.25 mL 10× PCR buffer, 1 mL of dNTPs, 0.75 mL of forward and reverse primers each (20 mM), and 0.1 µL of Taq polymerase. Place the reagent on ice to thaw and add the enzyme last. Prepare the master mix one more than the number of samples. A general rule is to add the most dilute reagents first and adding the most concentrated reagents at the end.

2. Dispense 1 µL of DNA template into tube. Include a negative control without DNA template. Extracted DNA concentration must be around 20–100 ng.

3. Program thermocycler with the following steps: initial denaturation, 35–40 cycles denaturation, annealing and extension steps and a final extension step. Conditions might vary according to chosen primers as listed in Table 4.

4. After amplification, analyze PCR reaction results via gel electrophoresis on 1% (w/v) agarose containing 1x loading dye. Check the correct size of amplicon by electrophoresis (Stock and Goodrich-Blair, 2012; Cimen et al., 2016a).

5. Send amplified PCR products for sequencing to companies that offer gene sequencing services.

Some factors that affect the success of PCR:
- Primer concentration: Very high primer concentration increases the likelihood of nonspecific PCR products forming.
- Template DNA concentration: Too little or too much DNA template amount affects the PCR reaction.
- Concentrations of dNTPs: Too high concentrations of dNTPs inhibit the PCR reaction.
- Annealing temperature: The optimal annealing temperature has to be determined experimentally.
- Number of cycles: The number of cycles necessary to obtain enough PCR product depends strongly on the concentration of the DNA template.

4. Isolation methods of the mutualistic bacterium

4.1. Isolation from infected Galleria mellonella hemolymph

When the IJs infect insects, they proceed to the hemocoel to release the symbiotic bacteria. Xenorhabdus or Photorhabdus proliferate in the hemolymph causing sepsis and/or toxemia and eventual death of host within 2 to 3 days while simultaneously eliminating other rival microorganisms (Boemare and Akhurst, 2006). The symbiotic bacteria can be isolated 28–36 h after inoculation (i.e. moribund infected host) by collecting the hemolymph under sterile conditions. The following procedure can be used.

1. Infect 10 G. mellonella larvae in 9-cm Petri dishes lined with two moist filter papers by adding 1 mL nematode suspension containing approximately 2000 IJs. The following steps 2–5 should be conducted under sterile conditions (e.g., laminar flow hood or biosafety cabinet).

2. After 28–36 h, collect live but moribund larvae and disinfect surface by submerging in PBS. Crush nematodes using a sterile motorized homogenizer.

3. Place insect between the index and thumb and the middle finger, bend the insect using index and thumb. Be careful not to over squeeze larva. Obtain insect hemolymph by either:
   a. cutting one of the insect’s false legs (prolegs) using a pair of sterile mini-dissecting scissors;
   b. injecting a 26G sterile syringe intrahemocoelically (just underneath the cuticle at a 25° angle but not too deep) (Figure 10); or
   c. cutting the head of insect and squeezing out insect contents with an L-shaped rod.

4. A clear hemolymph sample will ooze out. Do not use murky or cloudy hemolymph as this a sign of contamination with enteric bacteria of the insect.

5. Collect hemolymph sample using a sterile microbiological loop or micropipette and inoculate on appropriate culture medium, preferably, nutrient agar, NBTA (nutrient agar + bromothymol blue + 2,3,5-triphenyltetrazolium chloride) or T7 (see details in frequently used media and reagents section).

6. To increase success of isolation, use three or more insect larvae.

7. Incubate cultured plates in an incubator at 25–28°C.

4.2. Direct isolation of bacteria from nematode stages

Bacteria can be isolated directly from the infective juveniles (IJs) (Cimen, 2013) or from gravid females during endotokia matricida (Ulug et al., 2015).

Procedure:
1. Transfer 1 mL of IJs to a 1.5-mL sterile Eppendorf tube. Allow nematodes to sink to the bottom of tube. Centrifuge at 13,000 rpm for 3 min and decant supernatant, without disturbing IJs. Or collect females as described in subsection 3.1.

2. Surface sterilize nematodes using 0.4% Hyamine solution for 6 min. Mix thoroughly. Discard Hyamine solution after allowing nematodes to sink to the bottom.

3. Wash IJs with 1 mL sterile Ringer’s solution. Repeat twice.

4. Centrifuge at 13,000 rpm for 3 min, discard final Ringer’s solution and add 20 µL of phosphate-buffered saline (PBS). Crush nematodes using a sterile motorized homogenizer.

5. Inoculate 10 µL of homogenate on NBTA, incubate at 25–28°C for 24 h. Collect identified Xenorhabdus or Photorhabdus colonies and subculture in Luria-Bertani (LB) broth. Before storing, identify bacteria (method described below) and verify (phase) on NBTA. Store in 25% glycerol as stock at –80°C.

5. Characterization of bacteria (and variants)

Xenorhabdus and Photorhabdus belong to the same family, have similar lifestyles, and share most phenotypic characteristics. The culture and phenotypic features of their Phase-I forms need to be correlated with molecular analysis of DNA gene sequences to ensure accuracy in identification and reduce discrepancy in classification (Stackebrandt et al., 2002; Hazir et al., 2003a; Adams et al., 2006).
Bacteria in the *Xenorhabdus* genus are gram-negative motile rods with peritrichous flagella and motile. Phase-I forms absorb dye on NBTA and MacConkey agar thus forming dark blue convex, umbonate and mucoid colonies on NBTA that swarm and red colonies on MacConkey. They produce enzymes like lecithinase, proteases, and various other respiratory enzymes. *Xenorhabdus* spp. are catalase negative (no bubbles will form if a colony is transferred to a slide with H₂O₂) and they do not reduce nitrate to nitrite, a trait that distinguishes them from other enteric bacteria. Phase-I variants ferment various carbohydrates like glucose into acid but with no gas (Boemare, 2002; Boemare and Akhurst, 2006). In contrast, Phase-II forms do not absorb dye and do not produce antibiotics or protein inclusions; some forms may be mobile as Phase-I (Boemare, 2002).

*Photorhabdus* spp. are also gram-negative rods with peritrichous flagella that are motile. Phase-I form dark...
green or yellow, convex, umbonate, mucoid and gummy colonies on NBTA, red colonies on MacConkey agar, and hemolysis zones around colonies streaked on sheep or horse blood agar. They are catalase positive but oxidase negative and cannot reduce nitrate to nitrite. The bacteria ferment various carbohydrates (glucose, fructose, maltose, etc.) without gas production. Photorhabdus have the ability to bioluminescence and produces vibrant pink, red, orange, green or yellow pigmented colonies. Phase-II forms are less pigmented or produce pigmented colonies different from Phase-I form. They also do not absorb neutral red dye on MacConkey agar, or produce antibiotics (Brunel et al., 1997; Boemare, 2002; Boemare and Akhurst, 2006).

Molecular analysis of 16S rRNA and such like housekeeping genes can help distinguish between both bacteria but are now believed to be inept in detailing with the specification of these bacteria; therefore, detailed and accurate reclassification methods like whole genome sequencing or concatenation and analysis of multiple DNA loci can help further specialize congeneric and homogeneous bacteria, especially in Photorhabdus, thereby resolving discrepancies at the subspecies level (Brunel et al., 1997; Forst et al., 1997; Adeolu et al., 2016; Machado et al., 2018).

5.1. Phenotypic assessment of bacteria (biochemical tests and enzymatic activities)

Besides phenotypic assessments of Gram stain and colonial morphology, various rapid biochemical and enzymatic tests can provide discriminable characteristics of Xenorhabdus spp. and Photorhabdus spp.

Catalase test: This test is used to identify catalase-producing bacteria by bringing the organism into contact with hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) which will be broken down into water and oxygen. Enteric bacteria, except Xenorhabdus, are all catalase positive.

Dispense 1–2 mL of 3% \( \text{H}_2\text{O}_2 \) into a test tube. Collect a single colony from a culture that is not more than 24-h-old and immerse in \( \text{H}_2\text{O}_2 \). Observe for active bubbling which indicates positive for catalase production (Cheesbrough, 2006).

Indole test: This test is to assess the ability of bacteria to break down tryptophan amino acid into acid using tryptophanase enzyme. Grow Xenorhabdus or Photorhabdus in tryptophan broth for 24 h. Add a few drops of Kovac’s reagent to culture. Observe for color change; presence of a red or red-violet color shows positive result (Collins et al., 2004).

Carbohydrate fermentation: This test is used to assess whether a bacterium has the ability to use certain types of carbohydrates as an energy source. Acid, gas, or both are produced if bacteria can ferment the tested carbohydrates. Inoculate a pure culture of bacteria into test tubes with fermentation broths of different carbohydrates (glucose, lactose, sucrose, mannitol, etc.), a pH indicator (phenol red) and a Durham tube for gas collection. Incubate at 28 °C for 24 h. Color change of phenol from red to yellow and/or gas in Durham tubes indicate positive results (Collins et al., 2004).

Lecithinase activity: The purpose of this test is to check for the ability to produce lecithinase enzyme which digests lecithin protein found in most animal tissues. Streak a loopful of bacteria on egg yolk agar and incubate for 72 h. Lecithinase activity is confirmed by opaque zone around inoculum (Collins et al., 2004).

Casein hydrolysis: This test is used to identify protease-producing bacteria by assessing their ability to degrade casein protein. Inoculate bacteria in a straight line on skim milk agar and incubate for 3 days. Proteolysis is indicated by clear zone around inoculum (Collins et al., 2004).

Motility: Spot inoculate 5 µL of bacteria culture broth in the center of motility agar (LB broth supplemented with 0.3% agar). Incubate at 28 °C for 24 h and check for swarming (Easom and Clarke, 2008; Cimen, 2013).

Bioluminescence: Grow Photorhabdus on any suitable agar medium [LB, protease agar or Tryptic soy broth (TSB), etc.] for 24–72 h depending on species or isolate. Or infect G. mellonella with an overnight bacteria suspension and incubate for 24–72 h. Bioluminescence activity can be seen with the naked eye under totally dark conditions. Measure luminescence activity using a bioluminescence imaging like IVIS Spectrum (Stock and Goodrich-Blair, 2012).

Antibiotic production: Inoculate a loopful of Xenorhabdus or Photorhabdus from LB or TSB agar to broth medium and incubate overnight. Spot inoculate 5 µL of overnight culture on Mueller Hinton agar and incubate at 28 °C for 3–5 days; place Petri dishes under UV light for 5–10 min to kill the bacteria. Separately prepare Micrococcus luteus broth culture as an indicator, by adding 1% overnight bacterial culture to LB or TSB with 0.75% agar and mix homogenously, then overlay suspension on spot inoculated Xenorhabdus or Photorhabdus. Incubate at 30 °C overnight and check for zone of inhibition (Donmez Ozkan et al., 2019).

Pathogenicity: Incubate LB broth with pure cultures of Xenorhabdus or Photorhabdus for 24 h at 28 °C. Wash this overnight culture in PBS, then adjust suspension to OD_{600} to 1 (4 × 10^6 cfu/mL) (cfu = colony-forming units). Dilute 10 times to 2 × 10^6 cfu/mL. Inject 10 µL of diluted suspension into the hemocoel of a G. mellonella larva. Infect at least 10 larvae and as negative control inject with equal amount of PBS. Store larvae at room temperature (23–24 °C) in the dark. Check for mortality 48–72 h later (Easom and Clarke, 2008).

Oxidase test (Cytochrome oxidase): The principle of this test is to assess if a bacterium produces cytochrome
c oxidases, an enzyme of the bacterial electron transport chain. Add 2 or 3 drops of oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) to a piece of filter paper placed on a clean Petri dish. Smear a single colony of bacteria on filter paper. A deep blue color change on inoculated area on paper indicates positive results (Collins et al., 2004, Cheesbrough, 2009).

Lipolytic activity: This test is to check lipase enzyme production. Inoculate bacteria on nutrient agar supplemented with Tween-80, -60, or -20 (0.5% v/v). Incubate for 48 h at 28 °C. Lipase producing bacteria will have a clear zone around their colonies (Cimen, 2013).

Analytical profile index (API): The API identification systems (bioMerieux Inc. Hazelwood MO) are miniature biochemical test kits that can be used for the identification of bacteria by evaluation of enzymatic activity on or the fermentation of various dehydrate substrates on strips. They are easy to use and provide rapid results. For Xenorhabdus and Photorhabdus, API 20E or API Rapid 20E can be used for identification according to manufacturer’s instructions (Aslanzadeh, 2006).

5.2. Genomic DNA extraction from bacteria (Maniatis et al., 2012)

DNA can be extracted using commercial kits but if kits cannot be procured, the protocol described below can be used:

1. Grow a pure culture of bacteria in LB broth to obtain overnight culture. Pellet at 12,000 rpm for 1 min, decant supernatant to collect pelleted bacteria cells.
2. Resuspend cells in 600 µL of lysis solution (9.34 mL TE buffer, 600 µL of 10% SDS, 60 µL of protease K, 20 mg/mL) and vortex.
3. Add 5 µL of RNase. Incubate 37 °C for 30 min.
4. Add an equal volume of phenol/chloroform. Mix by inverting tube until two phases mix.
5. Centrifuge at 12,000 rpm for 5 min. Transfer upper phase to a new tube without disturbing the white protein layer of phenol/chloroform interface.
6. Add an equal volume of chloroform, mix well and centrifuge for 5 min at 12000 rpm. Transfer aqueous phase to a new tube.
7. Precipitate DNA by adding cold isopropanol (three times the volume of aqueous phase collected in step 5). Incubate at −20 °C for 30 min.
8. Centrifuge at 12,000 rpm for 15 min at 4 °C. Discard supernatant and rinse DNA with 70% ethanol.
9. Centrifuge for 2 min at 12,000 rpm. Discard supernatant and air dry for 5–10 min or overnight until you cannot smell ethanol any longer. Resuspend DNA in sterile distilled water or TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). Store until further use.
10. For PCR amplification, check subsection 1.2.2.

Table 8 lists some 16S primers used in the amplification of DNA in molecular identification of Xenorhabdus and Photorhabdus.

5.3. Obtaining bacterial growth culture, cell-free supernatant, and pellet

In recent years, there has been a multitude of studies investigating the biological activities of metabolites produced by Xenorhabdus and Photorhabdus. These studies used the bacterial growth culture, cell-free supernatant or pellet to suppress plant pathogenic fungi (Bock et al., 2014; Shapiro-Ilan et al., 2014b; Hazir et al., 2016; Chacon-Orozco et al., 2020; Cimen et al., 2021), bacteria (Purgani et al., 2008; Fodor et al., 2010; Donmez-Ozkan et al., 2019), mites (Bussaman et al., 2012; Eroglu et al., 2019; Cevizci et al., 2020; Incedayi et al., 2021), plant-parasitic nematodes (Kepenekci et al., 2016, 2018), insects (Da Silva et al., 2013; Wagutu et al., 2017; Vitta et al., 2018; Shah et al., 2021), etc.

The following method can be used to obtain the bacterial growth culture, cell-free supernatant, or pellet:
1. Inoculate bacteria from stock cultures onto LB (Merck, Darmstadt-Germany) agar plates at 28 °C for 24 h.
2. Transfer a single colony from these plates into flasks containing 10 mL sterile TSB (Merck, Darmstadt, Germany) and incubate the flasks at 30 °C and 150 rpm for 24 h. Measure the cell density of the bacterial culture using a spectrophotometer and adjust turbidity at OD_{600} as needed. Use this bacterial growth culture for experiments.
3. To obtain cell-free supernatant transfer this bacterial broth culture to Falcon tubes and centrifuge at 10,000 rpm at 4 °C for 20 min. Then filter the supernatant.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primers (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPl</td>
<td>ACGTTACCTTGTACGACTT</td>
<td>Weisburg et al., 1991</td>
</tr>
<tr>
<td>fDl</td>
<td>AGAGTTTGATCTGGCTCAG</td>
<td>Weisburg et al., 1991</td>
</tr>
<tr>
<td>16SP1</td>
<td>GAAGAGTTGGATCGATGCTC</td>
<td>Tailliez et al., 2006</td>
</tr>
<tr>
<td>16SP2</td>
<td>AAGGAGGTGATCCAGCGCAG</td>
<td>Tailliez et al., 2006</td>
</tr>
<tr>
<td>Universal 1 (U1)</td>
<td>ACG CGT CGA CAG AGT TTG ATC CTG GCT</td>
<td>James, 2010; Relman, 1993</td>
</tr>
<tr>
<td>Universal 2 (U2)</td>
<td>CGC GGA TCC GCT ACC TTG TTA CGA CTT</td>
<td>James, 2010; Relman, 1993</td>
</tr>
</tbody>
</table>
through a 0.22-μm Millipore filter. These supernatants can be stored at 4 °C for up to 2 weeks prior to use in the experiments (Hazir et al., 2016, 2017; Cimen et al., 2021).

4. The remaining pellet at the bottom of the Falcon tubes after centrifugation can be resuspended in sterile Ringer’s solution or physiological saline and turbidity can be adjusted as needed (Vitta et al., 2018).

Bacterial growth culture or cell-free supernatants can be incorporated into agar plates or liquid media to assess biological activity.

6. Enumeration of EPNs
When the number of nematodes to be enumerated is low (1–200), they can be counted directly after dispensing them onto a suitable arena using a microdispenser. However, if the suspension contains a greater concentration of nematodes, then dilution will be required (Stock and Goodrich-Blair, 2012).

Procedure:
1. Place a container (e.g., plastic beaker) with an aqueous suspension of IJs on a magnetic stirrer with a stir bar. Stir homogenously.
2. Collect 1 mL of suspension and transfer to a 9-cm Petri dish with 9 mL of water. Make grid line on the bottom of dish to help with direction and prevent recounting of IJs (Figure 11). Count the nematodes using a hand tally counter under a stereomicroscope.
3. Alternatively, a nematode counting slide can be used (Figure 11). Usually, 2 mL of suspension are loaded onto the slide and the area above the grid is 1 mL. Count all nematodes on the grid. If a nematode is on the grid line, the convention is that nematodes on the right or top side of the grid box are counted and those on the left or bottom side are not counted (this avoids counting the same nematode twice).
4. Repeat counting thrice and multiply the average number of IJs by the total volume in container.
5. Add more water or concentrated IJs and remove excess water and repeat until desired concentration is achieved.

7. Virulence bioassay of EPNs
Virulence is the disease-causing power of a pathogen (Shapiro-Ilan et al., 2005a). For EPNs, virulence is most often assessed based on host mortality. The principle of this procedure is to expose insects to IJs and determine host mortality after a standard period. Its objectives may be to (1) determine the baseline susceptibility of the test insects when EPN IJs are applied, (2) screen IJs to compare the compatibility and effectiveness of certain nematode species or strain against a target host for a given biological control project, (3) screen for relative susceptibility of different hosts or host stages, and (4) assess the quality level of IJs after a storage, shipment or after field application. There are various procedures for assessing EPN virulence using different experimental arenas and substrates (e.g., soil, sand, filter paper, etc.) (see Sims et al., 1992; Westerman, 1994; Ricci et al., 1996 and references therein for more information on the diversity of virulence approaches). A general approach is indicated below.

The procedure described below simulates the natural soil environments of nematodes (Mauleon et al., 1993).
1. Pour 0.5–1 g of sterile sandy soil to each well of a 24-well culture plate. Use sand instead of filter paper to mimic practical soil environment; however, filter paper may in some cases be more appropriate than sand or soil (e.g., arguably if testing against a wood boring insect). The size of arena (plastic containers, small pots, etc.) can vary according to the size of the insect host.
2. Prepare and adjust freshly obtained IJs to the desired concentration. Dispense aqueous IJ suspension to well. When soil or sand is used as substrate, moisture levels should be adjusted to field capacity.
3. Add desired target insect of a given age or stage to each well.
4. Cover the wells with tape to prevent escape of the insect and cover with lid (Figure 12). Place plate in plastic bags to prevent moisture loss and store at appropriate temperature in the dark. Generally, room temperature (23–24 °C) is used for many species, but this can be varied depending on the goals of the assay, e.g., if expecting to apply nematodes when soil temperature is 20 °C then the test should be run at 20 °C.
5. Monitor and record mortality at the 24-h intervals.
6. All bioassays should contain at minimum three full replicates (preferably four or more) and the entire assay should be repeated in time (resulting in a minimum of two complete trials).
8. Penetration bioassay of EPNs (Mauleón et al., 1993; Glazer and Lewis, 2000)

Invasion rate or penetration efficiency measures the number of IJs entering a host. It is a good measure of relative nematode fitness or quality.

1. Add 200 IJs/50 µL to each well of a 24-well plate. Each well should have 0.5 g of sterile and air-dried sandy soil.

2. Add G. mellonella larva (average weight of 200–300 mg) to each well and seal with nonadhesive tape and plate lid to prevent escape of insects. Prepare at least 10 replicates or wells.

3. Incubate setup at room temperature (23–24 °C) for 48 h. Rinse and place dead insects of the same treatment in Petri dishes for another 24 h to allow nematodes to molt (this will make visualization of the nematodes easier).

4. Collect each insect cadaver and place into clean glass Petri dishes. Add fresh Pepsin solution (see Appendix for recipe). Remove the head of the dead insect using a dissecting scissors. After the head of the cadaver is cut, the inner part is turned inside out with the help of a thick L-shaped metal rod and the tissues are separated from the cuticle. The insect tissue is then disintegrated into small pieces to increase contact with the pepsin solution. Incubate Petri dishes at 37 °C in a rotary incubator (100–120 rpm) for 1–2 h to allow for insect tissue solubilization. Generally, a heterorhabditid-killed insect needs a longer time to dissolve their tissues compared to a steinernematid-killed insect. Some researchers forgo the pepsin step if they feel confident the nematodes inside the cadaver can be determined accurately without it (it can depend on the host insect and the researcher’s experience).

5. Count the number of J4 and adult nematodes using a stereomicroscope at 50× magnification. Calculate the penetration efficiency as a percentage using the formula:

\[
\text{Penetration efficiency} = \left( \frac{\text{Average number of IJs in cadavers} \times 100}{\text{Number of IJs added to well}} \right)
\]

9. In vivo mass rearing of EPNs for small-scale application and laboratory studies

One of the benefits of Steinernema spp. and Heterorhabditis spp. as biocontrol agents is that they can be easily mass produced, formulated and marketed (Abate et al., 2017). For EPNs to be used effectively, large amounts of IJs need to be economically produced. EPNs are produced using in vivo or in vitro (solid or liquid fermentation) methods (Ehlers and Shapiro-Ilan, 2005). The bulk of commercially available EPNs is produced using in vitro liquid culture methods; a few small-sized companies still use in vivo methods.

In vivo methods are most used and preferred for small-scale use such as in research laboratories or smaller commercial companies. For in vivo production, the White trap is the most common method for harvesting the IJs – see the description above (in the baiting method). There are in vivo rearing methods of scaling up including Lotek (described below) and also a Tenebrio-based method described in Shapiro-Ilan et al. (2016a). Thus, in vivo production, a living insect host functions as a bioreactor that nematodes infect, kill, and multiply in. Afterwards, by taking advantage of the natural migration of emerging IJs away from the host-cadaver, IJs can be collected in trays or shelves that resemble the White trap system (Dutky et al.,...
1964; Gaugler et al., 2002). *Galleria mellonella* is mostly used as a host because it is widely available, easily reared, highly susceptible and an excellent host for nematode reproduction, though other hosts such as *Tenebrio molitor* (mealworms), *Plutella xylostella* (diamondback moth), *Spodoptera litura* (cotton leafworm), *Crocidolomia binotalis* (cabbagehead caterpillar), etc., can be used. In vivo production is suitable for laboratory work and/or small-scale field test because it requires relatively simple technology, low capital outlay, and produces of high quality IJs. Depending on nematode species and size, and host size, a single insect larva yields average 20,000 to 500,000 IJs (Dutky et al., 1964; Bedding, 2006; Shapiro-Ilan et al., 2012, 2016a). However, host density and inoculation rate can affect IJ yields (Han et al., 1992, 1993; Shapiro-Ilan et al., 2002). A complete description of in vivo production methods as well as approaches for scale-up (e.g., for niche-level commercialization) can be found in Shapiro-Ilan et al. (2016a).

**Procedure** (Lotek method) (Gaugler et al., 2002; Shapiro-Ilan et al., 2016a)

1. Dip a 30 × 26 × 4 cm perforated aluminum tray holding 500 insects into an aqueous suspension with 2.1 × 10^4 to 8 × 10^5 IJs/mL of EPNs. Insects can be restrained from leaving tray using a 20-gauge perforated aluminum sheet over the tray. Dimensions and size of tray can be adjusted according to one’s needs. This method (Gaugler et al., 2002) guarantees >95 infection of hosts which is 15% more compared to the pipette method (Dutky et al., 1964).

2. Transfer inoculated tray to a dark humidity-controlled chamber or room. Trays can be stacked on top of each other to maximize space and prevents insect escape (upper most tray must be covered). An aquarium pump can be used to deliver air, humidifier to maintain moisture and a water trap to eliminate excess water. After 2–3 days collect live insects as well as nonputrefying cadavers. Insects killed by nematodes would not putrefy or have a bad odor. Discard putrefying cadavers.

3. After 4–5 days, place tray with dead larvae underneath pipes with a water supply. Deliver water through a mist nozzle at a rate of 37 mL/min. Misting can be set at 3-min duration at the 6-h intervals for each tray. Mist delivered causes nematode emergence as well as rinses nematodes into an aerated collection tank.

4. IJs can be separated from wastewater and concentrated using vacuum filtration which would reverse suction and remove the wastewater. Centrifugation can also be used instead of a vacuum filtration. IJs from the collection tank are transferred into a tank with a reverse suction on an air pump which will pull away the water; an aquarium stone can be used to collect the nematodes (water strains through the stone). The remaining paste-like substance containing the IJs can be spread onto sponge or formulated with a suitable carrier like vermiculite, activated charcoal, polyacrylamide gels, clay, alginate gel, water dispersible granules, etc.

**10. In vitro mass rearing of EPNs**

Glaser (1931) was the first to attempt to produce EPNs outside of insect host and since then, several studies have successfully produced nematodes on different types of solid or liquid artificial medium to enhance commercialization and large-scale use (Gaugler and Han, 2002; Ehlers and Shapiro-Ilan, 2005; Bedding, 2006). Bedding (1981, 1984) made a breakthrough in solid-phase mass-production by growing nematodes in sterilized chicken offal medium entrenched in sponges in plastic bags with the symbiotic bacteria. He recovered the nematodes by washing them out of the sponges. There is no need for high technology inputs and large investments in in vitro solid rearing equipment; however, it has several disadvantages: it is labor-intensive, there is an instability in the production process which is quite long (2–3 weeks), and frequent contamination of rearing medium can occur (Ravensberg, 2011).

10.1. Establishing monoxenic cultures (Lunau et al., 1993)

The objective of establishing monoxenic cultures is to obtain pure cultures of EPNs and their respective *Xenorhabdus* spp. and *Photorhabdus* spp. for mass production. Establishing a monoxenic culture is a prerequisite to in vitro production methods.

**Procedure:**

1. Infect last instar of *G. mellonella* with nematodes in Petri dishes as previously described in section 4. Dissect infected larvae usually after 3–4 days for *Steinernema* and 5–7 days for *Heterorhabditis*. Collect preferably 100 gravid females (*Steinernema*) or hermaphrodites (*Heterorhabditis*) by tearing insect cadaver and homogenizing pieces by pipetting using a Pasteur pipette. Wash away insect remain from nematodes. If there are J1 and J2 stages with adults, sieve out adults using a 50-mm sieve. Recollect adults in a glass tube. Add small pieces of a razor blade to tube and vortex until suspension is turbid to release eggs from the adult bodies. Sieve again using a 50-mm sieve and collect filtrate with eggs. Centrifuge for 1 min at 2000 rpm and remove supernatant. Add Ringer’s solution and centrifuge again until suspension is clear.

2. Discard supernatant and surface sterilize eggs by adding 1 mL sterilization solution [0.5 mL sodium hypochlorite (12%), 1.5 mL NaOH (4 mol), 10 mL distilled water]. Mix gently for 4 min and centrifuge for 2 min at 2000 rpm. Do not centrifuge for more than 6 min otherwise, too many eggs will die. Remove supernatant, fill up with sterile YS-medium and centrifuge again. Repeat washing with YS-medium once.
Alternatively, rather than obtain the gravid females and eggs from infected hosts, eggs can be obtained from gravid females grown on bacterial lawns. Surface sterilize IJs in Hyamine as described above and then apply the nematodes onto nutrient agar plates with a lawn of pure bacteria. The plates are then observed daily for the appearance of gravid females or hermaproditodes. Gravid females are washed off in saline solution; the process can be accelerated by gently agitating such as using a pipette (Lunau et al., 1993). Once gravid females are lysed centrifuge and wash as indicated above.

3. Transfer eggs to sterile cell wells with 300 mL YS-medium or other transparent medium. Incubate for 72 h. Check for contamination. The medium should be clear and show no turbidity.

4. Two days after egg isolation inoculate 20 mL of YS-medium in an Erlenmeyer flask with primary variant of X. nematode strain. Incubate in a rotary incubator at 200 rpm and 25 °C in the dark for 24 h.

5. Then inoculate WOUTS-Agar in 6-cm Petri dishes with 2 drops of bacterial suspension from YS-medium and approximately 50–100 J1 from sterile cell wells. Try to minimize the transfer of liquid and if J1 swim on the agar surface restart this process as these cultures are usually unsuccessful.

6. Seal plates with parafilm and incubate at 25 °C. Check nematode development daily. Transfer IJ stage into subsequent cultures. Cultures can be stored at 4–7 °C and 25 °C in the dark for 24 h.

To obtain axenic nematodes do not expose nematodes to bacteria in WOUTS-Agar. EPNs can develop to the 3rd stage without their symbiotic bacteria and can penetrate insect host; however, their virulence and the number of emerging new generation IJs will be lower (Leite et al., 2016).

10.2. Rearing of EPNs in solid medium

10.2.1. Agar lawns

Below are solid culture media on which EPNs can be produced. Surface sterilized or monoxenic IJs are added a day after bacteria have grown; nematodes move around in agar, feed on bacterial lawns and reproduce. For short-term culturing surface sterilized IJs can be used but there is potential to introduce contaminants that are lodged behind the second cuticle of the IJs. Once the production cycle is complete IJs can be washed off the plates and washed repeatedly, or the Petri dishes can be transferred to White traps for harvesting (Stock and Goodrich-Blair, 2012).

**Dog-food agar**

- Dry dog food: 100 g
- Distilled water: 500 mL
- Agar: 1%

**Procedure:** Add the grinded dog food, water, and agar in a 1-L Erlenmeyer flask. Autoclave at 121 °C for 15 min. Pour into 6-cm Petri dishes.

**Liver-kidney agar**

This an enriched medium that is made up of pureed kidney and liver.

**Procedure:** Blend chopped-up pieces of kidney (50 g) and liver (50 g), 2.5 g NaCl, 500 mL of water and 7.5 g agar into a blender. Transfer puree to a larger container (1-L Erlenmeyer flask). Autoclave at 121 °C for 15 min. Allow to cool then pour into 6-cm Petri dishes (Poinar and Thomas, 1966; Stock and Goodrich-Blair, 2012).

**Lipid agar**

**Procedure:** Add 8 g of nutrient agar, 5 g of nutrient broth and 5 g of yeast extract to 890 mL of double distilled water in a 2-L Erlenmeyer flask. Add 10 mL of MgCl₂·H₂O (0.2 g/ml) and 1.5% agar, then mix thoroughly. Autoclave at 121 °C for 15 min. Further, add 4 mL of corn oil and 96 mL of corn syrup and mix homogeneously. Dispense aseptically into Petri dishes (Stock and Goodrich-Blair, 2012; McMullen and Stock, 2014).

**Nematode growth medium (NGM)**

**Procedure:** Dissolve 3 g of NaCl, 2.5 g of peptone, 20 g of agar in 1 L distilled water in a 2-L Erlenmeyer flask (for liquid medium do not add agar). Autoclave at 121 °C for 15 min. Allow to cool by keeping flask in a water bath at 55 °C. Add 1 mL of cholesterol (5 mg/mL in ethanol, do not autoclave), 1 mL of 1 M MgSO₄, 1 mL of 1 M CaCl₂, and 25 mL of 1 M (pH 6.0) KPO₄; mix after each addition. Dispense aseptically into Petri dishes (if possible, use an automated plate pourer to dispense a constant amount of agar; this reduces the need for refocusing the microscope when switching from one plate to another). Store NGM plates at 4 °C until use (Stiernagle, 1999).

10.2.2. Nematode production in sponge

Besides agar media, nematodes can be produced in three dimensional cultures with nutrients added to polyether-polyurethane sponge —this provides adequate ventilation and interstitial space for movement— and preinoculated with the primary form of symbiotic bacteria, which convert the culture medium into a suitable medium for nematode development and reproduction. Bedding (1981, 1984) used thinly coated crumbled polyurethane foam sponge with poultry offal homogenate. Due to unreliable results and difficulty in standardization, Bedding's approach has been improved upon and several other media consisting of yeast extract, corn oil, corn starch, dried egg solids, etc. (Wouts, 1981; Han et al., 1992, 1993; Leite et al., 2017).

**Procedure:**

1. Grow symbiotic bacteria in 250 mL-Erlenmeyer flasks with 50 mL of TSB + Y medium (4% tryptic-soy-broth + 0.5% yeast extract) or LB medium for 2 days at 25 °C and 280 rpm.
HAZIR et al. / Turk J Zool

2. Transfer 1 mL of culture (5 x 10⁹ cells) into another 250 mL-Erlenmeyer flasks with 2 g of sponge flakes and 50 mL liquid medium (23 g yeast extract, 6.25 g egg yolk, 6.25 g egg white, 25 g glucose, 5 g NaCl, 40 g peanut oil, 2 g agar and 1 L distilled water) (Leite et al., 2017). Another improved medium containing 15% soy flour, 5% wheat flour and corn oil, 1% yeast extract and egg yolk flour, and 10% crumbled polyether polyurethane foam can be used (Han et al., 1992, 1993).

3. Add nematodes (final concentration of 5000 IJs/mL) and incubate at 280 rpm and 25 °C.

4. Harvest nematodes from culture after 2–5 weeks by washing them out of the sponge in washing machines and then by separating the IJs via sedimentation, centrifugation, or sieving or by using centrifugal sifters (Ehlers and Shapiro-Ilan, 2005).

10.3. Rearing of EPNs in liquid medium

The principle is to provide a liquid growth medium with nutrients for bacteria on which nematodes will subsequently feed upon. Commercial production might require automated machines like conventional bioreactors, airlift, and internal loop bioreactors, etc., and technical know-how to maintain the equipment, but with the procedure described below in vitro liquid culture can be used to produce nematodes in flasks under laboratory conditions (Ehlers et al., 1998; Ehlers, 2001; Peters et al., 2017).

Procedure:

1. Extract symbiont bacteria of nematode (as described above) to be mass-produced from the hemolymph of G. mellonella. Culture bacteria on NBTA overnight then transfer single colonies to a suitable broth culture to establish stock.

2. Prepare and sterilize a nematode liquid culture medium (LCM) (with 10 g nutrient broth, 10 g tryptic soy broth, 5 g yeast extract, 5 g casein peptone, 0.35 g KCl, 0.21 g CaCl₂, 5.0 g NaCl, 30 mL vegetable oil) in a 2-L Erlenmeyer flask or larger container of choice depending on magnitude of production. Add 0.2% (v/v) of a Silicon emulsion to prevent foaming. Inoculate an overnight culture of bacteria into LCM medium. Several factors affect yield including media, inoculum age, and physical parameters such as aeration rate, agitation rate, etc. (see Leite et al., 2016, 2017).

3. Transfer newly obtained nematode IJs after the 24-h monoxenic postculture of bacteria. Incubate at 180 rpm and 25 °C in a rotary incubator (Ehlers et al., 1998).

After production, quality control of the nematodes (survival and virulence) produced is required before and after formulation. Sand-well assay (five-on-one assay for heterorhabditids and one-on-one assay for steinernematids) can be used as a standard quality control tool to assess IJ virulence. Other quality control parameters like assessment of energy reserves (dry weight or total lipid content) can also be assessed (Grewal, 2002; Kagimu et al., 2017).

11. Genetic improvement and stability

11.1. Strain improvement

Several approaches can be used to enhance biocontrol potential in EPN strains including discovery, directed selection, and hybridization (Shapiro-Ilan et al., 2017). These approaches can lead to improved biocontrol efficacy. Transgenic approaches (genetic engineering) may also have a potential for improving EPN biocontrol efficacy (Gaugler et al., 1997), yet methodology for genetic manipulation is beyond the scope of this paper.

Discovery simply entails finding new strains with superior biocontrol abilities. For example, following an intensive survey, S. riobrave strains with superior virulence to the citrus weevil, Diaprepes abbreviatus, were discovered; additionally, a mixture of strains was found to be the most virulent (Stuart et al., 2004). For procedures on collecting new strains of EPNs refer to subsection 2.2.2. (Collection and extraction of EPNs from soil).

Directed selection is used to enhance specific traits. In EPNs, selection has been used to improve host-finding (Gaugler and Campbell, 1991), dispersal (Bal et al., 2014a), nematicide resistance (Glazer et al., 1997) and other traits. When implementing directed selection, one should consider cost-benefit tradeoffs and the potential of trait reversion when selection pressure is removed (Shapiro-Ilan et al., 2017). Prior to implementing a selection regime, a diverse foundation population, made up of a collection of isolates, should be established (Gaugler et al., 1989). Subsequently, the foundation population is exposed to selection pressure for the desired trait (conceivably increase the selection pressure at each round). It can take 20 rounds of selection or more to produce substantial increases in the targeted trait (Gaugler and Campbell, 1991). To ensure success in selection approaches, the heritability of the desired trait can be estimated beforehand (Glazer et al., 1991).

Hybridization is accomplished through controlled crosses and subsequent screening of progeny for superior biocontrol traits; the approach has been implemented for both heterorhabditids (Shapiro et al., 1997) and steinernematids (Shapiro-Ilan et al., 2005b). Methods for hybridization are as follows:

1. Monoxenic cultures of the strains to be crossed are established based on the procedure described in subsection 13.1. Selection of the best strains to consider crossing (based on the merits of each) can be determined through bioassay and an analysis of pluses and minuses (Shapiro-Ilan et al., 2003c).
2. Early juvenile nematode stages are transferred individually to a fresh lawn of bacteria on 60 mm of nutrient agar. Early juvenile stages are chosen to ensure mating had not yet taken place. The bacteria chosen for the hybrid strain should be based on biocontrol capabilities of the strain (reproduction, virulence). If unsure, the hybridization procedures can be done on both bacteria lawns separately and the merits of final populations can be determined after.

3. Once nematodes mature to adulthood, crosses are then set up with five males from one strain and one female of the other; reciprocal crosses are also implemented (males and females from each strain reversed). At least 10 replicate crosses of each should be conducted. This approach is simple for steinernematids with only amphimictic forms. To ensure mating when crossing heterorhabditids, marker mutations may be used to assist (Shapiro et al., 1997).

4. Progeny populations are then reproduced (in vivo or in vitro), analyzed for specific traits and biocontrol potential in the lab (Shapiro-Ilan et al., 2005a) and verified further in greenhouse and field applications.

11.2. Strain stability

Beneficial traits of a suitable EPN strain such as virulence, environmental tolerance, reproductive capacity, and host finding can deteriorate after repeated subculturing as well as from inbreeding, drift, inadvertent selection, or arise from nongenetic factors, such as disease or nutrition. This might jeopardize biocontrol efforts; hence it is critical to secure the genetic stability of this population (Bai et al., 2005; Bilgrami et al., 2006; Chaston et al., 2011; Shapiro et al., 1996; Shapiro-Ilan et al., 2017) Trait deterioration can be avoided by cryopreservation, but several shortcomings exist with that approach such as genetic bottlenecking or potential for mechanical failure (Bai et al., 2005; Shapiro-Ilan et al., 2014b).

Trait deterioration can be deterred through the creation of homozygous inbred lines (Bai et al., 2005). The homozygous lines are inherently impervious to inbreeding depression and inadvertent selection. For heterorhabditids, diverse inbred lines are automatically created in liquid culture because heterorhabditids are unable to mate under these conditions (Anbesse et al., 2013); but this procedure is not applicable to steinernematids. To directly obtain stable strain(s) that have high biocontrol potential, an array of inbred lines is created (>15 lines), and subsequently the lines showing the highest levels of biocontrol traits are chosen for application and commercialization (Shapiro-Ilan et al., 2014b). The following is a procedure to create inbred lines (Glazer et al., 1991):

1. IJs are surface sterilized (e.g., using 1% hyamine) and inoculated onto 5-cm diam. Petri dishes containing nematode growth media (NGM) and preseeded with the nematode’s symbiotic bacteria. NGM is recommended because of its translucent nature; nematode development can be easily observed.

2. For heterorhabditids, single J4 nematodes are then transferred individually to new plates. It is best to start with more lines than desired as some will be lost over time (50 plates is a good number for each generation). The nematodes then reproduce hermaphroditically and the process is repeated (J4 or virgin female moved individually to new plates). This should be repeated for at least seven generations to achieve >95% homozygosity.

3. For steinernematids inbred lines can be created using the same procedures described above except through sibling mating rather than hermaphroditic reproduction. Consequently, more generations (>20) are required to establish homozygosity (>95%) (Wang et al., 2020).

12. Storage of EPNs

Most laboratory work, commercial products or sample sharing is done using the IJ stage because of its resistance to unfavorable conditions, tolerance to a wide temperature range, and ability to be refrigerated at temperatures between 4 °C and 10 °C for several months to a year in aqueous suspensions or formulated in various dry or semiliquid substrates (Campbell and Gaugler, 1993; Kaya and Gaugler, 1993; Koppenhöfer and Fuzy, 2003, Gungor et al., 2006). Temperature, oxygen level, and moisture are the most important parameters that can affect nematode survival (Glazer, 2002; Koppenhöfer and Fuzy, 2003; Stuart et al., 2015).

12.1. Aqueous suspensions

IJs can be stored in distilled water in various containers like tissue culture flasks or Tetra Pak containers (Figure 6). This method requires refrigeration at an optimum IJ concentration of 1000 to 5000 IJs/mL (Gulcu and Hazir, 2012; Stock and Goodrich-Blair, 2012). In larger tanks, IJs can be stored at concentrations up to 100,000 IJs/mL but in such situations these tanks need to be aerated with an aquarium air pump.

Procedure:

1. Assess the activity of freshly harvested nematodes from White traps. Live IJs actively move or maintain a J-shaped position. Probe straight nematodes to confirm if alive or dead (this procedure can also be used to determine proportion of live/dead nematodes for other applications such as in bioassays). Rinse and sieve out live IJs if there
are cadaver tissues, dead nematodes and/or other live nematode stages present in the suspension.

2. Adjust suspension to concentration of 1000 to 3000 IJs/mL. Lower concentration to 500 to 1500 IJs/mL for larger nematodes like *S. longicadum* and *S. glaseri*. Pour suspension into a flask and make sure the depth of water is less than 1 cm.

3. *Heterorhabditis* IJs tend to form clumps or rosettes in water. Add a few drops of sodium bicarbonate solution (1 g NaH$_2$CO$_3$/50 ml of H$_2$O). This solution has no negative effects on the IJs (Woodring and Kaya, 1988).

4. Place flask flatly in incubators or refrigerators at temperatures between 4 °C and 15 °C depending on isolates. Some species or isolates from places with warmer conditions die when incubated at ≤10 °C; these IJs can be stored at 15–20 °C. Therefore, storage temperature should be selected according to nematode origin.

### 12.2. Sponge storage

IJs can be stored efficiently in polyurethane, cellulose or melamine sponges and placed in Ziploc plastic bags (Figure 13) (Touray et al., 2020). This method is the cheapest and easiest of all formulation substrates. The porous structure of the sponges provides better aeration, moisture, and space for the movement of nematodes. Generally, sponges, especially polyether-polyurethane, are normally used when live IJ samples are stored or transported between laboratories, but they can be used to store IJs even at room temperature for 2–3 months and up to 8 months at colder temperatures. IJs can be retrieved or extracted by soaking and squeezing sponge in water (Grewal, 1998, 2002; Stock and Goodrich-Blair, 2012; Touray et al., 2020).

Besides aqueous suspension or sponge, several studies have aimed at developing techniques or methods that improve the storage duration of IJs by formulating in dry or semiliquid substrates such as charcoal, alginate, foam, clay, vermiculite, granules, polyacrylamide gels, etc. For details on these methods, check Bedding (1988), Connick et al. (1993, 1994), Silver et al. (1995), and Grewal (2000). Leite et al. (2018) explored preservation of *S. feltiae* in various substrates and found that some combinations of materials, such as vermiculite plus polyacrylamide gel provided the greatest preservation capacity.

### 12.3. Storage in liquid nitrogen

Long-term storage of EPNs can be achieved in liquid nitrogen. The percentage of glycerol used, and IJ densities in the cryovial and when thawing should be optimized for each species or strain to maximize survival (Bai et al., 2004). For example, Bai et al. (2004) found that optimum survival *H. bacteriophora* and *S. carpocapsae* was achieved with 12,000 IJ/mL in glycerol and 7500/mL in Ringer’s solution. For *S. carpocapsae*, maximum survival also was observed with 60,000 IJs/mL in glycerol and 25,000/mL in Ringer’s solution.

Below are basic procedures used for cryopreservation of *S. carpocapsae* and *H. bacteriophora* in liquid nitrogen (Bai et al., 2004).

1. One hundred milliliters of IJs are first filtered (Whatman filter paper No. 1) through a vacuum filtration system to remove water.

2. The IJs on the filter paper are immersed in Petri dishes (10-cm diam.) containing 18% glycerol solution for *S. carpocapsae* or 13% glycerol solution for *H. bacteriophora*.

3. After 48 h for *S. carpocapsae*, or 168 h for *H. bacteriophora*, the IJs were vacuum filtered on a filter paper disc, which was then dipped in prechilled 70% methanol (approximately −10 °C) for 10 min, and the IJ suspension was filtered again while rinsing with prechilled 70% methanol.

4. The filter paper was rolled and placed in prechilled cryogenic vials (in NaCl salted ice, about −5 °C), which were held in a prechilled cryogenic box and immediately plunged into liquid nitrogen.

5. To assess the success of the procedure, after 72 h sample vials can be removed from liquid nitrogen and

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![Figure 13. Sponge types which can be used for nematode storage and formulation.](natural-sponge-oasis-lysol-nano-scotchbrite.png)
thawed by pouring ca. 1.5 mL of Ringer’s solution into the vial. IJ survival is then determined based on nematode movement response when probed with a dissecting needle (see assessment of viability above). If survival is not satisfactory, further optimization may be required (glycerol concentration, period of incubation, IJ density).

13. Storage of mutualistic bacteria

13.1. Short-term storage

Working samples of *Xenorhabdus* and *Photorhabdus* used daily or weekly can be stored at 4 °C for not more than 2 weeks. Ageing cultures of these bacteria might result in a sudden phase change. Store sealed cultures of bacteria grown on agar plates or cotton-plugged agar slants in a refrigerator.

13.2. Long-term storage

Bacteria can be stored for long periods in cryoprotectants like glycerol, skim milk, dimethyl sulfoxide (DMSO), etc., by mixing with the bacterial suspensions prior to freezing. The cryoprotectants stabilize frozen concentration and protect cells from ice crystals that might damage cell membranes and mitigate the effects of changing solute concentration (Simione and Brown, 1991; Sanderson and Ziegler, 1991; Huba'lek, 2003). Extra care must be taken in the storage of *Xenorhabdus* and *Photorhabdus* because these bacteria are capable of spontaneously changing from the desirable Phase-I form to Phase-II (Leclerc and Boemare, 1991).

1. Inoculate a loopful of bacteria from a 24-h-old agar plate into an Erlenmeyer flask containing 5–10 mL of LB broth.
2. Prepare a 50% glycerol by diluting a 100% glycerol in distilled water. Autoclave and allow to cool. Dispense 500 µL in 2 mL cyrovials.
3. From the overnight culture transfer 500 µL of log-phase bacteria suspensions into 500 µL of glycerol solution, and vortex to evenly mix the bacterial suspension. Store at −20 to −80 °C or in liquid nitrogen at −150 °C. Frequently thawing and freezing reduces shelf life.
4. Recover by transferring a loopful of bacterial from glycerol stock and streak on appropriate culture medium. Incubate at 25–28 °C for 24 h.

14. Transportation of nematodes and bacteria

IJ samples can be shipped to other laboratories or institutes in infected-insect hosts, sterile soil, polyacrylamide gels, or sponges or in tissue culture flask with water (Stock and Goodrich-Blair, 2012). The nematode infected-insect hosts can be wrapped in moist tissue paper placed in Falcon tubes; upon arrival the cadavers can be placed on White traps for collection of IJs about to emerge. With the soil method, IJs are added to sterile and moist soil, and upon arrival the soil is baited with *G. mellonella* to retrieve the nematodes. Lastly, aqueous suspensions involve storing nematodes on sponge materials that provide nematodes with moisture and adequate aeration during transit (Touray et al., 2020).

In the case of *Xenorhabdus* or *Photorhabdus*, suspend an overnight bacterial culture in 15% glycerol in a test tube to make a dense suspension. Impregnate suspension on a sterile filter paper disk and place disk on a LB agar plate. Seal plate with Parafilm. Send samples using a fast courier with average arrival time of 2–5 working days. Upon arrival, the bacteria are cultured on LB broth (Sanderson and Ziegler et al., 1991; Spira et al., 2011).

15. Basics of field application

EPNs can be applied to fields using conventional agriculture and agronomic tools like mechanical sprayers, mist blowers, electrostatic sprayers, etc. that are used generally in pesticide and fertilizer application or through irrigation systems. Upon selection of a proven suitable EPN species for a target pest, IJs should generally be applied at a minimum of 25 IJs per cm² in early mornings or evenings so that IJs do not die from desiccation and ultraviolet light (Georgis, 1990; Grewal, 2002; Wright et al., 2005; Shapiro-Ilan et al., (2012, 2015). The volume, agitation system, pressure and recycling time, system environmental conditions, and spray distribution pattern during application need to be optimized according to the nematode species for successful application. EPNs are prone to stress from the effects of the different parts of the equipment like nozzle shape and pump type, and major improvements are required to ameliorate these negative impacts. Thus, increasing the viscosity of aqueous suspension can prevent death of IJs from sedimentation and oxygen deprivation, and tank mixing can ensure equal distribution of IJs during application (Grewal, 2002; Wright et al. 2005; Shapiro-Ilan et al., (2012; Hiltgold, 2015).

Besides these tools, EPNs can also be formulated in different substrates like alginate gel, polyacrylamide gels, and water dispersible granules (WDG) which are inert ingredients (Hiltgold, 2015). EPNs have also been formulated in activated charcoal or vermiculite. These formulations may be broadcast directly on or in soil and then are watered to assist in IJ dispersal.

The nematodes are generally applied in aqueous suspension to soil (their natural habitat). However, aboveground applications are also possible and gels or other protective formulations, or adjuvants (such as Barricade fire-gel) can be used to protect the nematodes from harmful UV radiation or desiccation (Shapiro-Ilan et al., 2016b). For example, Barricade gel can be applied separately after the nematodes are applied, or applied in a
diluted form (e.g., 2%) that allows the gel to be tank-mixed with the nematodes (Shapiro-Ilan et al., 2016b).

Another pioneering delivery system is the use of EPN-infected insects. In this approach, IJs emerge directly from the host cadaver and can pursue pests in cryptic habitats, flowerpots, greenhouses, or other settings. Such emerging IJs are highly dispersive and infective, have shown higher efficacy than aqueous-applied IJs, have extended survival rates, and are more tolerant to environmental stress compared to those collected from White traps (Shapiro-Ilan et al., 2003b; Gulzer et al., 2020). This method of delivery is quite easy and straightforward as it eliminates several application steps like IJ collection or storage. The insect cadavers are introduced into a suitable locality such as 5 cm below the soil surface. Cadavers can be coated with a substance (such as starch, kaolin–starch, calcitic calcareum, or a combination thereof, etc.) or placed in gelatin capsules to prevent rupture during handling (Shapiro-Ilan et al., 2001; Gumus et al., 2015; Dolinski et al., 2015; Raja et al., 2015). Gumus et al. (2015) controlled wood-boring Cossus cossus (goat moth) larvae in tunnels of chestnut logs using S. carpocapsae-infected but alive G. mellonella called “insect bomb”. In their experiments, they released infected G. mellonella larvae 16 h after post inoculation into tunnels created by C. cossus larvae. After two weeks, the previously infected G. mellonella larvae had died and were found deep within these tunnels. They found that the emerged IJs killed 86% of C. cossus larvae. The same method was highly effective against Spodoptera cilium (90% mortality) in turfgrass bioassays in the same study. The insect bomb method was significantly more effective than spraying of IJs or chemical pesticides on or into tree holes, turfgrass and other cryptic habitats and has a potential to be used against other wood boring insects.

16. Conclusion

The use of chemical pesticides has raised serious concerns about safety to humans and nontarget organisms, environmental pollution, and pest resistance. Thus, the search for safer alternatives, such as the EPNs and their symbiotic bacteria, has intensified. These nematode/bacterium complexes have the potential for huge economic impacts because they can be effective biological control agents against various soil insect pests as well as those that occur in cryptic habitats. Moreover, the species of symbiotic bacteria in the genera, Xenorhabdus and Photorhabdus, produce an arsenal of metabolites that have shown great potential as excellent sources of medical and agricultural antibiotics and insecticides (Donmez-Ozkan et al., 2019; Cimen et al., 2021; Incedayi et al., 2021; Gulsen et al., 2022). Also, from a research standpoint, EPNs are excellent model organisms for various studies in diverse disciplines such as ecology, genomics, pathology, and medicine. Accordingly, we have put together a manual that will be helpful for those initiating studies with these nematode/bacterium complexes from their isolation in the field to their identification and characterization in the laboratory. In addition, we have included mass-production methods for EPNs and their symbiotic bacteria, storage and transportation, and their application in the field against soil insect pests and those that occur in suitable cryptic habitats. Finally, we include an Appendix section on how to rear two insects that are often used for in vivo production of EPNs and bioassay studies and have provided recipes that are commonly used in EPN and their symbiotic bacteria research.

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HAZIR et al. / Turk J Zool


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HAZIR et al. / Turk J Zool


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HAZIR et al. / Turk J Zool


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Appendix

Rearing the insects

_Galleria mellonella_ (Lepidoptera: Pyralidae)

_Galleria mellonella_ is found worldwide where honeybees are found and are considered pests of honeybees. _G. mellonella_ is a holometabolous insect with four stages in its life cycle—egg, larva, pupa, and adult. After mating, females lay eggs in crevices and cracks of honeycombs, and upon hatching from the eggs, larvae feed on wax comb, pollen, cast-off skins of immature bee stages, propolis and honey causing huge economic losses to apiculturists (Gulati and Haushik, 2004; Kwadha et al., 2017). Despite this, _G. mellonella_ is used in research laboratories as a model organism (Harding et al., 2013) or sold as pet food for birds and reptiles and as bait for fishes (Finke, 2002).

Within entomopathogenic nematology, _G. mellonella_ larvae are used as baits in the isolation of nematodes from soil or for in vivo mass-production of IJs for research or small-scale commercial production. They have a short life span of 6 weeks to 6 months depending on rearing conditions and are highly fecund as the females that lay as many as 300–600 eggs in a lifetime (Gulati and Haushik, 2004). They are easily reared on artificial medium and are highly susceptible to nematode infection (Van Zyl and Malan, 2014). _G. mellonella_ reared on a diet composition listed in Jorjão et al. (2018) have a shorter larval phase, higher weight, and an improved immune system. Other artificial medium consisting of corn flour, wheat flour, powdered milk, honey, glycerin, beeswax and yeast as recommended by Haydak (1936) and Wiesner (1993) in 1-L glass containers can also be used. However, increasing the amount of beeswax will yield better larvae.

**Ingredients of _Galleria_ artificial diet (Jorjão et al., 2018)**

- Corn meal: 250 g
- Yeast extract: 150 g
- Soy flour: 100 g
- Powdered milk: 100 g
- Honey: 200 g
- Glycerol: 200 g
- Beeswax blocks

**Preparation:** Weigh and mix corn flour, wheat flour and powdered milk in a container. Measure and mix honey, yeast and glycerin in a beaker, place over a hot plate at 80 °C and mix until mixture is less viscous and homogenous. Melt beeswax at 80 °C or in a microwave. Mix all ingredients well. Medium can be stored at room temperature until needed.

**Culturing of _G. mellonella_.**

1. Place 20–25 adults or pupae in a 1-L glass container covered with wire mesh and filter paper or mix eggs with fresh feed. Place filter paper over mesh to collect eggs.

2. Fertilized female will lay eggs through mesh on filter paper. Cut out the parts with eggs and transfer to new medium. Replace old filter paper with new ones. Incubate containers in an insect room with temperature of 28–30 °C and relative humidity of 10%.

3. Transfer pupae to new containers to prevent escape of adults and for collection of eggs thus continuation of culture.

4. Last instar, prior to any bioassay use, can be immersed in water at 58–60 °C twice for 5–6 sec. This procedure inactivates the larvae and prevents spinning of silk cocoon as well as prevents the larvae from entering the pupal stage. After the hot water treatments, immediately transfer the larvae onto paper towels to remove water from the surface of the larvae. If some of the larvae are still very active, the same hot water procedure can be repeated. Water temperature and dipping period is very crucial. Be careful just after the heat treatment because the larvae appear dead, but after a few seconds, they become active. If a higher temperature than 60 °C is used and/or the larvae are kept longer than 5–6 s, a high percentage of the larvae may die. Inactivated larvae can be stored in coarse-grained sawdust at 4 °C for 1-2 months.

_Tenebrio molitor_ (Coleoptera: Tenebrionidae)

The beetle, _Tenebrio molitor_ (mealworm), is often used for research and as animal feed for pets and zoo animals. They are easy to rear and handle, do not require much attention like lepidopterans, and they can be fed an omnivorous diet of wheat flour, fresh vegetables and/or fruits (Tran et al., 2019). _T. molitor_ can be used for mass-production of nematodes because they have a hard cuticle which does not break up during handling of the cadaver. Although they are less susceptible to most EPN species and yield a smaller number of IJs compared to _G. mellonella_, they can be used to produce _S. scarabaei_, _S. kushidai_, and _S. scapterisci_ (Shapiro-Ilan et al., 2012, 2014b; Van Zyl and Malan, 2014; Prabowo et al., 2019).

The mealworm can be reared by placing 25 adults in a large plastic container with _ad libitum_ access to food (wheat flour) and water (soaked cotton in a 9-cm in Petri dish). All life stages can be kept in this container. More extensive descriptions of growing _T. molitor_ and scale-up can be found in Shapiro-Ilan et al. (2016a).

**Frequently used media and reagents**

**NBT A**

- Nutrient agar: 28 g
- Triphenyltetrazolium chloride (TTC): 4 mL
- Bromothymol blue (BTB): 0.025 g
- Distilled water: 1 L

**Preparation:** Measure nutrient agar, pour in to a 2-L flask with 1 L of distilled water. Add BTB and mix. Autoclave at 121 °C for 15 min. Upon cooling at around 42–43 °C, add filtered TTC and stir. Aseptically dispense
into Petri dishes at 50–55 °C before agar hardens.

**Luria-Bertani (LB)**
- Trypton: 10 g
- Yeast extract: 5 g
- NaCl: 5 g
- Agar: 1.5%
- Distilled water: 1 L

*Preparation:* In a 2-L flask add all ingredients and 1 L of distilled water. Autoclave at 121 °C for 15 min. Pour aseptically into Petri dishes at 50–55 °C. For liquid medium do not add agar and autoclave using liquid settings. Rather than using a 2-L flask, dispense into smaller flasks 250 mL in 500-mL flask before autoclaving.

**Tryptic soy agar**
- Casein peptone: 17 g
- Soy peptone: 3 g
- Glucose: 2.5 g
- NaCl: 5 g
- Dipotassium hydrogen phosphate: 25 g
- Agar: 1.5%
- Distilled water: 1 L

*Preparation:* Dissolve all the ingredients above in 1 L of distilled water. Autoclave at 121 °C for 15 min then dispense aseptically into Petri dishes. Better to pour at 42–43 °C.

**Pepsin digest solution**
- Pepsin: 8 g
- NaCl: 23 g
- Concentrated HCl: 20 mL
- Distilled water: 940 mL

*Preparation:* For 1 L pepsin solution, dissolve pepsin, HCl and NaCl into 940 mL sterile distilled water. Always use freshly prepared solution. However, prepared solution may be stored at 4 °C for a week.

**Egg yolk agar**
- Pancreatic digest of casein: 15 g
- Vitamin K₁: 10 g
- NaCl: 5 g
- Papae digest of soybean meal: 5 g
- Yeast extract: 5 g
- L-Cystine: 0.4 g
- Hemin: 5 g
- Egg yolk emulsion: 100 mL
- Agar: 20 g
- Distilled water: 900 mL

*Preparation:* Weigh out all the above ingredients except egg yolk emulsion and mix in 900 mL sterile distilled water. Adjust pH to 7 with NaOH or HCl. Autoclave at 121 °C for 15 min. Allow to cool to 50–55 °C. Under aseptic conditions, add egg yolk emulsion, mix homogeneously, and dispense into Petri dishes at 42–43 °C.

**Skim milk agar**
- Skim milk powder: 28 g
- Tryptone: 5 g
- Yeast extract: 2 g
- Dextrose (Glucose): 1 g
- Agar: 15 g
- Distilled water: 1 L

*Preparation:* Add all ingredients to 1 L of distilled water and autoclave at 121 °C for 15 min. Aseptically pour into Petri dishes at 42–43 °C.

**Fermentation broth**
- Trypticase: 1 g
- Carbohydrate: 0.5 g
- NaCl: 0.5 g
- Phenol red: 0.0189 mg
- Distilled water: 100 mL

*Preparation:* Weigh out and dissolve trypticase, NaCl, and phenol red in 100 mL distilled water in a 500-mL Erlenmeyer flask. Dispense 20 mL into each test tube. Plug with cotton wad. Add 0.5 g of different carbohydrates (glucose, lactose, sucrose, mannitol etc.) and vortex. Insert inverted Durham tubes into all tubes and make sure they fully filled with broth. Autoclave at 121 °C for 15 min.

**Tergitol 7 (T7) agar**
- Peptone: 10 g
- Yeast extract: 6 g
- Meat extract: 5 g
- Lactose: 20 g
- Bromothymol blue: 0.05 g
- Tergitol-7: 0.1 g
- Agar: 13 g
- Distilled water: 1 L

*Preparation:* Weigh out and dissolve the above ingredients in a 1-L Erlenmeyer flask. Adjust pH to 7.2 with NaOH or HCl and autoclave at 121 °C for 15 min. Aseptically pour into Petri dishes at 42–43 °C.