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Investigation of Indigenous Entomopathogenic Nematodes in Guangxi and Its Biological Control of *Spodoptera frugiperda*

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Abstract: *Spodoptera frugiperda* has caused serious economic damage to various crops. Entomopathogenic nematodes (EPNs) can be used as biological control agents for many pests, including lepidopteran insects. In this study, 218 soil samples were collected from 46 sites in Guangxi, and EPNs were detected in 15 samples. The ITS region of the rDNA gene was used for the molecular identification of isolated nematodes. In total, four and eleven identified populations belonged to *Heterorhabditis* and *Oscheius*, respectively. A series of bioassays were conducted to examine the virulence of EPN isolates from Guangxi to control the larvae and pupae of *S. frugiperda*. The mortality of the third-instar larvae caused by EPNs was concentration dependent. The same dose of EPNs was used to control the third and sixth-instar larvae of *S. frugiperda*, and the virulence was lower in the sixth-instar larvae. *S. frugiperda* pupae were treated with different EPNs strains, and the adult eclosion rate of the treated group was significantly lower than that of the sterile water control group (93.3%). Therefore, EPNs could significantly inhibit the eclosion of *S. frugiperda* pupae. This study provides important information for the biological control of *S. frugiperda* with EPNs.

Keywords: entomopathogenic nematodes; *Heterorhabditis*; *Oscheius*; molecular identification; *Spodoptera frugiperda*; biological control

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1. Introduction

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is known as one of the top ten plant pests in the world. It had spread rapidly in Africa and Asia recently, causing serious economic damage to various crops [1]. In 2019, *S. frugiperda* invaded Yunnan (97°31′–106°11′ E, 21°8′–29°15′ N, China) from Myanmar, and spread to more than 20 provinces in the Yellow River basin and its southern regions in less than one year [2,3]. Since *S. frugiperda* invaded Guangxi in 2019, it has become one of the major agricultural pests in corn fields. The invasive population of *S. frugiperda* in Guangxi was identified as a corn strain [4]. According to the National Bureau of Statistics, the corn export of China in 2021 was 272.55 million tons, the second largest corn-producing country in the world. Therefore, the control of *S. frugiperda* is urgent to ensure corn production [5].

Various chemical pesticides, such as chlorpyrifos and lufenuron, have been used to control *S. frugiperda*. However, *S. frugiperda* has developed resistance to chemical pesticides due to their widespread application [6–8]. Moreover, the large-scale application of pesticides not only pollutes the environment, but also poses a threat to the safety of humans and animals [9,10]. In terms of biological control, *S. frugiperda* has also developed a resistance to *Bacillus thuringiensis* (Bt) toxin-gene transgenic crops and Bt insecticides [11–15]. Therefore, the development of biological control agents is of great importance to the sustainable management of *S. frugiperda* [16,17].

Entomopathogenic nematodes (EPNs) are the natural enemies of underground pests [18,19]. EPNs penetrate the host insect through natural openings, such as the mouth, anus, and spiracles, and release the mutualistic bacteria that they carry. The bacteria multiply in

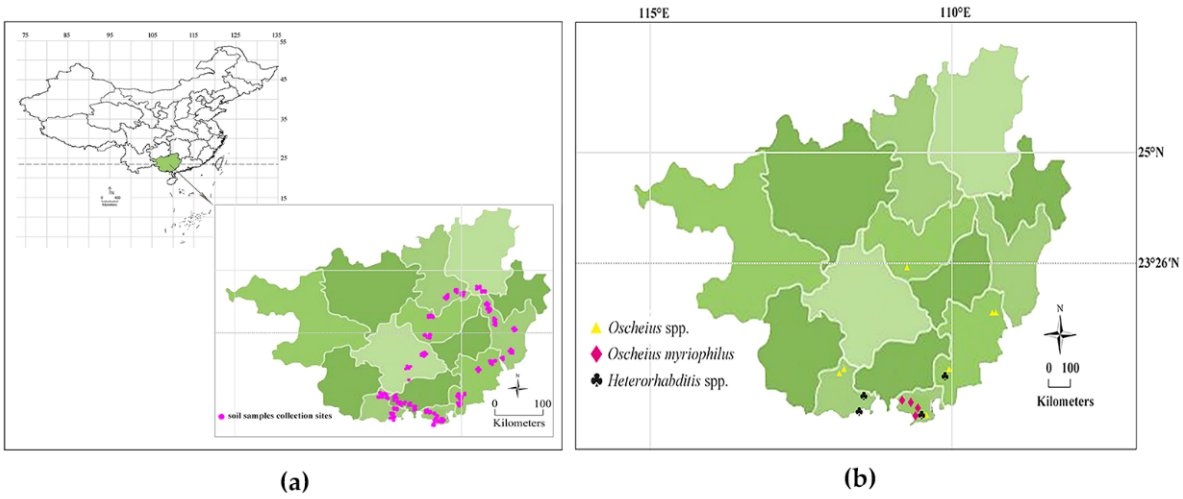
insects and produce a variety of toxins, causing the insects to die from septicemia [20,21]. It has a wide host range and an active search ability, does not pollute the environment, is safe for human and animals, is produced in large quantities, and can be used in combination with pesticides [19]. EPNs have been used to control several agricultural and forestry pests, including Scarabacidae, *Bradysia odoriphaga*, and *Agrotis ypsilon* [22,23]. Studies have shown that EPNs have potential as biological control agents for *S. frugiperda* [24–27]. Leyva-Hernandez et al. (2018) found that *Steinernema riobrave* had a 90% mortality rate for *S. frugiperda* [28]. Additionally, Yang et al. (2021) found that the mortality rate of the second-instar larvae of *S. frugiperda* infected with *S. feltiae* and *S. krussei* for 72 h was 100% and 80%, respectively [19]. Therefore, the exploration and development of more EPN resources to control *S. frugiperda* is promising.

The Guangxi Zhuang autonomous region is located in the south coast of China (104°26′–112°04′ E, 20°54′–26°20′ N). On the one hand, there is a great variety of biological resources in Guangxi because of the complex and diverse terrain. On the other hand, it is difficult for exotic EPNs to adapt to the local habitat due to the humid and hot climate in Guangxi. Li et al. (2016) reported that the annual average temperature in Guangxi is about 23.0 °C, and the RH is about 70% [29]. There are few studies on the virulence of indigenous EPNs to *S. frugiperda* in Guangxi. In order to explore the biocontrol potential of local EPNs for the control of *S. frugiperda*, a total of 218 soil samples were collected from Guangxi, and 15 EPN strains were isolated. To identify the indigenous EPN strains and evaluate their effectiveness to control *S. frugiperda*, the major objectives of this study were: (i) to determine the classification status of different strains by phylogenetic analysis of the ITS gene; and (ii) to test the virulence of the EPNs isolated from Guangxi against *S. frugiperda*.

2. Materials and Methods

2.1. Soil Sample Collection

Soil samples were collected from Guangxi in September 2021. A total of 218 soil samples were collected, covering most of Guangxi (Figure 1a). Three to five soil subsamples (each soil sample was about 500 g) of a 0–20 cm depth, at intervals of 8–10 m, were collected in the selected sampling points [30]. They were mixed evenly, put in a polyethylene bag, labeled with a marker, and sent to the laboratory as soon as possible. The trapping of EPNs was completed within two weeks. The sampling date, GPS coordinates, altitude, and soil type at each sampling site were recorded use the GPS toolbox software.



(a) (b)

Figure 1. Distribution map of soil samples and entomopathogenic nematodes collected in Guangxi, China: (**a**) soil sample collection sites; (**b**) entomopathogenic nematodes.

2.2. Isolation of EPNs with Galleria Mellonella

EPNs were isolated from the soil samples by a *Galleria mellonella* L. bait technique [31]. One steel tea leakage filled with damp gauze and containing five last-instar *G. mellonella* larvae was placed in a polyethylene bag filled with soil samples, which were kept at room temperature in the laboratory for two weeks. The survival of *G. mellonella* was checked every two days. If the *G. mellonella* died, the corpse was removed, and the surface soil was rinsed with distilled water. Additionally, infective juveniles (IJs) were collected by white traps [32]. The collected IJs were placed in cell-culture flasks (NEST Cell Culture Flask, Wuxi, CHN) and stored at 14 °C.

2.3. The Test Insects

S. frugiperda eggs were purchased from the Sichuan Academy of Agricultural Sciences (SAAS), CHN, and the eggs were incubated in an incubator (25 ± 2 °C, RH 60–70%, L/D 12/12). After the eggs of *S. frugiperda* hatched, fresh lab-grown corn leaves were clipped and used to feed the larvae of *S. frugiperda*. Bioassays were performed when they reached the corresponding age.

2.4. Molecular Identification of EPN Species

Genomic DNA was extracted with 10–15 isolated nematodes according to the protocol described by Holterman et al. (2006) [33]. Briefly, 10–15 isolated nematodes were selected and placed into a 200 µL tube containing a 20 µL buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl{\cdot}6H_2O$, 0.45% NP40, 1% Trition X-100 and 60 µg/mL proteinase K). The DNA solution was stored in a −20 °C refrigerator for use. The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was amplified by the polymerase chain reaction (PCR). The PCR primers were 18S: 5′-TTG ATT ACG TCC CTG CCC TTT-3′ (forward), and 28S: 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (reverse) [18,34].

Each PCR reaction was made in a total volume of 25 µL, containing 12.5 µL × Es Taq MasterMix (CWBIO, Taizhou, China), 0.5 µL and 10 µM of each primer, 2 µL genomic DNA, and 9.5 µL ddH_2O. The conditions for PCR amplification were as follows: pre-denaturation at 94 °C for 7 min; followed by 35 cycles of 94 °C/1 min, 50 °C/1 min, and 72 °C/1 min; and a final extension at 72 °C for 7 min [18]. The amplification of all products was examined by 1% agarose gel electrophoresis, and then sent to a company for sequencing (GENEWIZ Biotechnology, Tianjin, China). All the sequences were aligned by ClustalW with the default parameters in MEGA VII [35]. Sequences were edited using BioEdit v7.1.7 [36]. BLAST was used for the target sequence fragments in NCBI. Other related sequences of EPNs were downloaded from GenBank for sequence alignment. The phylogenetic tree constructed based on ITS genes was analyzed by the neighbor-joining (NJ) method in MEGA VII [35]. Bootstrap analysis was computed with 1000 replicates.

2.5. Virulence of EPNs against S. frugiperda

2.5.1. Screening of EPNs in Guangxi

A total of 15 EPNs were processed in a 24-well plate (LABSELECT Cell Culture Plate, Beijing, CHN). A total of 30 µL (100 IJs) EPN suspension was added to each well, equipped with filter paper. Five larvae were put into each 24-well plate as a replicate, and each EPN treatment was repeated five times. Fresh corn leaves were placed in the well plate as the food source for the larvae. Only sterile water was added to the control group, and an equal volume of the commercial EPN strain *Steinernema feltiae* was added to the positive control group. The 24-well plates were sealed with Parafilm (Bemis Parafilm, Neenah, WI, USA) and placed in an incubator (25 ± 2 °C, RH 60–70%, L/D 16/8). The number of dead larvae was recorded every 12 h, and the observation continued for 84 h. The experiment was repeated three times.

2.5.2. Virulence of Highly Effective EPNs to the Third-Instar Larvae of *S. frugiperda*

The selected high-efficiency EPNs (22835, 22855, 22896) were subjected to virulence assays at different concentrations. The experimental methods refer to the previous step described in the experimental screening of EPNs in Guangxi. Four quantitative gradients were set up for each EPN strain, so that the number ratio of EPNs and the third-instar larva were: 50:1, 100:1, 150:1, and 200:1. The same amount of commercial EPN strain *S. feltiae* and an equal volume of sterile water served as controls. The 24-well plates were then sealed with Parafilm and kept at 25 ± 2 °C, 60–70% RH, and L/D 16/8. Larval mortality was determined every 12 h, and the observation continued for 84 h. The experiment was repeated three times.

2.5.3. Virulence of Highly Effective EPNs to the Sixth-Instar Larvae of *S. frugiperda*

The virulence of highly effective EPNs (22835, 22855, 22896) to the sixth-instar larvae of *S. frugiperda* was determined. The bottom of each 24-well tissue culture plate was lined with a filter paper. Each plate was inoculated with 30 µL distilled water containing 100 IJs. The control plates contained 30 µL distilled water only, and the positive control plates contained 30 µL commercial EPN strain *S. feltiae*. One sixth-instar larva of *S. frugiperda* was released into each plate, then tender and fresh corn leaves were added as a food source for the larva. All the 24-well plates were incubated at 25 ± 2 °C and 60–70% RH. Larval mortality was determined every 12 h, and the observation continued for 84 h. Each treatment was replicated five times with five larvae per replicate (25 larvae for each EPN strain), and the assay was repeated three times at different times.

2.5.4. Virulence of Highly Effective EPNs to *S. frugiperda* Pupae

The high-efficiency EPNs (22835, 22855, 22896) were selected to determine their effects on the adult eclosion rate of *S. frugiperda* pupae. In a 90 mm Petri dish (NEST Petri dish, Wuxi, CHN), a layer of filter paper was laid on the bottom of the Petri dish, and an appropriate amount of distilled water was added to keep the filter paper moist. Five five-day-old *S. frugiperda* pupae were placed in a 90 mm Petri dish, EPNs suspension was added, and the ratio of EPN to *S. frugiperda* pupae was 1200:1. Each EPN strain had three replicates. The same amount of commercial EPN strain *S. feltiae* and sterile water served as controls. The Petri dishes were sealed with Parafilm and placed in an incubator (25 ± 2 °C, RH 60–70%, L/D 16/8). The adult eclosion rate was recorded for five days after treatment. The experiment was repeated three times at different times.

2.5.5. Pot Experiment

High-efficiency EPNs (22835, 22855, 22896) were used for pot experiments. Pots (22 cm diameter, 25 cm depth) were filled with nutrient soil, and each pot was planted with four corn seeds. The corn seeds were soaked for 12 h in advance and watered every 2–3 days. Pots were kept in a greenhouse (25 ± 1 °C, RH 75%). When the corn seedlings grew to approximately 15 cm, three third-instar larvae of *S. frugiperda* were released into the whorl of each corn seedling. After two days, the leaves and whorls of each corn seedling were sprayed with EPN suspension at 4800 IJs plant^{-1}. The same amount of commercial EPN strain *S. feltiae* and sterile water served as controls. Each treatment had six replicates. All the pots with the same treatment were randomly placed in the same insect net (50 cm × 50 cm × 50 cm) to prevent the larvae from escaping. The number of dead larvae was counted for five days after EPNs application. The death of larvae caused by EPNs was demonstrated by dissecting them in tap water with a stereoscopic microscope.

2.6. Statistical Analysis

Distribution maps were prepared using DIVA-GIS ver. 7.5.0 and output as TIF files that were edited subsequently in Adobe Photoshop 2021. To assess the virulence of EPN strains, one-way ANOVA was performed in *S. frugiperda* mortality, which was calculated as a percentage. The effect of EPN concentrations, EPN strains, and their interactions with

larval mortality was analyzed by two-way ANOVA. The lethal median time (LT_{50}) was calculated by Kaplan–Meier analysis in SPSS 19.0, and a log-rank test was used to compare the lethal time. Differences between treatments were determined using Tukey's test, with $p < 0.05$ as significance. Data from the repeated experiments were combined and there was no significance between them ($\alpha > 0.05$). All data are presented as mean ± standard error.

3. Results

3.1. Identification of EPNs from Soil Samples from Guangxi

A total of 218 soil samples at 46 sites (Figure 1a) were collected from different habitats, including forests (82), corn fields (56), orchards (32), croplands (22), watersides (14), and wastelands (12).

Fifteen EPN strains were screened from the collected soil samples. The EPN strains were identified and characterized based on ITS gene sequences. All DNA sequences were subjected to BLAST alignment in NCBI. Four isolated strains belonged to *Heterorhabditis* and the other 11 belonged to *Oscheius* (Figure 1b).

Phylogenetic tree constructed with ITS sequences showed that *Heterorhabditis* and *Oscheius* were significantly separated (Figure 2). Different species of EPNs could also be distinguished. The similarity between strain 22864 and *Heterorhabditis* sp. EPNKU59 was 99.85%, the similarity between strains 22848 and 22855 was 99.56% with *Heterorhabditis* sp. EPNKU59, and the similarity between strain 22861 and *Heterorhabditis* sp. EPNKU59 was 99.41%. This shows that strains 22848, 22855, 22861, and 22864 belonged to the genus *Heterorhabditis*. The similarity between strains 22719, 22800, 22801, 22833, 22896, and 22902 with *Oscheius* sp. EPNKU33 was 100%, and the similarity between strain 22811 and *Oscheius* sp. EPNKU33 was 99.59%. These results indicate that strains 22719, 22800, 22801, 22811, 22833, 22896, and 22902 belonged to the genus *Oscheius*. The similarity between strain 22835 and *Oscheius myriophilus* (MW618710.1) was 100%, and strains 22911 and 22926 had 99.86% similarity with *Oscheius myriophilus* (MW618710.1). The similarity between strain 22817 and *Oscheius myriophilus* (MW618710.1) was 99.72%, indicating that strains 22817, 22835, 22911, and 22926 belonged to *Oscheius myriophilus*.

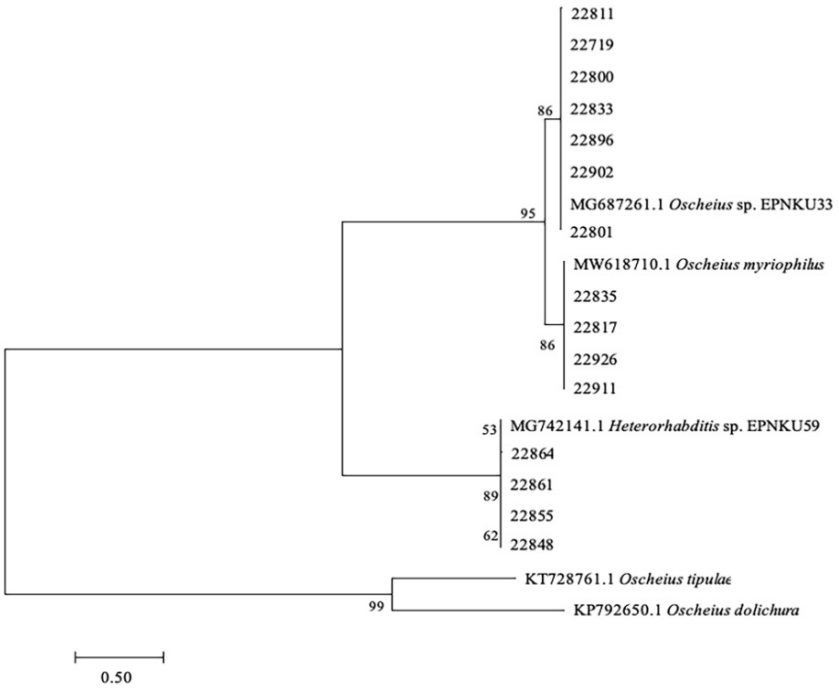


Figure 2. Phylogenetic trees based on ITS rDNA were constructed using neighbor-joining method to describe the *Heterorhabditis* and *Oscheius* EPN strains. Numbers on branches represent bootstrap support (>50%) based on 1000 replicates. Scale represents K2P genetic distance.

3.2. Screening of Highly Virulent EPNs in Guangxi

The mortality of *S. frugiperda* larvae differed significantly among the EPN strains (Figure 3, ANOVA, $F_{16, 84} = 13.205$, $p < 0.001$). The larvae had a certain mortality with different EPNs after 48 h, and the larval mortality of the commercial strain *S. feltiae* could reach 100%. After 60 h of treatment, the larval mortality increased significantly. After 72 h of treatment, the larval mortality was over 50%. After 84 h of treatment, the larval mortality was 76–92%, and strains 22835, 22855, and 22896 lead to the highest mortality for the third-instar larvae, reaching 92%.

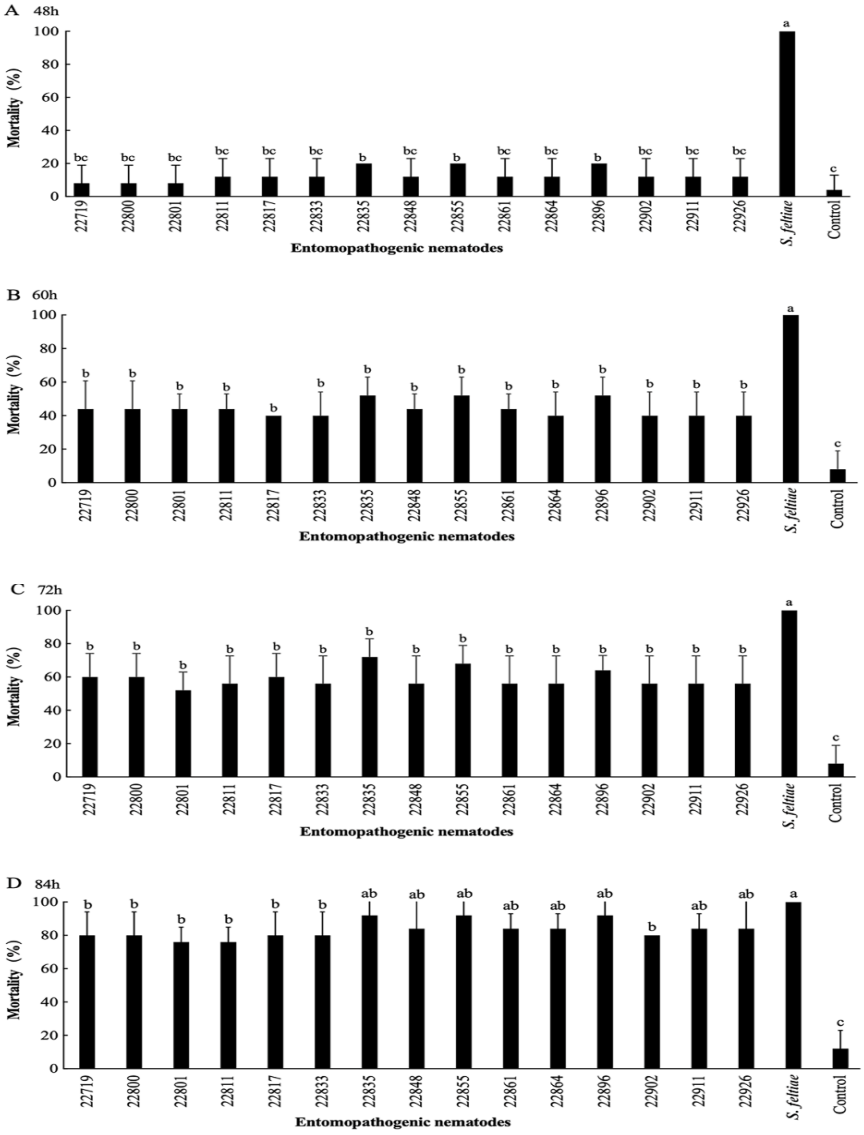


Figure 3. Mortality (mean ± SE) of the third-instar larvae of *Spodoptera frugiperda* treated with different entomopathogenic nematodes for 48, 60, 72, and 84 h ((**A**–**D**) 100 IJs larva^{-1}). Bars with different letters represent significant differences between treatments by Tukey's test ($p < 0.05$). *S. feltiae*: *Steinernema feltiae*; 22719, 22800, 22801, 22811, 22833, 22896, 22902: *Oscheius* spp.; 22817, 22835, 22911, 22926: *Oscheius myriophilus*; 22848, 22855, 22861, 22864: *Heterorhabditis* spp.

The LT_{50} values of all the EPN strains for the third-instar larvae of *S. frugiperda* are shown in Table 1. EPN strains 22835, 22855, and 22896 needed 58.634 h, 60.135 h, and 61.388 h, respectively, to reach a mortality of 50%. Based on the LT_{50} values and the mortality of isolated EPNs to the third-instar larvae, high-efficiency EPNs (22835, 22855, 22896) were screened and used in the following bioassays.

Table 1. Comparison of median lethal times (LT_{50s}) in the third-instar larvae of *Spodoptera frugiperda* by entomopathogenic nematodes (EPNs).

EPNs	LT_{50} (h)	95% FL	χ^2	*p*
22719	66.312	61.764–71.545	1.459	0.918
22800	67.231	62.613–72.706	0.371	0.996
22801	68.593	63.828–74.495	3.365	0.644
22811	67.054	62.089–73.206	1.498	0.913
22817	66.258	61.564–71.800	0.577	0.989
22833	66.846	62.075–72.621	0.86	0.973
22835	58.634	48.805–71.115	10.303	0.067
22848	65.116	60.165–71.121	1.504	0.913
22855	60.135	55.672–64.891	0.909	0.97
22861	66.217	61.769–71.291	1.83	0.872
22864	66.281	61.205–72.678	1.847	0.87
22896	61.388	57.068–65.917	2.311	0.802
22902	67.36	62.593–73.202	0.543	0.99
22911	66.717	62.290–71.815	1.141	0.95
22926	65.681	61.096–70.983	1.557	0.906

FL: fiducial limits. Corrected mortality was used to calculate LT_{50} values.

3.3. Virulence of EPNs at Different Concentrations to the Third-Instar Larvae of S. frugiperda

The virulence of different concentrations of three screened EPNs (22835, 22855, 22896) to the third-instar larvae of *S. frugiperda* was determined (Figure 4). Larval mortality differed significantly among different EPN strains, and it was significantly affected by the concentration of EPN strains (Table 2). The mortality of the third-instar larvae increased with the increase in EPNs concentration, and the larval mortality increased significantly after 60 h treatment. Strain 22835 with larva ratios of 200:1, 150:1, 100:1, and 50:1 led to a mortality of 64%, 52%, 40%, and 24% for 60 h, respectively, and 92%, 84%, 84%, and 68% after 84 h treatment, respectively. Strain 22855 with larva ratios of 200:1, 150:1, 100:1, and 50:1 led to a mortality of 60%, 52%, 36%, and 20% for 60 h, respectively, and 92%, 88%, 84%, and 68% for 84 h, respectively. Strain 22896 with larva ratios of 200:1, 150:1, 100:1, and 50:1 led to a mortality of 56%, 52%, 36%, and 20% for 60 h, respectively, and 92%, 88%, 84%, and 68% after 84 h treatment, respectively.

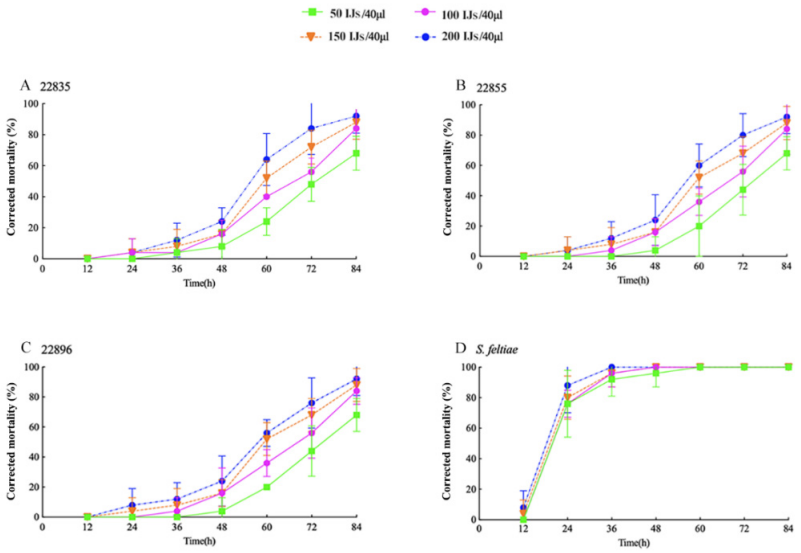


Figure 4. Corrected mortality of the third-instar larvae *Spodoptera frugiperda* treated with different concentrations of entomopathogenic nematodes for 12, 24, 36, 48, 60, 72, and 84 h. Mortality was corrected with control. Significant differences among different treatments by Tukey's test ($p < 0.05$) are shown in Table S1. Error bars represent standard error. (**A**) 22835: *Oscheius myriophilus*; (**B**) 22855: *Heterorhabditis* spp.; (**C**) 22896: *Oscheius* spp.; (**D**) *S. feltiae*: *Steinernema feltiae*.

Table 2. ANOVA parameters for the effects of entomopathogenic nematode strains, concentration, and their interactions on the mortality of *Spodoptera frugiperda* larvae over 84 h.

Source	DF	F Value	*p*
EPN strains (S)	3	10.446	0.003
Concentration (C)	3	9	0.005
S × C	9	1.287	0.262
Error	64		
Corrected total	79		

3.4. Virulence of Highly Effective EPNs to the Sixth-Instar Larvae of S. frugiperda

The mortality of the three screened EPNs (22835, 22855, 22896) to the sixth-instar larvae of *S. frugiperda* was determined (Figure 5). The mortality of the sixth-instar larvae was significantly different among different treatments (ANOVA, $F_{4,24} = 60.7$, $p < 0.001$). The sixth-instar larvae had a certain mortality by different EPNs after 48 h treatment. Commercial EPN *S. feltiae* had the highest mortality, with mortality reaching 100% at 60 h. Compared to a mortality of 12% in the sterile water control, strains 22835, 22855, and 22896 had a mortality of 56%, 56%, and 52% after 84 h, respectively.

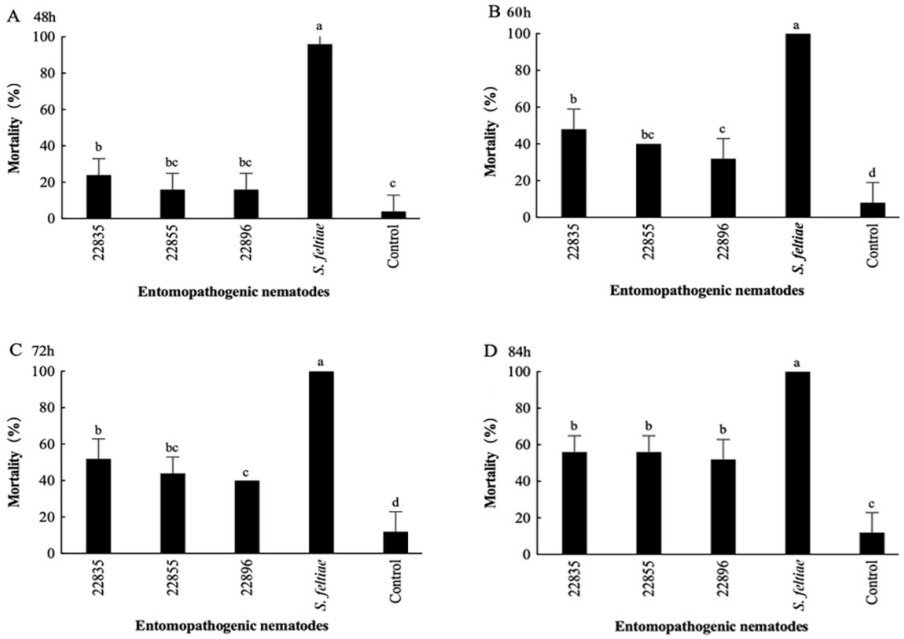


Figure 5. Mortality (mean ± SE) of the sixth-instar larvae of *Spodoptera frugiperda* treated with different entomopathogenic nematodes for 48, 60, 72, and 84 h ((**A**–**D**) 100 IJs larva^{-1}). Bars with different letters represent significant differences between treatments by Tukey's test ($p < 0.05$). *S. feltiae*: *Steinernema feltiae*; 22835: *Oscheius myriophilus*; 22855: *Heterorhabditis* spp.; 22896: *Oscheius* spp.

The LT_{50} values of the 22835, 22855, and 22896 strains' treatment against the sixth-instar larvae of *S. frugiperda* were calculated (Table 3). The LT_{50} value was 73.821 h for strain 22835, while the LT_{50} values for 22855 and 22896 were 82.603 h and 80.415 h, respectively.

Table 3. Comparison of median lethal times (LT_{50s}) in the sixth-instar larvae of *Spodoptera frugiperda* by entomopathogenic nematodes (EPNs).

EPNs	LT_{50} (h)	95% FL	χ^2	*p*
22835	73.821	61.145–100.628	3.291	0.655
22855	82.603	67.903–117.839	6.825	0.234
22896	80.415	69.855–104.185	0.554	0.99

FL: fiducial limits. Corrected mortality was used to calculate LT_{50} values.

3.5. Virulence of Highly Effective EPNs to S. frugiperda Pupae

The adult eclosion rate of *S. frugiperda* pupae was determined by all three EPN strains (22835, 22855, 22896) (Figure 6). The adult eclosion rate of *S. frugiperda* pupae had significant differences among EPN strains ($F_{4,14} = 6.857$, $p < 0.05$). Compared to an adult eclosion of 93.3% in the sterile water control, significantly lower adult eclosion rates were observed in strains 22835 (26.7%), 22855 (26.7%), and commercial EPN species *S. feltiae* (26.7%). The adult eclosion rate for strain 22896 was 33.3%. The results showed that different EPNs could cause the death of pupae and inhibit the adult eclosion of pupae in different degrees compared to the sterile water control.

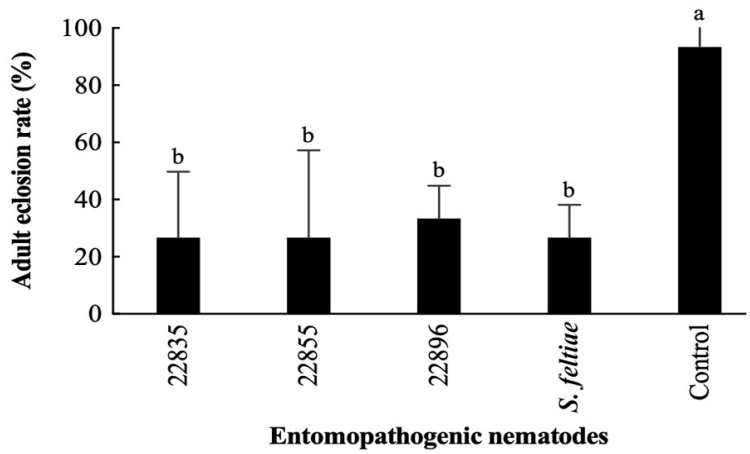


Figure 6. Adult eclosion rate of *Spodoptera frugiperda* pupae by entomopathogenic nematodes. Pupae (five pupae per Petri dish) of *S. frugiperda* were treated with EPNs (1200 IJs) for five days. Bars with different letters represent significant differences between treatments by Tukey's test ($p < 0.05$). *S. feltiae*: *Steinernema feltiae*; 22835: *Oscheius myriophilus*; 22855: *Heterorhabditis* spp.; 22896: *Oscheius* spp.

3.6. Pot Experiment

Five days after EPNs application, the mean mortality of the third-instar larvae of *S. frugiperda* differed significantly between treatments (Figure 7, ANOVA, $F_{4,29} = 111.41$, $p < 0.05$). Compared to the 0.5 dead larvae per pot in the sterile water control group, the number of dead larvae per pot were 4, 3, 2.5, and 8 for strains 22835, 22855, 22896, and *S. feltiae*, respectively.

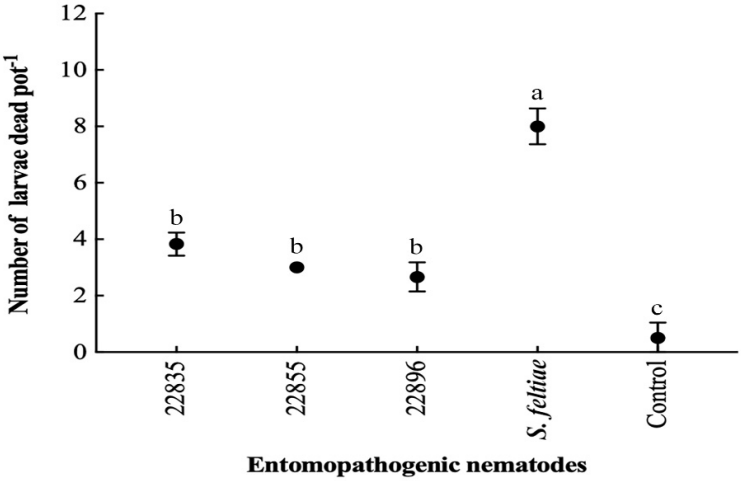


Figure 7. Mean number of dead larvae of the third-instar *Spodoptera frugiperda* in pot experiments after five days of different treatments (4800 IJs plant^{-1}). Bars with different letters represent significant differences between treatments by Tukey's test ($p < 0.05$). *S. feltiae*: *Steinernema feltiae*; 22835: *Oscheius myriophilus*; 22855: *Heterorhabditis* spp.; 22896: *Oscheius* spp.

4. Discussion

This study is the first to investigate EPNs in Guangxi. In September 2021, 218 soil samples were collected from 46 sampling sites in Guangxi, and 15 strains were isolated and confirmed as EPNs [18]. Four isolates belonged to *Heterorhabditis* spp. and 11 isolates belonged to *Oscheius* spp. Among the 11 strains, four of them were identified as *Oscheius myriophilus*, and the remaining seven strains were identified *Oscheius* spp., but no specific species were identified. We speculated that this might be due to few sequences of the genus *Oscheius* in GenBank. The genus of *Oscheius* has been determined as EPNs [37]. The genus *Oscheius* is less virulent against pests than the genera *Heterorhabditis* and *Steinernema* and tends to take longer to kill pests [38,39]. In some areas, the genera *Steinernema* and *Heterorhabditis* are absent, and the genus *Oscheius* may play a very important role, especially in the regulation of soil insect populations. Our results indicate that the genus *Oscheius* is widely distributed in Guangxi, and EPNs in *Oscheius* have the potential to be used as biological control agents in this area.

Our study demonstrated that the virulence of EPNs differed significantly depending on the EPN strains and the developmental stage of the host. The same dose of EPNs (100 IJs) treated the third- and sixth-instar larvae, the mortality of the third-instar larvae treated with two *Oscheius* spp. (22835, 22896), and one *Heterorhabditis* spp. (22855), which were all 92% for 84 h, while the mortality of the sixth-instar larvae was 56%, 52%, and 56% for 84 h, respectively. The LT_{50} values of different EPN strains for the third-instar larvae were lower than those of the sixth-instar larvae. Additionally, we found that the virulence of different EPN strains to the third-instar larvae was better than that of the sixth-instar larvae. Our results are consistent with previous studies. For examples, Fallet et al. (2022) reported that *S. carpocapsae* strain RW14-G-R3a-2 could cause 100% mortality in the second- and the third-instar larvae and close to 75% mortality in the sixth-instar larvae of *S. frugiperda* [40]. Additionally, Patil et al. (2022) found that the third-instar larvae of all tested EPN isolates were more sensitive than the fourth-instar larvae [41]. Therefore, this study concludes that the virulence of EPNs against larvae and pupae depends not only on the intrinsic properties, but also on the developmental stage of the host insect [8,42–45]. In future studies, different EPNs will be used to evaluate the virulence of host pests at different stages, which is of great significance for the application of EPNs in fields for the precise control of different stages of target pests.

The mortality of the third-instar larvae of the two *Oscheius* spp. (22835, 22896) and the one *Heterorhabditis* spp. (22855) were 64%, 56%, and 60%, respectively, when the infection ratio was 200:1. Liang et al. (2020) found that, after exposing one *S. frugiperda* larva to 50 *S. carpocapsae* AII and 50 *S. longicaudum* X-7 for 36 h, respectively, the mortality of the second-instar larvae of *S. frugiperda* was 92% and 80%, respectively [46]. By comparison and analysis of the results, the virulence of the EPNs isolated from Guangxi was significantly lower than that of *S. carpocapsae* AII and *S. longicaudum* X-7. The results showed that different species and strains had a different pathogenicity to the same host insect, which might be related to the infection mode of different EPNs. Further research on the infection mode of the genus *Oscheius* could be conducted in the future.

In this study, we found that the mortality caused by EPNs to the third-instar larvae was concentration-dependent; that is, the mortality of the third-instar larvae increased with the increase in EPNs concentration. However, there was no significant difference in the mortality of the third-instar larvae in the infection ratios of 200:1 and 150:1 for 60 h among the three EPN strains. Patil et al. (2022) reported that the mortality of third-instar larvae was not significantly different for all the tested EPNs, especially at higher infection concentrations (200 or 400 IJs $larva^{-1}$) [41]. There might be a threshold concentration for mortality. Therefore, increasing the concentration of EPNs could not increase mortality once the threshold concentration was reached.

Due to the last-instar larvae pupate in soil, the virulence of different EPN isolates against *S. frugiperda* pupae was determined in this study. Additionally, various studies have reported that lepidopteran pupae are sensitive to EPNs [8,44,45,47–49]. For example,

Yan et al. (2020) reported that *H. indica* and *S. carpocapsae* were highly virulent to the pupae of *S. litura* [45]. Similarly, Acharya et al. (2020) reported that *H. indica* and *S. carpocapsae* could make the mortality of *S. frugiperda* pupae reach 60% and 67%, respectively [8]. In this study, the EPN strains 22835, 22855, and 22896 isolated from Guangxi and the commercial strain *S. feltiae* were used to infest *S. frugiperda* pupae at high doses, and the adult eclosion rates of *S. frugiperda* pupae after five days were 26.7%, 26.7%, 33.3%, and 26.7%, respectively. There were significant differences between the EPNs treatments and the sterile water control ($F_{4, 14} = 6.857$, $p < 0.05$), indicating that EPNs isolated from Guangxi could inhibit the adult eclosion of *S. frugiperda* pupae. This is consistent with the previous studies, showing that EPNs were virulent to *S. frugiperda* pupae. Fuxa et al. (1988) revealed that *S. feltiae* could cause 7–20% mortality of *S. frugiperda* pupae [50]. However, there is no report on the virulence of *Oscheius* on *S. frugiperda* pupae. The inhibitory effect of *Oscheius* on the *S. frugiperda* pupal eclosion in this study provided a basis for follow-up studies on the role of *Oscheius* in pest biological control.

S. frugiperda is one of the foliar pests, and its larval stage mainly damages the green leaves. Therefore, the virulence of different EPNs in the pot experiment was compared with that in laboratory conditions using 24-well plates. After five days, the virulence of EPNs to the third-instar larvae of *S. frugiperda* was much lower than those experiments conducted with 24-well plates. This may be due to that the exposure of the EPN suspension sprayed on the foliage to unfavorable environments, such as UV radiation, desiccation and extreme temperatures, which make it unable to exert its virulence effect on the larvae [44,51]. Acar and Sipes (2022) reported that desiccant agents, such as Barricade, and chemicals, such as P-amino benzoic acid (PABA) or octyl methoxycinnamate (OMC), can effectively protect against UV radiation and improve the survival of *S. feltiae*, thus making EPN more widely used for foliar pest control [52]. Subsequent research could develop more adjuvants to improve the effect of spraying EPNs on the ground. It is also critical to screen out EPNs from soil samples that are resistant to desiccation and UV radiation.

The present research focused on screening native EPN strains with high virulence and explored effective control strategies against *S. frugiperda* in maize. This study also showed that the virulence of different EPN strains to *S. frugiperda* larvae and pupae was significantly different, so it is necessary to screen more EPN strains with high virulence to target pests. In fact, natively isolated EPNs are better adapted to local environmental conditions [18]. Therefore, in the pest control process, we give priority to the use of natively isolated species, which greatly reduces the damage to the local environment [40,53]. In this study, the virulence of different EPNs against the larvae and pupae of *S. frugiperda* was determined only under laboratory conditions, and future studies should compare the virulence of natively isolated EPNs and commercial strains against pests through field experiments [18]. Some natively isolated EPNs are extremely infectious and can be used in combination with commercial strains. The genus *Steinernema* was not isolated in this survey, so further investigation will be needed in Guangxi to isolate more beneficial EPNs for future field applications.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12102536/s1, Supplementary Table S1. Comparison of mortality of the third instar larvae *Spodoptera frugiperda* treated with different concentrations of entomopathogenic nematodes for 60, 72, 84 h.

Author Contributions: W.R. designed the research; W.R. conducted the investigation; A.W. and J.S. performed the molecular biology experiments; A.W., M.F. and J.S. carried out subsequent bioassay experiments; A.W. and M.F. conducted the statistical analysis; A.W. wrote the manuscript; X.W. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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