

Research Article

Entomopathogenic Nematode (Nematoda: Rhabditida) Survey and their Occurrence in Soil of District Lakki Marwat, Khyber Pakhtunkhwa Province, Pakistan

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Authors' Contributions

SK planned the study, executed surveys, collected and analyzed the data and prepared the manuscript. SJ supervised research and thoroughly revised the manuscript. TAK guided in basic features of nematodes. NK scrutinized data.

Keywords

Entomopathogenic nematodes, Survey, *Steinernema*, Occurrence, District Lakki Marwat

Abstract | Entomopathogenic nematodes are one of the perfect biological control agents against many insect pests all around the world. In this regard, to collect data about entomopathogenic nematodes regarding their presence and distribution in remote areas such as one of the southern district Lakki Marwat of Khyber Pakhtunkhwa province. Extensive surveys carried out in the months of October 2019 to February 2020. Total 73 soil samples retrieved from multiple fruits, vegetables, ornamental flowers, medicinal plants and other commercially important trees from different four sites of district Lakki Marwat and subsequently assessed the presence of EPNs using last instar larva of greater wax moth *Galleria mellonella* L. as baited host. After completion of requisite process, resultantly the corresponding recovery rate of EPNs was found 10.9% which includes only genus *Steinernema* comprising two isolates of *S. balochiense* (24%), two isolates of *S. siamkayai* (24%) and four unidentified *Steinernema* spp (52%). However, genus *Heterorhabditis* was not identified from collected soil samples. Characterization of these previously reported isolates made possible by using morphometric scrutiny. The recovered nematodes further compared with original strains. This research established occurrence result of EPNs from four sites of Lakki Marwat district. In future, these discovered nematodes may be applied in captioned district as biocontrol agents against insect pests of agricultural systems. One of the alternative method against insecticides for better management of plant health under greenhouse and field circumstances in integrated pest management program. Being potential of these nematodes as biopesticides may be distributed to farmers to reduce use of insecticides already in practice and to control hazardous effect of chemical pesticides on both crops and human beings.

Novelty Statement | The present research effort was elaborated to conduct surveys and to ascertain the evidences of entomopathogenic nematodes from one of the southern districts i.e., Lakki Marwat, Khyber Pakhtunkhwa province of Pakistan.

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Introduction

Nematode and insects could be believed as utmost successful groups of invertebrate animals in the nature.

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Generally, one of the unique class of nematodes (entomopathogenic nematodes) under the family Heterorhabditidae and Steinernematidae are bacterial feeders, survive in soil and few of the same have phoretic connections with insects (Morris *et al.*, 2020). Staggeredly, numbers of countries are being used successfully to control variety of agricultural and stored product insect pests (Aatif

et al., 2019; Yuksel *et al.*, 2019; Ahuja *et al.*, 2020; Mokrini *et al.*, 2020; Torrini *et al.*, 2020; Salma *et al.*, 2020; Dichusa *et al.*, 2021) fall under the orders Lepidoptera, Coleoptera, and Diptera (Burnell and Stock, 2000). Essential parameters such as moisture, soil texture, potential of Hydrogen (PH) and organic matters are vital aspects that affecting the virulence, existence and perseverance of these nematodes (Sandhi *et al.*, 2020).

Entomopathogenic nematodes upsurge predation success via inducing cadaver volatiles that fascinate healthy herbivores (Zhang *et al.*, 2019). It penetrates into host via natural openings, injecting symbiotic bacteria as *Xenorhabdus* for Steinernematids and *Photorhabdus* for Heterorhabditids (Leite *et al.*, 2019), reproduces quickly and ultimately responsible for the death of host within 24 to 48 hours due to septicemia, creating the situation amiable for growth and reproduction of nematodes (Chandra *et al.*, 2019). Upon occurrence of food storage in the cadaver, thereafter, millions of EPNs emerged from dead insect body for the search of new hosts (Kaplan *et al.*, 2020; Shapiro-Ilan *et al.*, 2018) (Figure 1).



Figure 1: Emergence of EPNs and their presence on surface of dead cadaver.

A native EPN species is more adaptive to local environmental conditions. The biocontrol tactics in combined pest mechanism are of rising attention in the background of documented hazard effects of chemical composition on ecologies and human health. Consequently, finding EPNs species and also defining their insecticidal efficiency is chief scientific consideration (Bhat *et al.*, 2019; Askary and Abd-Elgawad, 2021).

Lakki Marwat is an important southern district of Khyber Pakhtunkhwa Province of Pakistan and geographically situated at 32-17 to 32-53 N latitude and 70-23 to 71-16 E longitude. The characteristics of area has sand dunes, burning heat and dry weather bearing hot summer and moderately cool winter. Soil profile is considered to be loamy sand, sandy clay and clay loam. Furthermore, temperature commonly noted from 27-42°C in summer whereas 4-20°C during winter season (Samreen *et al.*, 2020). However, the area unfortunately facing acute shortage of water for agriculture and entirely depends upon rain water though, rare and recurrent rainfall usually arises in the months of July and August (ESA, 2012).

Pakistan containing high diversity of ecosystem and rich in fauna adapted to various agroclimatic region. The occurrence and distribution of entomopathogenic nematodes from different agricultural sites including efficiency against various insect pests have been studied from Pakistan. However, existence of EPNs in soil of district Lakki Marwat is relatively un-explored. In this regard, this study is designed for the first time to conduct surveys of entomopathogenic nematodes occurrence and identification from soil of district Lakki Marwat.

Materials and Methods

Research site

An extensive nematological surveys were executed in four sites/ villages of District Lakki Marwat, Khyber Pakhtunkhwa which were previously unexplored i.e., Aba Khel, Aghzar Khel, Sarai Naurang and Tajori.

Sampling material

For the study of entomopathogenic nematodes, a total of 73 soil samples (1000 gm from each sample) were collected around the roots of various fruits, vegetables, ornamental flowers, medicinal plants and other commercially important trees up to 25 cm depth with the help of garden tool i.e., hand trowel. All samples were retained/kept in zip lock plastic bags with labeling flags comprising of relevant information (host, locality, time and date of collection) (Figure 2A) and subsequently shifted safely to main entomopathogenic nematodes research laboratory at National Nematological Research Centre, University of Karachi, Pakistan for their analysis.

Recovery method of EPNs

Recovery of entomopathogenic nematodes from soil were ensured by *Galleria* trap method as suggested by Bedding and Akhurst, 1975. Approximately 500 gm of soil were put into sterilized plastic pots (28 x 16 x 8 cm) and then six Greater wax moth *Galleria mellonella* L. larva (Lepidoptera: Galleridae) were included in each pot (Figure 2B). Pots were enclosed with a fitted perforated cap, placed upside down and incubated for period of week at

room temperature ($25 \pm 2^\circ\text{C}$). Furthermore, observations were noticed on daily basis. Dead larvae were removed and sanitized with distilled water for elimination of soil particles. Subsequently for isolation of 3rd stage juvenile's, dead cadavers were shifted to White trap method (White, 1927) by utilizing small plastic containers (28 x 16 x 8 cm). Plastic cavity block (4.5 x 4.5 cm) was placed inverted at the bottom of container having filter paper on the top of cavity block. Distilled water was filled at the depth of 1 cm. Thereafter, containers were covered with lid and incubated at $30 \pm 5^\circ\text{C}$. The progenies were migrated to the surrounding water that emerged from cadaver. Subsequently, progenies/ infective juveniles (IJs) collected on the fourth day of emergence were used for identification.

Collected IJs were further stored in a storage chamber maintained at temperature i.e., $17\text{--}20^\circ\text{C}$ in 100 ml flask having distilled water mixed with a drop of Triton X-100.

For life stages of adult nematodes, five *G. mellonella* were parasitized by infective juveniles in 10 ml beaker having moist sterilized soil. In 1 ml water suspension approximately 100 IJs was applied on the top of the soil. After that, beaker was wrapped with aluminum foil and incubated at 25°C (Figure 2C-E). Dead larva was rinsed with distilled water and accordingly placed in petri dishes (Figure 2F). Hence, first and second generation of male and female were achieved by dissecting cadaver every day (up to 10 days) in Ringer's solution under stereomicroscope (Homonick *et al.*, 1997).

Processing of specimens for morphological study (light microscopy)

Obtained specimens were further heat killed, preserved in TAF, afterward proceeded with solution I (3–4 ml) containing 95% ethanol (20 ml), glycerin (1 ml), distilled water (79 ml) and kept in incubator at $35\text{--}40^\circ\text{C}$ for 12 hours. Thereafter, solution II (3–4 ml) (5 ml glycerin, 95 ml ethanol) was added and subsequently again placed in incubator at about 40°C for next 3 hours (Seinhorst, 1959). For permanent mounting, the processed nematodes were transferred to microscopic glass slide ($25.4 \times 76.2 \times 1$ mm) having a drop of glycerin. A small glass bits were used to evade destruction of nematode bodies. Approximately, 10 IJs, male and female of each were morphologically identified according to Nguyen and Hunt (2007) and subsequently measured under compound microscope.

Cross section

Cross section of lateral lines was performed in a drop of glycerin on microscopic slide and then nematode was sliced with a razor blade or sharp needle under stereomicroscope. Subsequently, slices were transferred to a drop of melted glycerin jelly in the center of slide. Cover slip (19 mm) was placed with pieces of glass fiber to prevent the glycerin jelly from touching the slide. Thereafter, lateral lines were viewed/ studied under high resolution compound microscope (Homonick *et al.*, 1997).

Data analysis

The occurrence percentage and relative frequency of nematodes was assessed as follows:

$$\text{Occurrence (\%)} = \frac{\text{Total number of positive samples}}{\text{Total number of samples}} \times 100$$

$$\text{Relative Frequency (\%)} = \frac{\text{Frequency of species in a sample}}{\text{Sum of frequencies of all species}} \times 100$$

Results and Discussion

EPNs survey and their recovery rate

To explore the biodiversity related to entomopathogenic

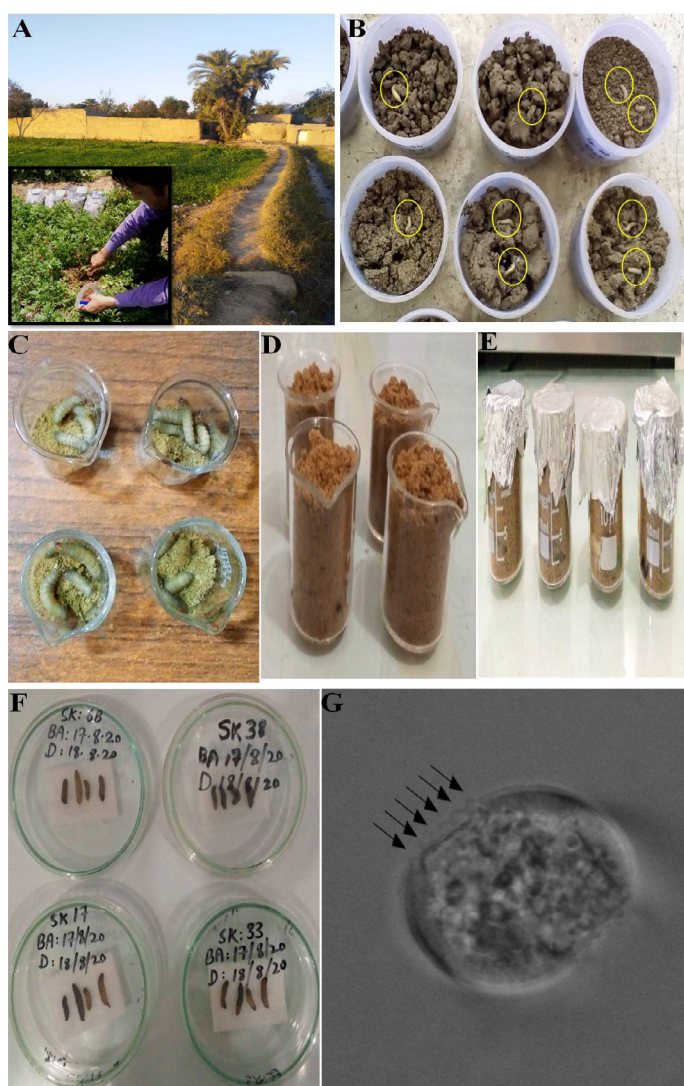


Figure 2: (A) Collection of soil sample from field of village Sarai Naurang, KPK. (B) Soil trap method in pots using wax moth larvae shown by circle line (C) 5 *G. mellonella* larva in sterilized 10ml beakers. (D) Beakers filled with sterilized soil. (E) Beakers sealed with aluminium foil and incubated at 25°C . (F) Dead larvae into petri dishes collected from sand bioassay for dissection. (G) Cross section of nematode body showing longitudinal ridges by arrows.

nematodes, 73 soil samples that resultantly revealed 8 positive samples (recovery rate is 10.9%). The current study reported first time EPNs surveys and occurrence in above cited four villages of district Lakki Marwat, Khyber Pakhtunkhwa province of Pakistan. The targeted areas for surveys and the specimens from where they were surfaced from its specific area are shown in map (Figure 3). Further, detailed information on EPNs status from each site is given in Table 1. During this survey, only genus *Steinernema* was surfaced comprising two isolates of *Steinernema balochiense* (Shahina *et al.*, 2015) (LM-38 and LM-17) (24%), two isolates of *Steinernema siamkayai* (Stock *et al.*, 1998) (LM-33 and LM-06) (24%) and four unidentified *Steinernema* spp. (52%) under the family Steinernematidae (Figure 4). These species were previously reported from Pakistan but during current survey these were surfaced from new geographical locations with some variations in their morphometry. Hence, after collected corresponding data the *Steinernema balochiense* (LM-38 and LM-17) was found as 3.4% from Sarai Naurang and 4.5% from Aghzar Khel, respectively. Contrarily, *Steinernema siamkayai* (LM-33 and LM-06) was found as 8.3% from Tajori and 10% from Aba Khel respectively. However, four unidentified *Steinernema* spp were originated only from village Sarai Naurang with 13.7%. Their vegetation type and relative frequencies was provided in Table 1. It has been assessed that the recovery rate of positive samples was greater in village Sarai Naurang (6.8%) followed by villages Aba Khel, Aghzar Khel and Tajori (1.3% each) or the recovery rate of EPNs from Sarai Naurang was five times greater as compared to other villages (Figure 5).

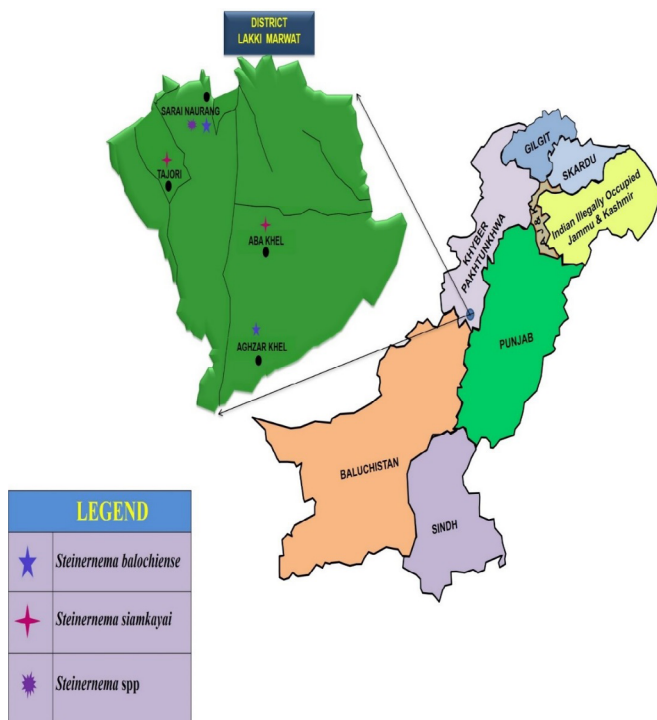


Figure 3: Location of District Lakki Marwat in KPK province showing surveyed sites and entomopathogenic nematode species encountered from specific locations.

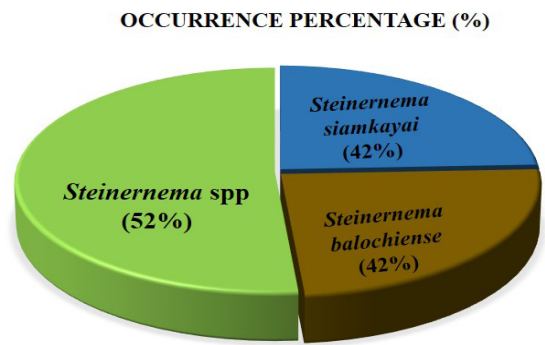


Figure 4: Occurrence percentage of *Steinernema* species.

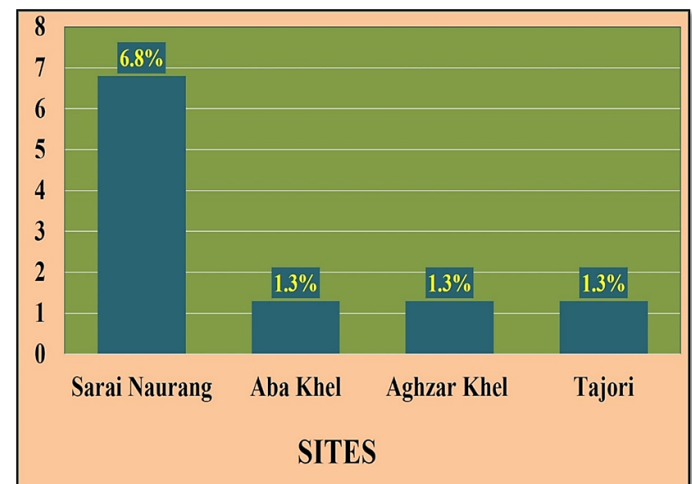


Figure 5: Site wise occurrence percentage of EPNs species.

Life cycle

Life cycle with respect to temperature and humidity of current isolates *Steinernema balochiense* and *Steinernema siamkayai* are presented in Table 2. Both isolates LM-38 and LM-17 completed life cycle (IJs-IJs) in 9-10 days. Whereas LM-33 and LM-06 completed within 7-8 days as previous Pakistani isolate completed life cycle within 5-6 days and though the original strain of *S. siamkayai* from 8-10 days.

Morphological characterization and comparison with original strains

Key morphological characterization was made with respect to 3rd stage infective juveniles' length. Upon conducted morphometric values/scrutiny, IJs of both current isolates belong to *carpocapsae* group as IJs length $\leq 600 \mu\text{m}$ (Nguyen and Hunt, 2007) and their lateral field which has six longitudinal ridges (Figure 2G). Next, these were further morphologically and morphometrically identified by first and second-generation males and females as *Steinernema balochiense* (Shahina *et al.*, 2015) and *Steinernema siamkayai* (Stock *et al.*, 1998) and subsequently each isolate compared between each other and presented in Tables 3, 4 and 5. Morphological similarities found in line with original strains but some variations have been detected in the following characters due to differences in geographical locations and other biotic or abiotic factors:

Table 1: Details of surveyed sites and entomopathogenic nematodes status.

Geographical origin	EPNs STATUS								
	GPS coordinates	Elevation (m)	NS	NPS	Genus	Species (Isolate code)	Vegetation	Occurrence %	RF %
Aba Khel	32°34'09"N 70°49'39"E	265	10	01	Steinernema	<i>S. siamkayai</i> (LM-06)	Pomegranate	10	25
Aghzar Khel	32°23'37"N 70°44'16"E	471	22	01	Steinernema	<i>S. balochiense</i> (LM-17)	Sarkanda	4.5	11.2
Sarai Naurang	32°49'43"N 70°46'33"E	299.10	29	01	Steinernema	<i>S. balochiense</i> (LM-38)	Paper flower	3.4	8.5
				04	Steinernema	<i>Steinernema</i> spp.	Sheesham, Karir, Guava, Grapes	13.7	34.3
Tajori	32°37'51"N 70°34'59"E	319	12	01	Steinernema	<i>S. siamkayai</i> (LM-33)	Date Palm	8.3	20.8

NS, Number of samples; NPS, Number of positive samples; RF, Relative frequency.

Table 2: Life cycle of current EPN isolate highlighted during study.

Species	Temperature (°C)	Humidity (%)	Life cycle (Days)
<i>S. balochiense</i> (LM-38; LM-17)	28.7-30	61-66	9-10
<i>S. siamkayai</i> (LM-33; LM-06)	30-33.7	38-40	7-8

Table 3: Comparative morphometric data of 3rd stage infective juveniles of *Steinernema balochiense* and *Steinernema siamkayai*. All measurements are in μm in the form of Mean $\pm$ SD and range in paranthesis.

Characters	<i>Steinernema balochiense</i> (LM-38)	<i>Steinernema balochiense</i> (LM-17)	<i>Steinernema siamkayai</i> (LM-33)	<i>Steinernema siamkayai</i> (LM-06)
Infective Juveniles (IJs)				
L	424.5 $\pm$ 46.46 (330-485)	466.7 $\pm$ 33.38 (412-535)	402.2 $\pm$ 39.99 (358-452)	458.8 $\pm$ 14.7 (444-486)
a	19.2 $\pm$ 1.12 (17.7-22)	19.1 $\pm$ 1.58 (17.1-23.2)	19.0 $\pm$ 0.53 (18.6-19.8)	19.7 $\pm$ 0.78 (18.5-20.5)
b	4.4 $\pm$ 0.40 (3.6-4.9)	4.6 $\pm$ 0.27 (4.3-5.3)	4.3 $\pm$ 0.31 (4.0-4.9)	4.8 $\pm$ 0.13 (4.7-5.1)
c	10.6 $\pm$ 0.58 (9.8-12.1)	10.3 $\pm$ 1.06 (9.1-13)	10.64 $\pm$ 0.60 (10.1-11.6)	10.6 $\pm$ 0.18 (10.5-11)
c'	3.37 $\pm$ 0.27 (3.0-3.7)	3.6 $\pm$ 0.37 (3-4.1)	3.4 $\pm$ 0.17 (3.2-3.6)	3.5 $\pm$ 0.19 (3.3-3.8)
Body width	21.9 $\pm$ 1.64 (18-24)	24.4 $\pm$ 0.91 (23-26)	21 $\pm$ 2.52 (18-24)	23.2 $\pm$ 0.97 (22-24)
Excretory pore (EP)	35.2 $\pm$ 2.6 (30-40)	38.4 $\pm$ 1.95 (35-41)	33.6 $\pm$ 0.8 (32-34)	36.6 $\pm$ 1.2 (35-38)
Nerve ring (NR)	72.2 $\pm$ 1.83 (70-75)	69.2 $\pm$ 2.63 (65-75)	56 $\pm$ 4.56 (52-56)	64.8 $\pm$ 0.97 (64-66)
Esophagous (ES)	94.9 $\pm$ 3.93 (90-100)	100.1 $\pm$ 3.36 (92-105)	90.8 $\pm$ 4.48 (84-98)	93.4 $\pm$ 0.8 (92-94)
Tail	39.9 $\pm$ 4.18 (30-45)	45.3 $\pm$ 3.63 (40-50)	37.8 $\pm$ 5.23 (32-44)	42.8 $\pm$ 1.6 (42-46)
Anal body width	11.7 $\pm$ 1.00 (10-13)	12.5 $\pm$ 0.80 (11-14)	10.8 $\pm$ 0.97 (10-12)	11.9 $\pm$ 0.48 (11-12.5)
D%	37.0 $\pm$ 2.52 (33.3-42.1)	38.3 $\pm$ 1.13 (36-40)	36.9 $\pm$ 1.24 (34.6-38)	39 $\pm$ 1.17 (38-40.8)
E%	88.6 $\pm$ 4.63 (83.5-100)	85.1 $\pm$ 6.64 (76-97.5)	90.3 $\pm$ 11.09 (77.2-103)	85.5 $\pm$ 2.73 (82.6-90.4)
H%	37.3 $\pm$ 9.63 (37-50)	45.1 $\pm$ 3.94 (39-50)	39.2 $\pm$ 6.26 (31.8-48.4)	48.5 $\pm$ 1.86 (47.6-52.3)
Hyaline	14.9 $\pm$ 4.3 (10-20)	21 $\pm$ 3.28 (16-25)	14.6 $\pm$ 1.49 (12-16)	20.8 $\pm$ 0.97 (20-22)

Note: a, body length/width; b, body length/esophagus; c, body length/tail; c', tail/anal width; D%, EP/ES $\times$ 100; E%, EP/TL $\times$ 100; H%, Hyaline/TL $\times$ 100.***Steinernema balochiense* (LM-38 and LM-17)**

IJs: They are different from original strain with respect to body length only.

1st and 2nd generation male: They are dissimilar from original strain by body width, esophagus, excretory pore, a and b ratios, SW%, D%, E% and anal width.1st and 2nd generation female: They are different from original strain by body length, width, esophagus, excretory

pore, D%, E%, b ratio, and tail length.

***Steinernema siamkayai* (LM-33 and LM-06)**

IJs: They are different from original strain with respect to nerve ring position and E%.

1st and 2nd generation male: They are different from original strain by body length, esophagus, nerve ring, excretory pore, anal width, D%, SW% and spicule length.

Table 4: Comparative morphometric data of *Steinernema balochiense* (Isolates LM-38 and LM-17). All measurements are in μm in the form of Mean $\pm$ SD and range in paranthesis.

Characters	LM-38				LM-17			
	1 st Gen. Male	1 st Gen. Female	2 nd Gen. Male	2 nd Gen. Female	1 st Gen. Male	1 st Gen. Female	2 nd Gen. Male	2 nd Gen. Female
L	1460 $\pm$ 179.7 (1234-1716)	4922.8 $\pm$ 444.3 (4558-5756)	1098.6 $\pm$ 213.12 (778-1414)	1896.8 $\pm$ 127.2 (1708-2060)	1478.8 $\pm$ 119.5 (1296-1610)	4270 $\pm$ 670.7 (3469-5212)	791.8 $\pm$ 33.43 (741-838)	114.4 $\pm$ 387.2 (1175-1497)
Body width	107 $\pm$ 17.2 (88-130)	282 $\pm$ 19.64 (250-305)	81 $\pm$ 24.16 (45-115)	95 $\pm$ 7.07 (85-105)	112.4 $\pm$ 11.27 (95-127)	153.6 $\pm$ 8.82 (140-165)	51.4 $\pm$ 4.40 (45-58)	69.6 $\pm$ 3.55 (65-75)
Excretory pore (EP)	80 $\pm$ 7.07 (70-90)	17.4 $\pm$ 11.53 (102-135)	66.8 $\pm$ 7.75 (56-75)	71 $\pm$ 8.60 (60-85)	66.6 $\pm$ 5.16 (60-75)	95.4 $\pm$ 6.5 (87-105)	58.4 $\pm$ 2.15 (55-61)	66.6 $\pm$ 4.02 (60-72)
Nerve ring (NR)	83 $\pm$ 20.3 (50-105)	147.2 $\pm$ 2.13 (144-150)	90.8 $\pm$ 4.26 (85-97)	119.8 $\pm$ 14.4 (99-140)	96.2 $\pm$ 12.56 (81-115)	130 $\pm$ 7.07 (120-140)	8.6 $\pm$ 4.67 (92-105)	118.6 $\pm$ 5.57 (110-126)
Esophagus (ES)	140.2 $\pm$ 7.35 (130-151)	225 $\pm$ 14.14 (205-245)	134 $\pm$ 10.67 (120-150)	175.2 $\pm$ 7.67 (165-187)	159.4 $\pm$ 17.7 (136-183)	220.2 $\pm$ 23.0 (190-251)	125.2 $\pm$ 6.79 (116-135)	157.2 $\pm$ 9.55 (145-171)
Tail	26.8 $\pm$ 4.30 (20-23)	44.6 $\pm$ 5.81 (37-53)	26.2 $\pm$ 3.54 (21-31)	40.6 $\pm$ 1.85 (38-43)	28.6 $\pm$ 2.41 (25-32)	41.2 $\pm$ 8.18 (28-52)	23.8 $\pm$ 0.74 (23-25)	49.4 $\pm$ 3.26 (45-54)
Anal width	37.8 $\pm$ 5.74 (30-47)	84.6 $\pm$ 13.72 (69-104)	32.2 $\pm$ 4.53 (26-39)	38.4 $\pm$ 9.26 (26-51)	36 $\pm$ 2.28 (32-40)	50.4 $\pm$ 10.13 (40-68)	26 $\pm$ 0.89 (25-27)	27 $\pm$ 2.8 (23-31)
Spicule	73 $\pm$ 2.82 (69-77)	-	59 $\pm$ 1.41 (57-61)	-	75.4 $\pm$ 1.01 (74-77)	-	59.2 $\pm$ 1.72 (57-62)	-
Gubernaculum	51.2 $\pm$ 1.16 (50-53)	-	43.2 $\pm$ 1.16 (42-45)	-	50.8 $\pm$ 3.12 (46-55)	-	42 $\pm$ 1.41 (40-44)	-
SW%	183.8 $\pm$ 10.94 (169-200)	-	145.2 $\pm$ 3.31 (140-149)	-	208.8 $\pm$ 18.35 (185-234.3)	-	162.6 $\pm$ 1.85 (160-165)	-
GS %	66.3 $\pm$ 2.85 (62.5-70)	-	63 $\pm$ 2 (60-66)	-	65.94 $\pm$ 3.74 (61.3-71.4)	-	67.5 $\pm$ 33.37 (63.4-73.1)	-
Vulva	-	2649 $\pm$ 185.7 (2395-2900)	-	1056.6 $\pm$ 77.13 (956-1177)	-	2319.2 $\pm$ 334.0 (1945-2851)	-	752.2 $\pm$ 60.5 (671-837)
V%	-	51 $\pm$ 2.0 (48.8-54.2)	-	56.5 $\pm$ 0.43 (55.9-57.1)	-	552 $\pm$ 1.49 (53.4-57.6)	-	56.8 $\pm$ 0.81 (56-58.2)
a	12.2 $\pm$ 1.28 (10.9-14.3)	22.8 $\pm$ 4.16 (17.2-28.8)	15.4 $\pm$ 2.50 (12-19.3)	19.12 $\pm$ 0.60 (18.2-20)	12.16 $\pm$ 1.42 (10.4-14.4)	27.12 $\pm$ 2.94 (23.1-31.5)	14.78 $\pm$ 0.99 (13.5-16.4)	17.92 $\pm$ 1.06 (16.8-19.8)
b	10.7 $\pm$ 1.06 (9.2-12.2)	24.0 $\pm$ 1.18 (22.2-25.2)	8.1 $\pm$ 1.33 (6.4-10.1)	10.3 $\pm$ 0.60 (9.7-11.4)	9.18 $\pm$ 0.40 (8.7-9.8)	20.32 $\pm$ 1.96 (17.6-23)	6.04 $\pm$ 0.30 (5.6-6.5)	7.7 $\pm$ 1.03 (6.9-9.6)
c	50.3 $\pm$ 8.37 (38.5-63)	122.5 $\pm$ 17.9 (99-143.9)	39.5 $\pm$ 3.56 (35.3-45.6)	44.6 $\pm$ 2.09 (42.1-47.9)	52.32 $\pm$ 7.38 (43.2-64.4)	117.0 $\pm$ 17.8 (92-140.5)	42.2 $\pm$ 4.59 (36-49.2)	36.2 $\pm$ 6.8 (29.3-47.7)
D%	54.9 $\pm$ 3.20 (50.3-59.6)	52.6 $\pm$ 4.49 (47.4-60)	48 $\pm$ 1.35 (46.2-50)	43.5 $\pm$ 6.02 (34.3-51.5)	41.29 $\pm$ 7.45 (32.7-53.5)	47 $\pm$ 3.56 (41.8-51.2)	47.9 $\pm$ 2.74 (44-51.7)	43.8 $\pm$ 2.11 (41.3-47.3)
E%	232.2 $\pm$ 5.38 (225-240)	174.7 $\pm$ 16.72 (153.8-200)	160.1 $\pm$ 42.65 (100-220.8)	153.4 $\pm$ 5.42 (145-160)	271.3 $\pm$ 27.82 (234.3-312.5)	262.6 $\pm$ 41.01 (201.9-321.4)	265.7 $\pm$ 3.36 (260.8-270)	176.2 $\pm$ 3.8 (170-181)

Note: SW%, spicule length/anal width x 100; GS %, gubernaculum length/spicule length x 100; V%, vulva length/body length x 1000.

1st and 2nd generation female: They are different from original strain by body length, width, esophagus, nerve ring, excretory pore, anal width and V%.

Search and identification of native entomopathogenic nematode species adapted to local agroclimatic condition, being as a biocontrol agent counter to major insect pests, display a critical component in developing integrated pest management (IPM) policy of a particular pest. The present investigations/surveys have been conducted to recover the native entomopathogenic nematodes species in various villages of district Lakki Marwat, KPK, Pakistan. In this

current survey only genus *Steinernema* was surfaced from all positive samples from each selected area including two isolates of each *Steinernema balochiense* and *Steinernema siamkayai* with four unidentified *Steinernema* spp. Such result is supported to the fact that there is a greater diversity of Steinernematid species than Heterorhabditid worldwide (Nguyen *et al.*, 2007; Hunt and Nguyen, 2016). The presence of higher proportion of nematodes in village Sarai Naurang than other villages is the fact of good irrigation system that providing better moisture level for survival and persistence of these nematodes in soil as moisture is the most crucial factor.

Table 5: Comparative morphometric data of *Steinernema siamkayai* (Isolates LM-33 and LM-06). All measurements are in μm in the form of Mean $\pm$ SD and range in paranthesis.

Characters	LM-33				LM-06			
	1 st Gen. Male	1 st Gen. Female	2 nd Gen. Male	2 nd Gen. Female	1 st Gen. Male	1 st Gen. Female	2 nd Gen. Male	2 nd Gen. Female
L	1190 $\pm$ 158.3 (992-1450)	2529 $\pm$ 282.8 (2215-2985)	973.6 $\pm$ 29.18 (938-1020)	2408 $\pm$ 497.6 (1660-3000)	1140 $\pm$ 161.7 (900-1390)	2624 $\pm$ 118.4 (2440-2780)	802 $\pm$ 68.79 (709-902)	2486 $\pm$ 241.6 (2210-2890)
Body width	81 $\pm$ 8.60 (70-95)	128.2 $\pm$ 9.94 (111-140)	54.4 $\pm$ 3.26 (50-59)	130.6 $\pm$ 20.21 (101-157)	80.8 $\pm$ 6.71 (70-90)	122.8 $\pm$ 10.66 (107-135)	63.6 $\pm$ 2.41 (60-67)	190 $\pm$ 0.07 (180-200)
Excretory pore (EP)	61.6 $\pm$ 3.55 (57-67)	80 $\pm$ 7.07 (70-90)	56.2 $\pm$ 5.11 (49-64)	68.8 $\pm$ 6.24 (60-77)	71.2 $\pm$ 5.49 (62-78)	71.4 $\pm$ 4.40 (65-78)	55.4 $\pm$ 3.55 (50-60)	66.8 $\pm$ 4.3 (60-73)
Nerve ring (NR)	104.8 $\pm$ 11.66 (89-120)	125.8 $\pm$ 16.4 (103-151)	140 $\pm$ 7.07 (130-150)	139 $\pm$ 3.40 (134-144)	105 $\pm$ 3.40 (100-110)	90.6 $\pm$ 7.96 (80-103)	128.6 $\pm$ 5.16 (122-137)	121.4 $\pm$ 4.27 (115-127)
Esophagus (ES)	134.4 $\pm$ 17.92 (107-160)	186 $\pm$ 10.67 (170-200)	148.8 $\pm$ 8.08 (137-160)	189.2 $\pm$ 19.64 (157-210)	139 $\pm$ 21.54 (110-165)	142.4 $\pm$ 2.15 (139-145)	152.2 $\pm$ 12.15 (133-168)	168 $\pm$ 10.29 (150-180)
Tail	26.4 $\pm$ 3.55 (21-31)	28.4 $\pm$ 5.53 (20-36)	22.2 $\pm$ 1.72 (20-25)	56 $\pm$ 4 (50-61)	32.4 $\pm$ 2.41 (29-36)	25.6 $\pm$ 3.72 (20-30)	19 $\pm$ 1.41 (17-21)	46.6 $\pm$ 3.44 (42-51)
Anal body width	51.2 $\pm$ 6.27 (43-61)	55.4 $\pm$ 4.02 (50-62)	27.2 $\pm$ 2.13 (24-30)	36.8 $\pm$ 2.56 (33-40)	32.8 $\pm$ 2.56 (30-35)	62.2 $\pm$ 1.72 (60-65)	29 $\pm$ 2 (26-32)	52.8 $\pm$ 4.7 (46-60)
Spicule	64.8 $\pm$ 3.12 (60-69)	-	55.2 $\pm$ 3.12 (51-60)	-	71 $\pm$ 1.41 (69-73)	-	52.6 $\pm$ 1.85 (50-55)	-
Gubernaculum	53.4 $\pm$ 2.15 (50-56)	-	37.8 $\pm$ 1.72 (35-40)	-	53.2 $\pm$ 1.72 (51-56)	-	38 $\pm$ 1.09 (37-40)	-
SW%	1.54 $\pm$ 0.10 (1.4-1.7)	-	1.54 $\pm$ 0.04 (1.5-1.6)	-	1.48 $\pm$ 0.07 (1.4-1.6)	-	1.66 $\pm$ 0.04 (1.6-1.7)	-
GS %	0.73 $\pm$ 0.02 (0.71-0.77)	-	0.8 $\pm$ 0.08 (0.7-0.9)	-	0.70 $\pm$ 0.007 (0.70-0.72)	-	0.8 $\pm$ 0.08 (0.7-0.9)	-
Vulva	-	1714.4 $\pm$ 86.3 (1612-1860)	-	1206 $\pm$ 155.0 (977-1399)	-	1601.8 $\pm$ 64.15 (1522-1697)	-	983.6 $\pm$ 54.80 (910-1058)
V%	-	55 $\pm$ 3.40 (50-60)	-	54.8 $\pm$ 3.12 (50-59)	-	53.2 $\pm$ 1.16 (52-55)	-	55.4 $\pm$ 3.72 (50-60)
a	12.2 $\pm$ 0.74 (11-13)	19.9 $\pm$ 6.07 (12.5-28.8)	16.4 $\pm$ 0.86 (15-17.5)	16.6 $\pm$ 1.85 (14-19)	11.6 $\pm$ 1.01 (10-13)	18 $\pm$ 3.03 (14-23)	16.8 $\pm$ 0.74 (16-18)	18 $\pm$ 1.09 (17-20)
b	12 $\pm$ 2 (9-15)	16.6 $\pm$ 5.2 (7-22)	8.24 $\pm$ 0.18 (8-8.5)	13.4 $\pm$ 2.15 (10-16)	12.2 $\pm$ 1.32 (10-14)	13.6 $\pm$ 2.41 (10-17)	7.82 $\pm$ 0.59 (7-8.8)	10.2 $\pm$ 0.74 (9-11)
c	41 $\pm$ 5.40 (34-49)	58 $\pm$ 2.82 (54-62)	32.6 $\pm$ 1.85 (30-35)	56.8 $\pm$ 2.56 (53-60)	36.6 $\pm$ 5.53 (29-45)	54 $\pm$ 2.8 (50-58)	32 $\pm$ 3.4 (27-36)	45.6 $\pm$ 3.44 (40-50)
D%	0.54 $\pm$ 0.03 (0.49-0.060)	0.39 $\pm$ 0.04 (0.33-0.46)	0.44 $\pm$ 0.01 (0.42-0.47)	0.34 $\pm$ 0.04 (0.3-0.4)	0.47 $\pm$ 0.01 (0.45-0.50)	0.34 $\pm$ 0.05 (0.30-0.42)	0.46 $\pm$ 0.008 (0.45-0.47)	0.37 $\pm$ 0.06 (0.3-0.4)
E%	2.02 $\pm$ 0.51 (1.9-2.6)	2.56 $\pm$ 0.38 (2-3.1)	2.26 $\pm$ 0.04 (2.2-2.3)	1.28 $\pm$ 0.07 (1.2-1.4)	2.28 $\pm$ 0.07 (2.2-2.4)	2.38 $\pm$ 0.23 (2.1-2.7)	2.2 $\pm$ 0.14 (2.0-2.4)	1.36 $\pm$ 0.10 (1.2-1.5)

Note: SW%, spicule length/anal width x 100; GS %, gubernaculum length/spicule length x 100; V%, vulva length/ body length x 100.

Steinernema balochiense originally isolated from Pakistan and described by morphologically, morphometrically as well as by DNA sequence polymorphisms by (Shahina *et al.*, 2015) from guava (*Psidium guajava* L.) while *Steinernema siamkayai* was formerly identified and described by (Stock *et al.*, 1998) from sandy clay loam soil under sweet tamarind (*Tamarindus indicus* L.) at Lohmsak district, Petchabun Province, Thailand. *S. siamkayai* is warm adapted and widely distributed nematode species in Pakistan. As per

previously held record, from Pakistan the said species (*S. siamkayai* isolate Pak-157) was collected from soil samples of wheat from NWFP (Tabassum *et al.*, 2007). Mehreen and Shahina (2013), studied detail intraspecific variations and phylogenetic relationship of twenty-eight *S. siamkayai* strains which were obtained from various areas of Pakistan by utilizing molecular markers and mitochondrial genes (12S mtDNA). Notwithstanding above, heat tolerance infectivity and reproduction were also measured at different temperatures under laboratory

circumstances and concluded that they caused maximum mortality at high temperature zones.

Although, the first ever authentic report on entomopathogenic nematodes survey and their occurrence in Pakistan was conducted as well as studied with the utmost efforts of (Shahina and Maqbool, 1996). For further exploration, different habitats (agricultural fields, experimental plots, lawns, parks, turf grass and sea shores) were explored for soil sampling (500 samples) from Karachi, Thatta and Badin districts of Sindh which exhibit 95% genus *Heterorhabditis* and 5% genus *Steinernema* (Shahina *et al.*, 1998). Subsequently, Tabassum *et al.* (2005) conducted an extensive survey from Dera Ghazi Khan, Bahawalpur, Vehari, Larkana, Sukkar, Nawab Shah, Hederabad, Tando Jam and Mirpurkhas areas of Sindh and Punjab, Pakistan and collected 603 samples with low i.e., 2.5% recovery rate for EPNs. Afterward frequent surveys have been collected time to time from different habitats of Pakistan and subsequently in these research periods, up till now fifteen species of EPNs including twelve species of *Steinernema* and three species of *Heterorhabditis* has been identified (Shahina *et al.*, 2019). Our result found low recovery rate of nematodes which may be the influence of any climatic factor of this particular region. The result of low recovery is also coincided or similar to another research conducted throughout the world as (Abate *et al.*, 2017) conducted survey in different sites of South Africa and accordingly found 4% EPNs from 28/640 soil samples. de Brida *et al.* (2017) isolated EPNs in 16 soil samples corresponding to 8% (16/201 samples) collected from agricultural sites with annual, fruit and forest crops in Brazil. A survey was conducted in nine provinces and three cities of Korea in which 23 soil samples (4.6%) were made optimistic for entomopathogenic nematodes out of 499 (Choo *et al.*, 1995). Similarly, (Kour *et al.*, 2020) detected EPNs from 35/478 sites (7.3%) around Viti Levu, Fiji Islands. Huma *et al.* (2019) first time explored six districts of Khyber Pakhtunkhwa, Pakistan including Swat, Kohat, Haripur, Chitral, Mardan, Peshawar and found positive vegetations i.e., 23.1% out of 108 samples from grassy land, tomato field, forest land and poplar trees land for EPNs (*Heterorhabditis* and *Steinernema*) existence. Another EPNs survey were also commenced from all over the world provided evidences on their occurrence (Kary *et al.*, 2009; Valadas *et al.*, 2013; Yuksel and Canhilal, 2019; Canhilal *et al.*, 2016; Laznik *et al.*, 2009; Edgington *et al.*, 2010; Ma *et al.*, 2010; Hazir *et al.*, 2003; Chaerani and Indrayani, 2018; Dichusa *et al.*, 2021). Global surveys were also supported for the presence of these valuable species and have to be commercially used for insect controlling. Devindrappa *et al.* (2019) isolated *S. siamkayai* from five districts of Uttar Pradesh, India with 8% retrieval from 150 samples and applied against soil dwelling stage pupae of *Helicoverpa armigera* (Hub.). Khatri-Chhetri *et al.* (2010) isolated *S. siamkayai* and *S. abbasi* from Nepal and

tested their parasitic potential against 3rd instar larvae of Scarabaeid beetle (*Chiloloba acuta*) (Pokhrel *et al.*, 2016). In another research, (Gowda *et al.*, 2020) conducted survey from districts of Purvanchal and Bundelkhand region of India and found 2.3% rate for EPNs out of 130 soil samples. Later on, *Spoladea recurvalis*, *Spodoptera litura*, *Spilosoma obliqua* and *Myllocerus subfaciatus* were checked for mortality response by applied EPNs (*S. siamkayai* and *Heterorhabditis indica*).

Worldwide surveys demonstrated that EPNs found in 0.7–50 percent soil samples (Bruck, 2004; Pillay *et al.*, 2009). Although extensive surveys professed only 2–11 percent EPN from greater than one thousand soil samples (Yoshida *et al.*, 1998; Hazir *et al.*, 2003; Hatting *et al.*, 2009; Ma *et al.*, 2010). Variation in mode of sampling, type of insect used for baiting as well as soil type can fluctuating the rate of EPNs existence. Not only these reasons but also vary by other parameters such as soil moisture, soil quality, potential of Hydrogen (pH), temperature and biotic factors (Cheruiyot *et al.*, 2013). Non existing results of EPN doesn't mean that they are entirely absent. However, they are enormously reliant on insect aggregation and have a tendency to be aggregated in soil more willingly than randomly distributed (Hominick, 2002; Campos-Herrera *et al.*, 2013). More EPNs detection and their higher diversity may be increased by huge number of samples collected from more sites (Hominick, 2002; Erbas *et al.*, 2014).

Though the main aim of the present survey was to collect the data about insect pathogenic nematodes which was restricted and implemented only in District Lakki Marwat for the first time in the history of previously conducted surveys in Pakistan.

Conclusions and Recommendations

Present survey conducted in four villages of District Lakki Marwat where no such research focused yet. In order to recover substantial diversity of native entomopathogenic nematodes species, numbers of soil samples were collected from agricultural sites and subsequently passed through soil trapping technique baited with host *Galleria mellonella* Linnaeus. Eight positive samples revealed species of *carpocapsae* group of nematodes belong to the genus *Steinernema* as *Steinernema balochiense*, a widespread *Steinernema siamkayai* and four *Steinernema* spp. with some morphometric variations from type specimens due to different agroclimatic environment of the district.

From present study it ascertained that environment of Lakki Marwat district rich in enormous diversity of entomopathogenic nematodes. This research is part of Ph.D. dissertation of principal author and based on data collection about EPNs distribution at district level. The

recorded numbers of native species of entomopathogenic nematodes in few surveyed sites does not exemplify the full array of occurrence and may be possibility of presence of genus *Heterorhabditis*. In future, further research will explore occurrence, distribution, discoveries of new species and their insect controlling strategies against agricultural pest at captioned sites and other parts of district. These native beneficial nematodes be applied at laboratory and field level against insect's pest which caused serious damage to major crops of the area including wheat, gram and other plantation. Hence, it is recommended that a sound policy may be designed and implemented at government level and the same be circulated to farmers to reduce the practice and hazardous effect of chemical pesticides on both crops and human beings.

Conflict of interest

The authors have declared no conflict of interest.

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