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Phylogenetic analysis and biological studies of entomopathogenic nematode *Steinernema abbasi* isolates

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Abstract

In this study seven entomopathogenic nematode *Steinernema* isolates were examined using genetic analysis by ITS rDNA and 12S mtDNA. Phylogenetic analysis of these isolates was inferred by using four different methods i.e., Maximum Evolution (ME), Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbor Joining (NJ) based on the two makers. Sequence composition and phylogenetic analyses of these isolates showed closeness with *Steinernema abbasi*. On the basis of ITS rDNA region these seven Pakistani isolates were compared with seven worldwide isolates of *S. abbasi* and species of *bicornutum* group. While one isolate PAK.S.*S*.15 (JN599140) was analyzed using 12S mtDNA with other known species. In all four trees, isolate PAK.S.*S*.15 form monophyletic group with *S. abbasi* (AY944002). In laboratory experiment, biological life cycle of three isolates of *S. abbasi* PAK.P.*S*.9 (EF469773), PAK.S.*S*.15 (JN571086) and PAK.S.*S*.16 (JN571096) and one other isolate of *Heterorhabditis indica* PAK.S.*H*.56 (GU130179) were applied against fungus growing termite *Microtermes mycophagus* (D.). The study were also influenced by mortality; days to death, infection cycle length and reproductive potential. Highest mortality 100% was obtained on PAK.S.*S*.16 @ 75 IJs/cm^2. The strains PAK.S.*S*.16 and PAK.P.*S*.9 showed shortest 1.21 and 1.2 days to death in termites, while the isolate PAK.P.*S*.9 showed the shortest infection cycle length (4.65). Moreover, the strain PAK.S.*S*.16 showed highest rate of reproductive potential (830) than other three strains. The study was influenced by mortality; days to death, infection cycle length and reproductive potential. To elaborate biological life cycle, the results were presented using dendrogram of the association groups hierarchical cluster analysis.

Keywords: Entomopathogenic nematode, phylogenetic analysis, ITS rDNA, 12S rDNA gene and *Steinernema abbasi*.

The Entomopathogenic nematodes (EPN) in the Steinernematidae and Heterorhabditidae families are considered interesting candidates for use in biological control programs since they have a mutualistically symbiotic association with enteric bacteria that makes them strongly virulent to insects (Adams *et al*., 2006; Kaya *et al*., 2006). These nematodes are symbiotically associated with entomopathogenic bacteria *Xenorhabdus* (Thomas & Poinar, 1979) and *Photorhabdus* (Boemare *et al*., 1993). These nematodes have been used successfully as biological control agents of insect pests. It is worth mentioning that a hundred laboratories in more than 60 countries are currently studying EPN and their bacterial symbionts. The research is diverse, covering the fields of taxonomy, phylogenetics, biogeography, biology, ecology, molecular biology, genetics and genetic improvement, physiology, biochemistry, behavioral ecology, formulation production and application technology (Stock, 2005). Currently, the most popular genetic markers for classifying entomopathogenic nematodes are the 28S and ITS rDNA. These markers have been used widely for the molecularly characterized analysis and phylogenetic relationships (Uribe-Lorío *et al*., 2007; Stock & Gress, 2006). *S. abbasi* Elawad *et al*., (1997) was first reported from Pakistan based on morphometric and molecular studies of isolates 507 (*S. abbasi*) Shahina *et al*., (2007). Originally, *S. abbasi* was isolated from the alfalfa field soil in Sultanate of Oman and has been shown to carry a bacterium, *Pseudomonas* (*Flavimonas*) *oryzihabitans* (Anzai *et al*., 1997; Elawad *et al*., 1999). According to Tsai *et al*., 2008 and Tailliez *et al*., 2006 symbiotic bacterium were determined to be a species of *Xenorhabdus* based on its physiological and biochemical characteristics. Both of these characteristics were determined to be similar to *X. indica* of *S. abbasi*

(Oman isolate) based on sequence analyses of 16S rDNA.

In this paper, molecular characterizations of ITS rDNA sequences of seven Pakistani isolates are mainly focused. They were studied in comparison with seven worldwide strains of *S. abbasi*. Phylogenetic relationships among these strains were inferred with four different methods; Maximum Evolution (ME), Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbor Joining (NJ). Entomopathogenic nematodes and subterranean termites can thrive in similar environments. This has led investigators to test the capability of specific nematode species to infect and kill termites (Yu *et al*., 2008). Furthermore, the mortality rate was determined using different concentrations of nematodes against fungus growing termites *Microtermes mycophagus* D. (Isoptera: Termitidae: Macrotermitinae) which is also known as desert termite. The fungus growing termites are important in agriculture due to their feeding characteristics to dead organic materials (Salihah *et al*., 1988). Various treatments have been adopted to control termites, in which chemicals are used on first priority as control measures. However, they are expensive and have many harmful effects on human health as well on environment (Forschler & Townsend, 1996). Previously, few studies have been conducted in which nematodes were used as biological agent to control termites (Yu *et al*., 2006; Wang *et al*., 2002; Trudeau, 1989). In the present study, possible biological life cycle of *S. abbasi* isolate was conducted to evaluate termite mortality, time to death, days to first IJs emergence and reproductive potential (no. IJs/mg insect).

Materials and Methods

Nematode extraction: The seven isolates of EPN were collected using the *Galleria* baiting technique Bedding & Akhurst, 1975 (Table 1). Nematodes were cultured in last-instar larvae of wax moth *G. mellonella* which were exposed to 3,000 infective juveniles (IJs) in a 9 cm diam. Petri dish, lined with moist filter paper (Whatman No.1) and sealed with Para film. The Petri dishes were incubated at 35 ± 2 °C. Larvae died after 24 h of exposure. The insect cadavers were transferred to white trap (White, 1927) and incubated at 30 ± 2°C for 4-5 days for the recovery of new generations of IJs. The recovered IJs were preserved in 1.5 ml Eppendorf tube (70 % ethanol) and stored at -20 °C before use.

DNA isolation: Total genomic DNA was isolated with worm buffer lysis according to Shahina & Mehreen (2014). First the ethanol was removed by washing nematode three time with double distill water. For each isolate, 100 µl of nematode sample (ca. 2,000-3,000 IJs) was used for DNA extraction in 1.5 ml Eppendorf reaction tube. After extraction, isolated DNA was stored at -20°C until use.

PCR amplification: PCR amplification was performed according to Mehreen & Shahina 2013, with some modifications. The standard 50 µl PCR mixtures contained: 5µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.4, 15 mM $MgCl_2$, 1 % Triton X-100), 1 µl of both reverse and forward primer (10 µM), 2 µl of dNTP mixture (5 mM for each), 1 µl Tag DNA polymerase (Promega), 30 µl distilled water and 10 µl of DNA extract. Primers used for internal transcribed region ITS rDNA were as follows: TW81 5'-GTTTCCGTAGGTGAACCTGC-3' forward primer and AB28 5'-ATATGCTTAAGTTCAGCG-3' the reverse primer (Joyce *et al*., 1994) and for mitochondrial gene 12S rDNA 505 5'-GTTCCAGAATAATCGGCTAGAC-3' forward and 506 5' TCTACTTTACTACTACAACTTACTCCCC-3' reverse primer (Nadler *et al.*, 2006). The cyclic conditions were 4 min., at 94 °C, 35 cycle of 30 sec., at 94 °C, reannealing 30 sec., at 48 °C and extension at 72 °C for 2 min., followed by 10 min incubation at 72 °C. The reaction products separated in a 1% agarose gel containing 0.5l g of ethidium bromide per ml and were visualized under UV light with an imager. PCR products were purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into pGemT easy vector System I (Promega, Madison, WI) according the manufacturer's instructions. The plasmids were transformed into

Escherichia coli strain JM109 competent cells (Promega, Madison, WI). Clones containing putative rRNA inserts were identified through blue/white color selection. The plasmid preparations were extracted using the QIAquick Spin Miniprep Kit. From the positive results plates, two clones for each individual nematode were sequenced using an ABI Prism 377 sequencer (PE Applied Biosystem, Foster City, CA, USA) and dye terminator sequencing reagents at the University of Karachi, Karachi.

Phylogenetic analyses: The sequences of ITS rDNA of Pakistani strains (GenBank accession number: PAK.S.*S*.15-JN57108, PAK.S.*S*.16-JN571096, PAK.K.*S*.251-JX068812, PAK.K.*S*.254-JX068813, PAK.K.*S*.261-JX144735 and PAK.K.*S*.262-JX232283) were compared with already worldwide published *S. abbasi* sequences available in NCBI GenBank database using Blast (http://www.blast.ncbi.nlm.nih.gov) (Table 1). While, 12S mtDNA sequence PAK.S.*S*.15 (GenBank accession number JN599140) used for phylogenetic analysis (Nadler *et al*., 2006). Sequences of studied species were aligned using the default parameters of Clustal X (Thompson *et al*., 1997), then optimized manually in MacClade 4.05 (Maddison & Maddison, 2000). Phylogenetic analyses were performed by four methods: Maximum Evolution (ME), Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbor Joining (NJ) trees using MEGA 5.0 (Tamura *et al*., 2011) and PAUP, version 4.0b10 software package (Swofford, 2001). *Caenorhabditis elegans* (X03680) were treated as the outgroup taxon to resolve the relationships among other species for both markers. Branch support was estimated by bootstrap analysis (1000 replicates) using the same parameters as the original search. By using MrEnt 2.0 (Zuccon & Zuccon, 2014), the resulting trees were envisioned.

Table 1. Isolates designation and origin used in this study and their GenBank accession number.

Nematode isolates	Location	GenBank No.	Source (NCBI)*
*Steinernema abbasi***	Oman	AY248749	Nguyen & Adams (2003)
*S. abbasi***	Oman	AY230158	Spiridonov, Reid, Podrucka, Subbotin & Moens (2004)
GDe134	China	AY170343	Qiu, Fang, Zhou & Pang (2002)
SGas1	India	FJ715946	Ganguly & Kumar (2009)
SGkr1	India	FJ715947	Ganguly & Kumar (2009a)
PAL-S-09	Palestine	DQ655675	Iraki & Dar-Issa (2006)
PAL-S-08	Palestine	DQ655676	Iraki & Dar-Issa (2006a)
PAK.P.*S*.9	Pakistan	EF469773	Shahina, Nguyen, Kazmi, Tabassum & Firoza (2007)
PAK.S.*S*.16	Pakistan	JN571096	Shahina, Salma & Mehreen (2012)
PAK.S.*S*.15***	Pakistan	JN571086	Shahina, Salma & Mehreen (2012a)
PAK.K.*S*.251	Pakistan	JX068812	Shahina, Mehreen & Salma (2012b)
PAK.K.*S*.254	Pakistan	JX068813	Shahina, Mehreen & Salma (2012c)
PAK.K.*S*.261	Pakistan	JX144735	Shahina, Mehreen & Salma (2012d)
PAK.K.*S*.262	Pakistan	JX232283	Shahina, Mehreen & Salma (2012e)
S. bicornutum	Serbia	AY171279	Tallosi, Peters & Ehlers (1995)
S. ceratophorum	China	AY230165	Jiang, Reid & Hunt (1997)
S. pakistanense	Pakistan	AY230181	Spiridonov, Reid, Podrucka, Subbotin & Moens (2004)
S. riobrave	United States	DQ835613	Cabanillas, Poinar & Raulston (1994)
S. yirgalemense	Ethiopia	AY748450	Nguyen, Tesfamariam, Gozel, Gaugler & Adams (2004)

* NCBI = National center for biotechnology information; ** No code have found so that why species name is given; *** JN599140 (PAK.S.S.15) =12S rDNA (mitochondrial DNA).

Biological control assays

Collection and maintenance of termite culture: The termites (workers) of *Microtermes mycophagus* were collected from Botanical garden, University of Karachi. The termites were kept in cylindrical plastic containers (15.5 cm diam., 4 cm h) with 1-2 cm deep vermiculite and sand (1:1 by volume). Corrugated cardboard was added as food (Wang *et al*., 2002).

Bioassays: Virulence tests of three Pakistani isolates of *S. abbasi* strains PAK.P.*S*.9 (EF469773), PAK.S.*S*.15 (JN571086), PAK.S.*S*.16 (JN571096) and one strain of *H. indica* PAK.S.*H*.56 (GU130179) were conducted against *M. mycophagus* workers in laboratory at 25 ± 2º C and 75-80% relative humidity (Yu *et al*., 2006). The EPN strains were stored at 10-15 ºC before use. Experiments were conducted in 9 cm Petri dishes which were lined with moistened filter paper and placed ten termites. Three different suspensions of infective juveniles @ 25, 50 and 75 IJs/cm^2 applied. There were four replicates for each strain treatment and the entire experiment repeated three times. Mortality of termite was determined after 72 hrs post-inoculation. To evaluate time to death, days to first IJs emergence and reproductive potential (number of IJs/mg insect), IJs suspensions of 75 IJs/cm^2 was prepared and applied in 600 µl mQ water. In control treatments only water was applied. All experiments were replicated 4 times and were repeated twice. Dead termites were washed and individually placed in white traps at room temperature. After 10 days, IJs in distilled water were collected and fresh distilled water was added, the process was repeated (Kaya & Stock, 1997) until emergence stopped. The number of IJs produced per insect was determined through dilution counts and because the insect size correlated to number of nematodes produced. The length of the infection cycle was estimated by the number of days from start of infection to the first emergence. Nematode reproductive capacity was measured in termite based on procedures described by Campos *et al*., (2007) which was expressed as number of IJs/mg insect.

Statistical analysis: A multifactor analysis of variance (ANOVA) was generated to determine the difference in mortality (%), average time to death, length of the infection cycle and reproductive capacity. Mortality data was corrected according to Abbott's formula (Abbott, 1925) and normalized using the arcsine square-root transformation. Tukey's test at $P \leq 0.05$ was used to separate mean difference among parameters. The combined variables, percentage mortality, time to death, first IJs emergence and reproduction potential were subjected to hierarchical cluster analysis performed by establishing associated groups among EPN strains; squared Euclidean distance and values were standardized with Z transformation. All the data analysis was performed by using SPSS (SPSS Inc., Chicago, IL, USA) 14.0 computer application software for window XP.

Results and Discussion

Molecular characterization and phylogenetic analysis: The nematode ITS rDNA sequences for all seven isolates showed high similarity with *S. abbasi* in the NCBI GenBank database. The isolates were deposited in GenBank under accession number of ITS rDNA: JN571086 (PAK.S.*S*.15), JN571096 (PAK.S.*S*.16), JX068812 (PAK.K.*S*.251), JX068813 (PAK.K.*S*.254), JX144735 (PAK.K.*S*.261) and JX232283 (PAK.K.*S*.262). ITS sequences of the nematode isolates varied in the length of the aligned region. For isolates with similarity to *S. abbasi*, the length of the aligned ITS region excluding the gap were ranged from isolate PAL-S-09 (Palestine) 482 bp to SGas1 (India) 1043 bp. While Pakistani isolates similar to *S. abbasi* was isolate PAK.K.*S*.251 803 bp to PAK.S.*S*.15 856 bp as shown in Table 2. On the bases of ITS rDNA sequences the number of amino acid differences per site among sequences of *S. abbasi* isolates were calculated is shown in Table 3. The results showed that isolates

previously identified isolate PAK.P.*S*.9 showed closeness with original S. *abbasi* (Oman), PAK.K.*S*.254 show similarity with PAL.S.09, PAK.S.*S*.15 show closeness with PAL.*S*.08, while PAK.K.*S*.251 show difference among other isolates of *S. abbasi*. In case of 12S mtDNA only one isolate was sequenced which was further deposited to GenBank under accession number of JN599140 (PAK.S.*S*.15) 503 bp. Pakistani isolate showed 99% similarity with S. *abbasi* AY944002 (462 bp) with the 41 bp difference.

Table 2. Sequence length and composition of *S. abbasi* isolates based on the ITS rDNA gene sequences.

Species	A (%)	T (%)	C (%)	G (%)	Size (bp)
Steinernema abbasi (Oman)	24.9	37.5	15.4	22.2	837
S. abbasi (Oman)	25.0	35.4	16.6	23.0	988
S. abbasi SGas1 (India)	24.8	35.4	16.9	22.9	1043
S. abbasi GDe134 (China)	25.3	35.2	17.0	22.6	990
S. abbasi SGkr1 (India)	25.1	35.7	15.9	23.4	990
S. abbasi PAL-*S*-09 (Palestine)	23.9	32.6	17.4	26.1	482
S. abbasi PAL-*S*-08 (Palestine)	23.5	32.7	18.7	25.2	493
Steinernema sp. PAK.P.*S*.9 (Pakistan)	23.9	37.6	15.8	22.7	852
Steinernema sp. PAK.S.*S*.15 (Pakistan)	25.7	37.5	15.5	21.3	856
S. abbasi PAK.K.*S*.251 (Pakistan)	25.7	35.1	15.3	23.9	803
Steinernema sp. PAK.K.*S*.254 (Pakistan)	25.8	37.3	15.1	21.8	841
Steinernema sp. PAK.K.*S*.262 (Pakistan)	25.9	37.7	14.9	21.5	843
Steinernema sp. PAK.S.*S*.16 (Pakistan)	25.4	37.3	15.5	21.8	853
S. abbasi PAK.K.*S*.261 (Pakistan)	25.8	37.0	15.5	21.7	840

For the phylogenetic relationship of tree based on ITS rDNA with Maximum Evolution method Pakistani isolates JX232283 (PAK.K.*S*.262), JX068813 (PAK.K.*S*.254), JN571086 (PAK.S.*S*.15), JN571096 (PAK.S.*S*.16), EF469773 (PAK.P.*S*.9) and JX068813 (PAK.K.*S*.254) form cluster with FJ15946 (SGas1), *S. abbasi* (Oman) AY248749; AY230158 (Fig. 1A). The tree based on Maximum Likelihood Pakistani isolates JN571096 (PAK.S.*S*.16), JX144735 (PAK.K.*S*.261), JN571086 (PAK.S.*S*.15), JX232283 (PAK.K.*S*.262), JX068813 (PAK.K.*S*.254) and EF469773 (PAK.P.*S*.9) form sister clad with *S. abbasi* (Oman) AY248749, AY170343 (GDe134) China (Fig. 1B). The tree based on Maximum Parsimony method isolates JN571096 (PAK.S.*S*.16), JN571086 (PAK.S.*S*.15), JX232283

(PAK.K.*S*.262), JX144735 (PAK.K.*S*.261), JX068813 (PAK.K.*S*.254) and EF469773 (PAK.P.*S*.9) form sister clade with *S. abbasi* (Oman) AY248749; AY230158 and AY170343 (GDe134) China (Fig. 1C). The tree based on Neighbor Joining Pakistani isolates JN571096 (PAK.S.*S*.16), JX144735 (PAK.K.*S*.261), JX068813 (PAK.K.*S*.254), JN571086 (PAK.S.*S*.15), JX232283 (PAK.K.*S*.262) and JX068812 (PAK.K.*S*.251) form clade with *S. abbasi* (Oman) AY248749, AY170343 (GDe134) China and Pakistani isolate EF469773 (PAK.P.*S*.9) form clade with DQ655675 (PAL-S-09), DQ655676 (PAL-S-08) and FJ715946 (SGas1) as shown in Fig. 1D. Whereas, in case of Pakistani isolate JX068812 (PAK.K.*S*.251) other than Neighbor Joining tree form separate branch than other Pakistani isolates which showed some variation or difference than other Pakistani isolates. Pakistani isolates PAK.S.*S*.15 sequences used in phylogenetic analyses were same used by Nadler *et al*., (2006) and in all four trees JN599140 (PAK.S.*S*.15) formed monophyletic group with *S. abbasi* (AY944002) which showed maximum similarity with *S. abbasi* as shown in Fig. 2 A, B, C and D.

Table 3. The number of amino acid differences per site between sequences of *S. abbasi* isolates based on the ITS rDNA gene sequences.

Species	1	2	3	4	6	7	8	9	10	11	12	13	14	15
Steinernema abbasi	-													
S. abbasi	0.00													
SGas1	0.00	0.00												
GDe13	0.01	0.01	0.01											
SGkr1	0.01	0.01	0.01	0.02										
PAL.S.09	0.06	0.05	0.05	0.05	0.06									
PAL.S.08	0.08	0.07	0.07	0.07	0.08	0.08								
PAK.P.*S*.9	0.00	0.00	0.00	0.01	0.01	0.05	0.07							
PAK.S.*S*.15	0.08	0.07	0.07	0.08	0.08	0.13	0.15	0.07						
PAK.K.*S*.251	0.30	0.30	0.30	0.29	0.30	0.34	0.36	0.30	0.29					
PAK.K.*S*.254	0.06	0.05	0.05	0.06	0.06	0.10	0.13	0.05	0.07	0.30				
PAK.K.*S*.262	0.04	0.04	0.04	0.04	0.05	0.09	0.11	0.04	0.05	0.28	0.05			
PAK.S.*S*.16	0.05	0.05	0.05	0.05	0.06	0.10	0.12	0.05	0.06	0.29	0.05	0.03		
PAK.K.*S*.261	0.05	0.05	0.05	0.06	0.06	0.10	0.12	0.05	0.07	0.28	0.05	0.04	0.03	-

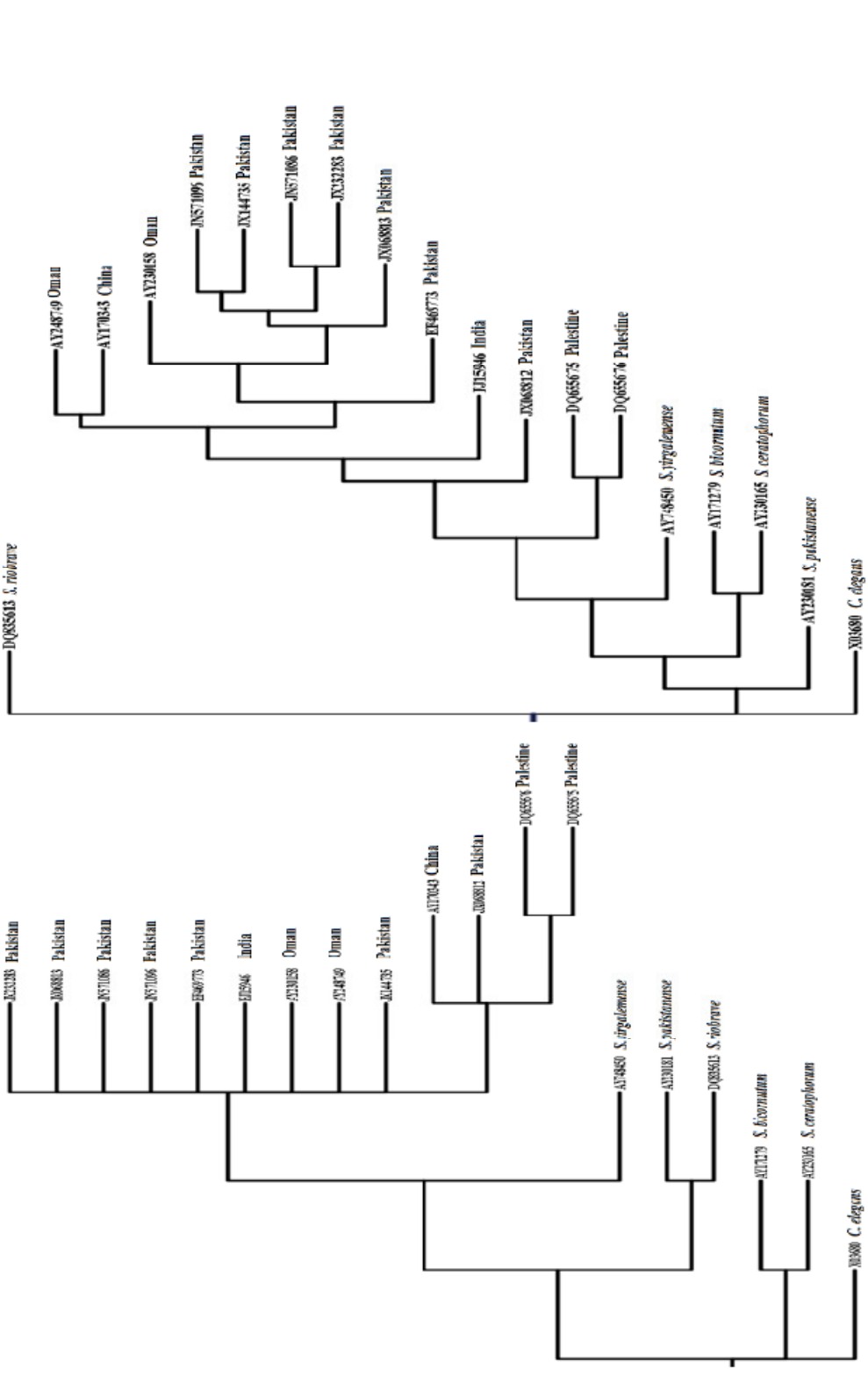

Fig. 1A. The phylogenetic tree Maximum Evolution (ME) of *S. abbasi* isolates inferred from the partial ITS rDNA gene sequences. *Caenorhabditis elegans* (X03680) was used as outgroup.

Fig. 1B. The phylogenetic tree Maximum Likelihood (ML) of *S. abbasi* isolates inferred from the partial ITS rDNA gene sequences. *Caenorhabditis elegans* (X03680) was used as outgroup.

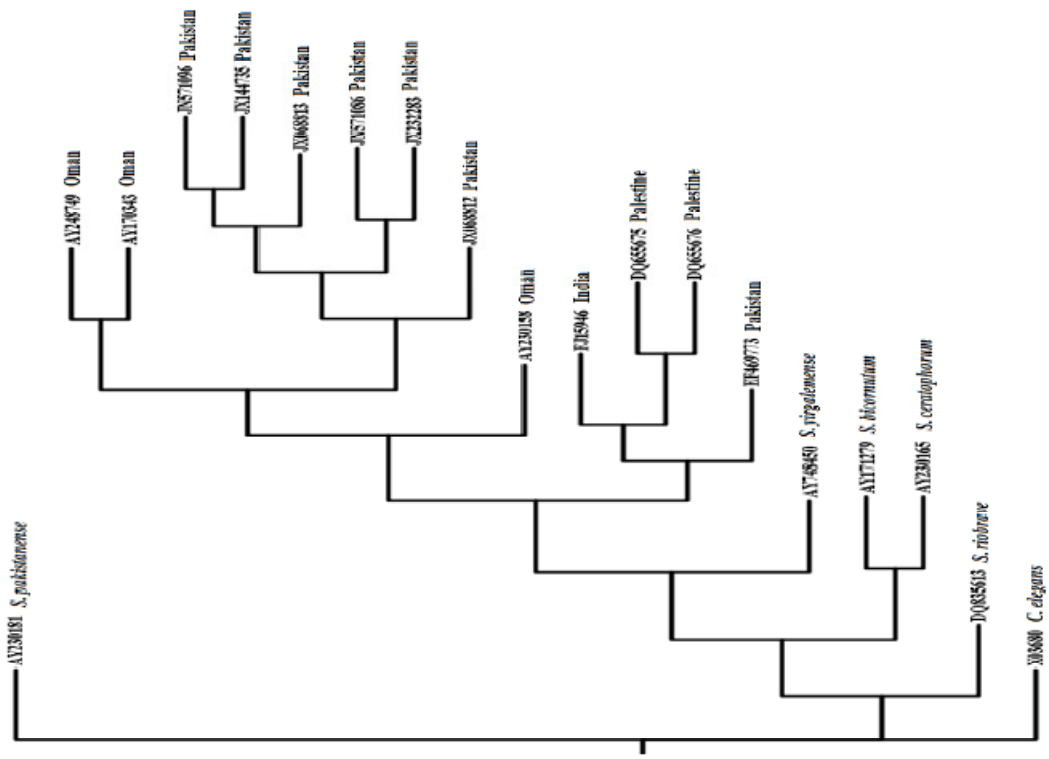

Fig. 1D. The phylogenetic tree Neighbor Joining (NJ) of *S. abbasi* isolates inferred from the partial ITS rDNA gene sequences. *Caenorhabditis elegans* (X03680) was used as outgroup.

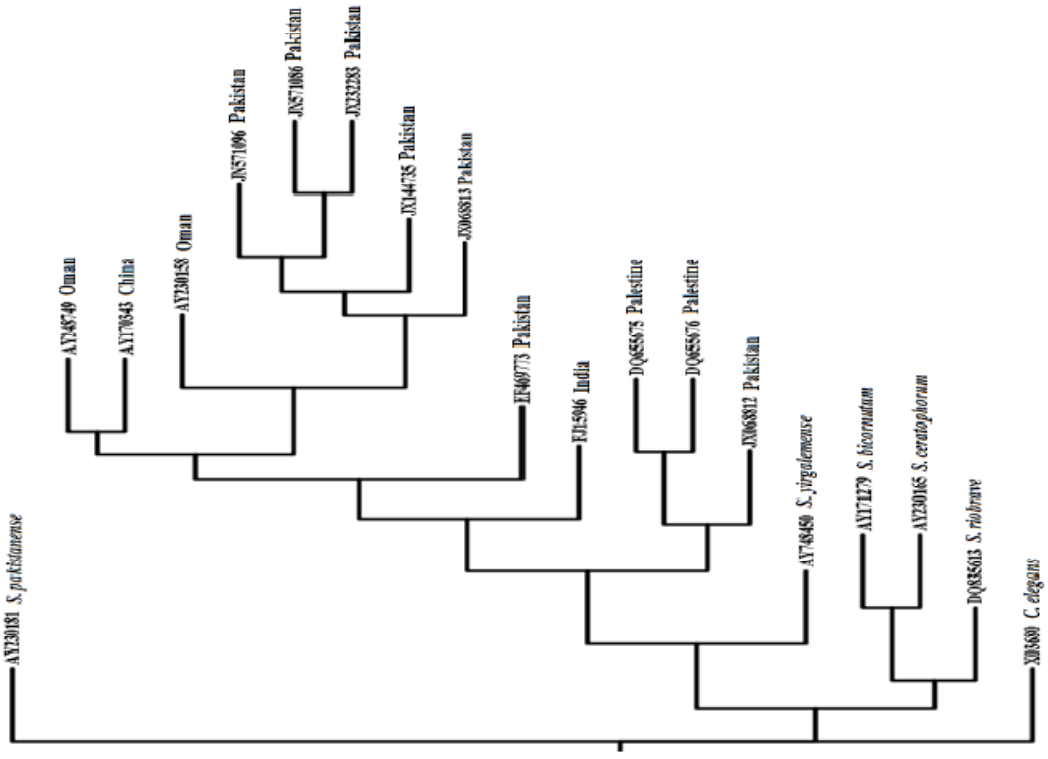

Fig. 1C. The phylogenetic tree Maximum Parsimony (MP) of *S. abbasi* isolates inferred from the partial ITS rDNA gene sequences. *Caenorhabditis elegans* (X03680) was used as outgroup.

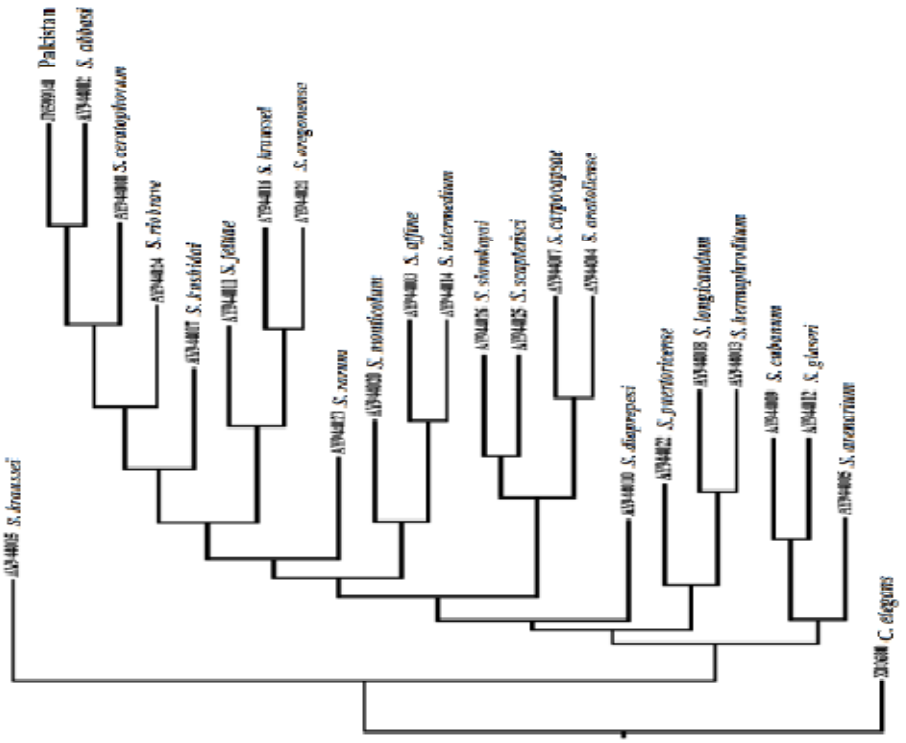

Fig. 2A. The phylogenetic tree Maximum Evolution (ME) of *S. abbasi* isolates inferred from the partial 12S rDNA gene sequences. *Caenorhabditis elegans* (X03680) was used as outgroup.

Fig. 2B. The phylogenetic tree Maximum Likelihood (ML) of *S. abbasi* isolates inferred from the partial 12S rDNA gene sequences. *Caenorhabditis elegans* (X03680) was used as outgroup.

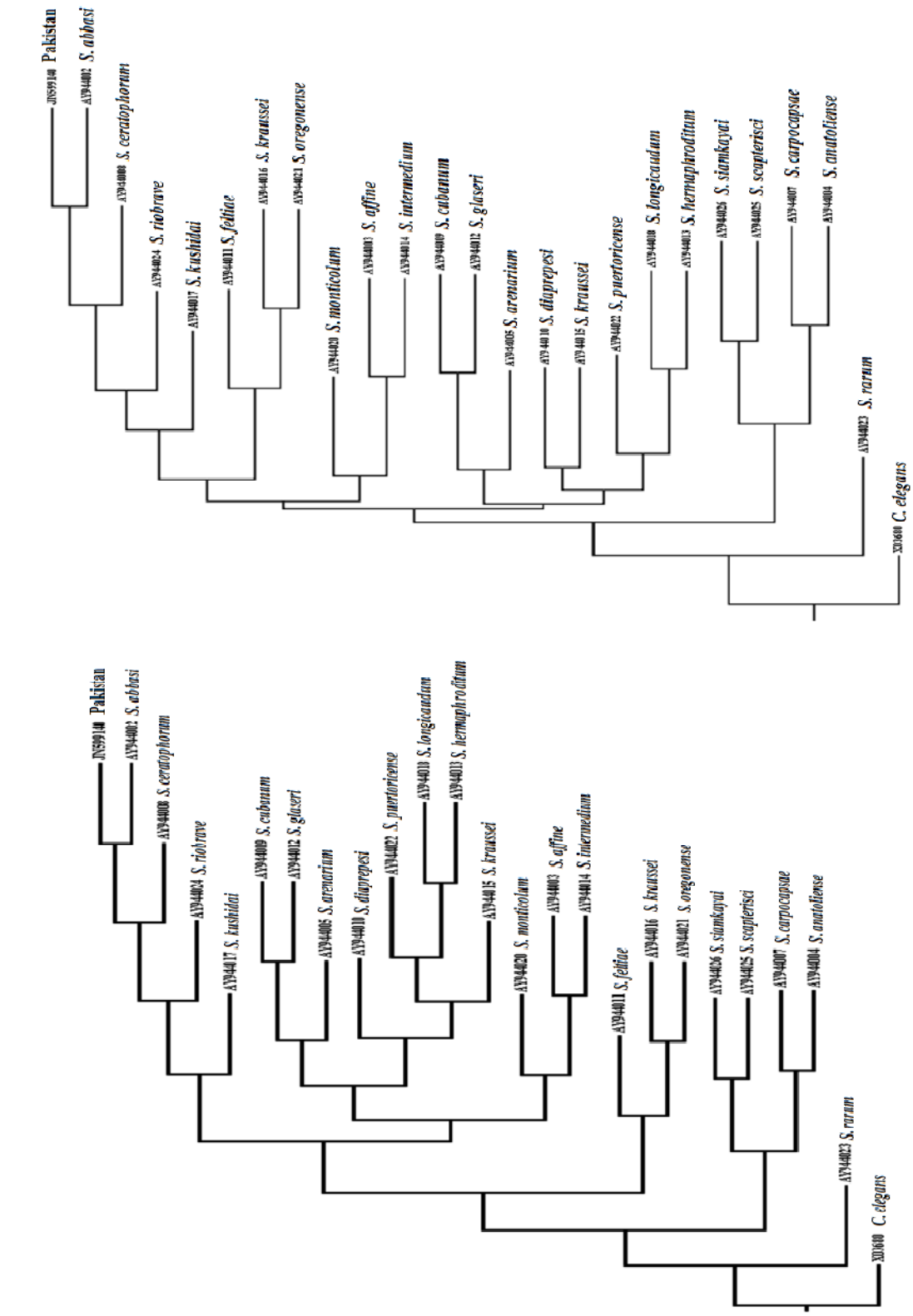

Fig. 2C. The phylogenetic tree Maximum Parsimony (MP) of *S. abbasi* isolates inferred from the partial 12S rDNA gene sequences. *Caenorhabditis elegans* (X03680) was used as outgroup.

Fig. 2D. The phylogenetic tree Neighbor Joining (NJ) of *S. abbasi* isolates inferred from the partial 12S rDNA gene sequences. *Caenorhabditis elegans* (X03680) was used as outgroup.

Biological control assays: There was highly significant difference in three applied doses @ 25, 50 and 75 IJs/cm^2 (F = 57.29; df = 2, 23; $P <$ 0.001) for all data sets. Tukey's test ($P \leq 0.05$) detected a significant difference between *S. abbasi* isolates and *H. indica* (Fig. 3). The nematode strain (PAK.S.*S*.16) @ 75 IJs/cm^2 caused significantly higher mortality (100%) compared to the other three strains, while it shows 65% mortality @ 25 IJs/cm^2. Similarly, strain (PAK.S.*S*.15) @ 75 IJs/cm^2 caused significantly higher 96.6 % mortality than other two isolates on the same dose. While the strain PAK.S.*H*.56 showed minimum mortality (80%) on highest dose (@ 75 IJs/cm^2).In all applied doses the total mortality was significantly higher than the mortality in control treatment. Maximum mortality (100%) was obtained when termites were treated with *S. abbasi* (PAK.S.*S*.16) at 75 IJs/cm^2. There was no significant difference in control treatment. The corrected mortalities of termites were significantly influenced by nematode isolates (F = 37.41; df = 3, 8; $P \leq 0.001$) with mean mortalities ranging from 80 to 100%. Four isolates (three *S. abbasi* and one *H. indica*) cause more than 55 and 73% mortality when the termites were exposed to 50 IJs/cm^2. The isolates PAK.S.*S*.15 and PAK.S.*S*.16 (96 and 100%) caused highest mortality than PAK.S.*H*.56 (80%) when termites were exposed to 75 IJs/cm^2. Isolates had a significant difference on the time of termites death (F = 410.42; df =3, 8; $P \leq 0.001$), the shortest time to death were observed in PAK.S.*S*.16 and PAK.P.*S*.9 (Fig. 4). Progeny production (days from infection of IJs emergence) were also significantly influenced in all isolates (F = 1003; df =3, 8; $P \leq 0.001$) the shortest emergence time of IJs was observed in *S. abbasi* strains (PAK.S.*S*.16 and PAK.P.*S*.9) 4.7 and 4.65 days, respectively (Fig. 5). Reproduction rate were also significantly different among the nematode isolates (F = 2293.70; df = 3,8; $P \leq 0.001$) the highest progeny production was observed in PAK.S.*S*.16 isolate 830 IJs/cm^2; while the lowest reproduction rate was recorded in *H. indica* PAK.S.*H*.56 700 IJs/cm^2 (Fig. 6).

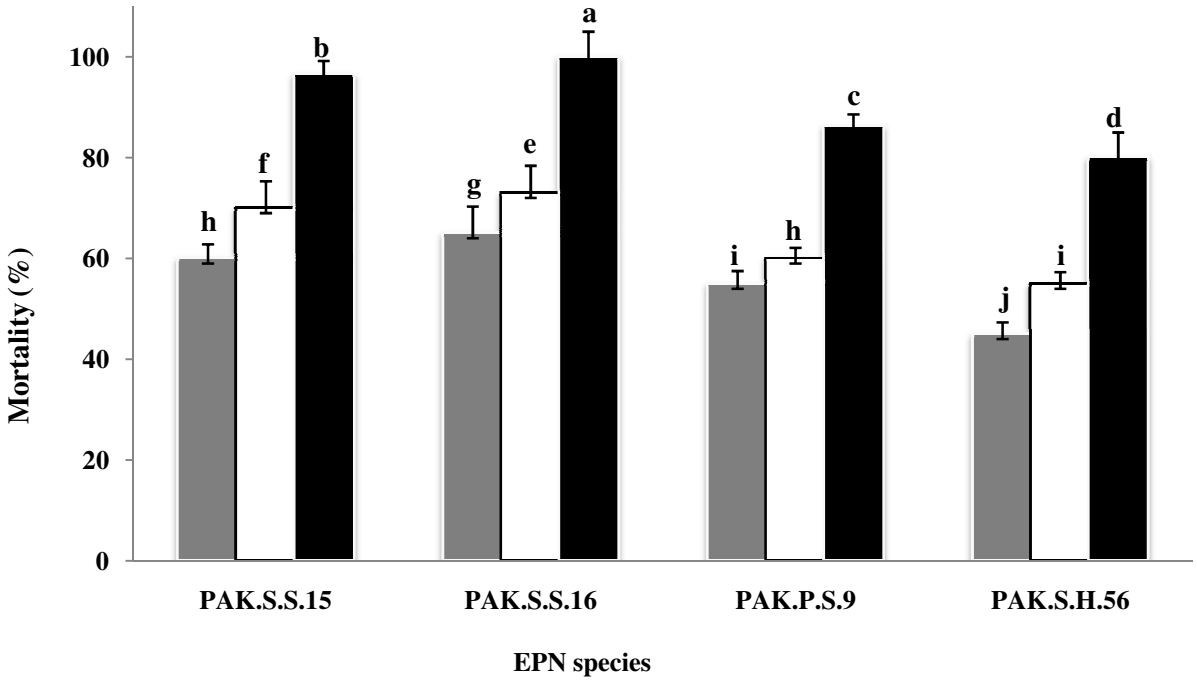

Fig. 3. Mean mortality (%) of *Microtermes mycophagus* treated with *S. abbasi* and *H. indica* after 25, 50 and 75 IJs/cm^2.

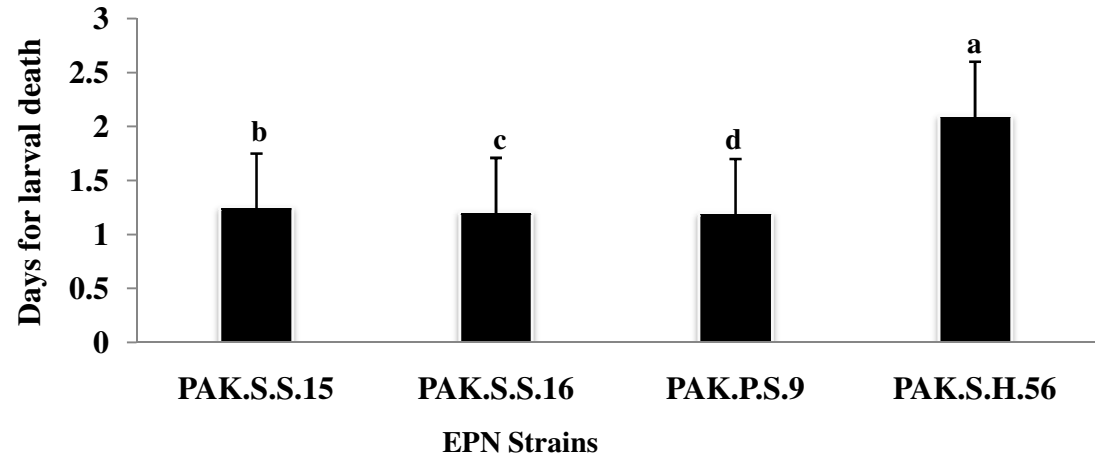

Fig. 4. Days to the larval death of *Microtermes mycophagus* after treated with *S. abbasi* and *H. indica* strains.

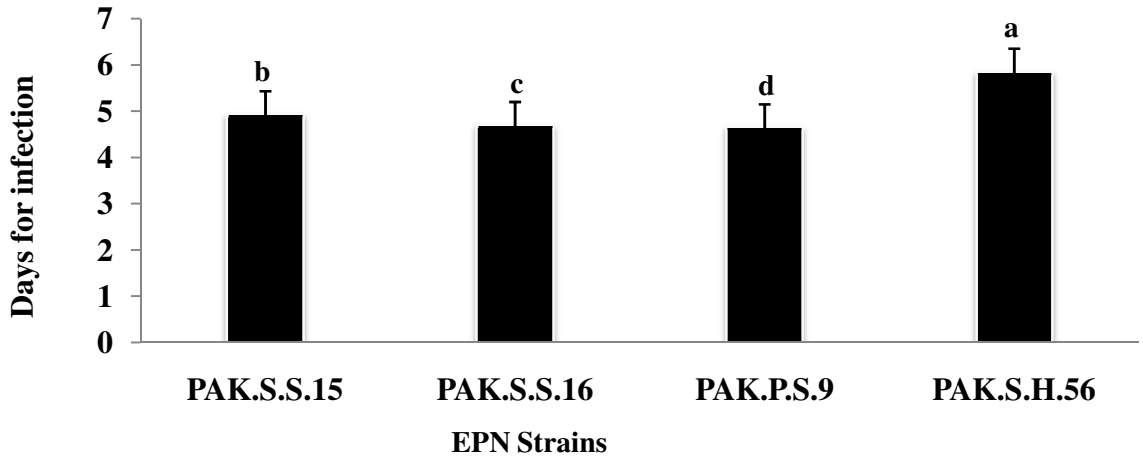

Fig. 5. Average number of days from infection to the emergence of IJs from *Microtermes mycophagus* treated with *S. abbasi* and *H. indica* strains.

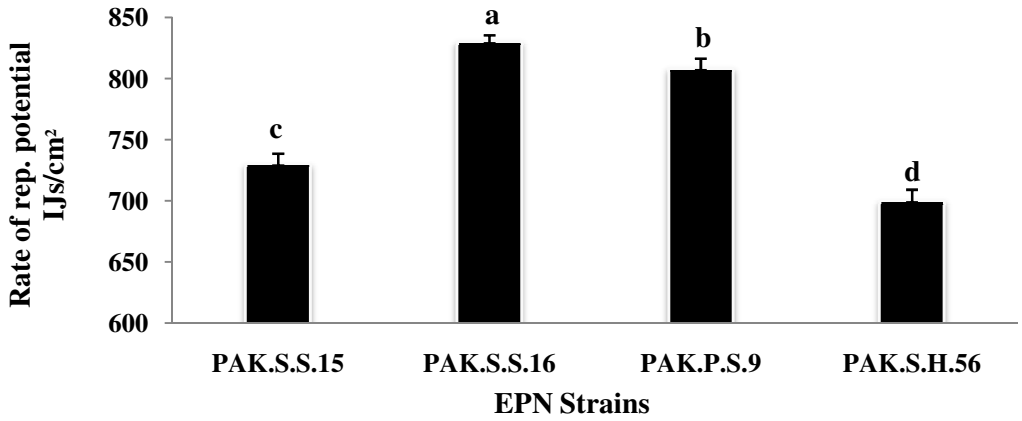

Fig. 6. Mean number of infective juvenile nematodes (IJs) produced per *Microtermes mycophagus* or per gram insect treated with *S. abbasi* and *H. indica* strains.

In addition results were also presented on the basis of dendrogram groups association (hierarchical cluster analysis). They were categorized between four strains based on the biological variables and determined through bioassay (Fig. 7). The group 1 includes *S. abbasi* (PAK.P.*S*.9 and PAK.S.*S*.16) with 86.3 and 100% termite mortality; 1.2 and 1.21 days to dead; 4.65 and 4.7 days to emergence of IJs and 808 and 830 IJs/mg insect for reproductive potential. Group 2 includes only one strain *S. abbasi* (PAK.S.*S*.15) which was slightly changed in biological bioassay than Group 1 as: 96.6 % termite mortality, 1.25 days to dead, 4.93 days to emergence of IJs and 730 IJs/mg insect for reproductive potential. While, Group 3 includes *H. indica* (PAK.S.*H*.56) which showed lowest biological variables than the strains of *S. abbasi* as shown: 80% larval mortality, 2.1 days to dead, 5.85 days to emergence of IJs and 700 IJs/mg insect for reproductive potential.

Entomopathogenic nematodes are tremendous biocontrol agents for insect pests. Whenever EPN is used against a pest insect, it is important to match the right nematode species against the target pest. It can be more effective if the applied agent is capable of killing the insect in short period of time and have ability to reproduce more progeny. According to our observation, nematode strain PAK.S.*S*.16 @ 75 IJs/cm^2 caused (100%) significantly highest mortality. In other experiment, time to death of insect was observed in which the strain PAK.P.*S*.9 showed shortest death period (1.2 days). In case of IJs emergence from termite (days from infection to the emergence of IJs) strain PAK.S.*S*.16 showed (4.93 days) moderate result among other three strains. In addition, the strain PAK.S.*S*.16 showed highest reproduction rate of 830 IJs/cm^{2}among all other strains. Recently Yu *et al*., 2010 checked virulence of *S. riobrave* strains 355 (=TX) 3-8b, 7-12, and TP against commercially available US pest termites *Heterotermes aureus* (Snyder), *Reticulitermes flavipes* (Kollar), and *Coptotermes formosanus* (Shiraki). An overall analysis of corrected mortality specified a significant interaction between *S. riobrave* strains and termite species. After 7 d of exposure, *H. aureus* exhibited a high level of susceptibility to all the *S. riobrave* strains while the termites were dead in all nematode treatments after 4 days. The TP strain of *S. riobrave* caused greater mortality in *R. flavipes* and *C. formosanus* compared to the other nematode strains. Previously, Yu *et al*., 2006, indicated a high level of virulence to *H. aureus* using the 355 strain of *S. riobrave*. Numerous studies showed that laboratory experiment for virulence among entomopathogenic nematode strains has been useful in selecting appropriate applicants for biological control (Shapiro & McCoy, 2000). Though, nematode species or strains that show the highest virulence for a particular pest in the laboratory may not necessarily prove to be the most efficacious under field conditions Shapiro *et al*., 2002). Therefore, the biocontrol potential of *S. abbasi* strains indicated in the laboratory must be confirmed in studies conducted under field conditions. In this aspect, current study of *S. abbasi* phylogenetic analysis and biological screening may provide new valuable information on possible use of nematodes for termite control.

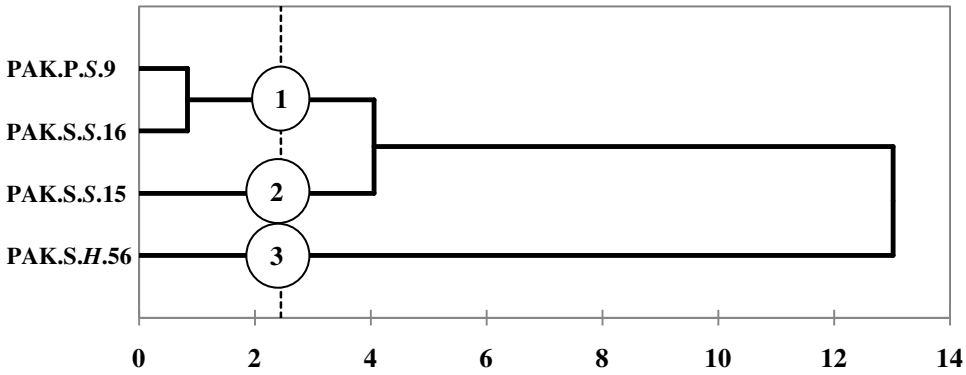

Fig. 7. Hierarchical cluster analysis between EPN species and biological data of virulence percentage larval mortality, days to dead *Microtermes mycophagus* days to IJs emergence and reproductive potential.

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