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RESEARCH ARTICLE

Frequency, Diversity and Living Strategies of Earthworms in Relatively Low and High Input Sugar Cane Fields of Faisalabad, Pakistan

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Abstract

Earthworms are important for top soil fertility. High Input Crop Fields - HIP (cultivation with intensive farming using pesticides and synthetic fertilizers) and Low Input Crop Fields - LIP (cultivation using relatively low doses of synthetic fertilizers and organic manures) of sugarcane were selected at random, each from an area of 10 acre, for the collection of earthworms. A total of 173 specimens were collected. Random Amplified Polymorphic DNA (RAPD-PCR) technique was applied to find out genetic differences and molecular characterization of earthworm's species. Total genomic DNA from nine earthworms specimens were extracted for RAPD analysis by using 15 random primers. Of these molecularly characterized isolates, a total of 123 loci were amplified by 15 primers with an average of 8.2 loci per primers. A total of 724 fragments were amplified out of which 89 fragments were found to be polymprhic. These results indicated that the level of DNA variation was high among these earthworm. The genetic similarities were ranging from 60.0% to 87.62%. The *P. morrisi* (LIP) and *P. posthuma* (LIP) had greatest similarity (87.62%) and minimum similarity was observed between *P. elongate* (LIP) and *P. hawayana* (HIP) which is 60.0%. The *P. houlleti* (LIP) show 75% genetic similarity with *P. posthuma* (LIP). The earthworm's relative diversity and abundance in sugarcane fields varied with months. June, September and November showed the less diverse earthen fauna with smaller sample of specimens. Whereas July, August and October samples were large and more diversified with respect to the occurrence of different species.

Keywords: Earthworm, RAPD-PCR, HIP, LIP, Biodiversity, Polymorphism

Introduction

Earthworms are perhaps the most important soil organisms in term of their influences on the organic matter breakdown, soil structural development, and nutrient cycling, especially in productive ecosystem (Rana et al, 2000). Despite the vast increase in scientific literature on earthworms in recent years, much remain to be known of their basic biology and ecology. It is lamentable that, very few studies on the identification and abundance of the earthworms in some habitats of the Punjab are available (Rafique and Rana, 2001).

With the changed scenario of agriculture with respect to the intensive practices, attempts to know the effects of chemicals, on earthworms are also few

(Edwards and Lofty, 1972; Stenerson, 1979; Hans et al, 1990; Edwards and Bohlen, 1992). Accordingly, many sublethal chronic toxicity symptoms in the earthworms exposed to different pesticides were recorded. Some of which were serious and short term while others which were minor but involved long term effects on the earthworm and their function. Important studies with regard to the effect of pesticides on earthworm, are those by (Zorum et al, 1986; Zang et al, 2000).

The recent developments in molecular biology have made it possible to apply DNA based technologies for genomic analysis in a variety of animal species (Jerry et al, 1997; Caterino et al, 2000). Among the several DNA based technologies, Random Ampli-fied Polymorphic DNA (RAPD) (Welsh and Mc Clelland, 1990) gained importance due to its simplicity, efficiency and no requirement of sequence information (Karp et al, 1997). Due to the technical simplicity and speed of RAPD methodology (Embrapa and Rural, 1997). RAPD markers have been successfully used for the generation of genetic similarities and phylogenetic analysis (Well and Sperling, 1999; Cognato and Sperling, 2000; Caterino et al, 2001; Krzywinski and Besanki, 2003).

Amplification in RAPD analysis occur anywhere in a genome that contain two compli-mentary sequences to the primer that are within the length limits of the PCR (Williams et al, 1990). It is fast and easy method for identifying DNA polymer-phism generated from several region of the genome (Hwang et al, 2001).

Moreover, it provides an opportunity to estimate relatedness within and among species based on DNA variation (Smith et al, 1996). RAPD markers are successfully used for the identification of different species (Wells and Sperling, 2001). The development and application of methods alternative to morphological identification is neither cheap nor trivial .Morphology remains the simplest, fastest and least expensive mean of identification for many species (Krzywinski and Besansky, 2003).

Earthworms are important for top soil fertility. Their specific abundance and diversity in any field cannot be ruled out because of diversified vegetation in the crop field which can also be associated with the presence of diversified organic matter and soil bio data. Thus, the present study has been planned to know.

Firstly number and diversity of earthworms in two types of sugarcane field viz; relatively organic (low input) and conventional (high input). Secondly their microhabitats in the low input field as well as high input sugarcane fields.

The conventional farming methods in our cropland are damaging most of the top soil biota and macro-organisms like earthworms. The phenotypic anomalies as reported earlier may have some genetic basis. The present study will also deal with the characterization of molecular changes in the genetic makeup of the earthworm species under pesticidal stress.

Materials and Methods

High Input Crop Fields (HIP) and Low Input Crop Fields (LIP) of sugarcane were selected at random, each from an area of 10 acre for the collection of Earthworms. Extraction of earthworms was made from the soil samples. These samples were taken from three different locations in the sugar cane fields; one from the middle of the field by using core samplers and other two samples from the edge of the cane field, of which one was collected from underneath the tree/shrub/herb along the edge and the other from the open edge of the crop by using an iron rectangle of one ft. sq. up to one ft. deep in the soil.

Sampling was initiated at earlier stage of growth of cane plants (about 2-3 feet height) till harvesting for the total duration of 6 months. Soil samples were packed in plastic bags and properly labeled of their location and date, and were taken to the laboratory for earthworm's extraction and further analysis. Soil was analyzed for its moisture contents. The record of plants other than sugar cane occurring in the sampling site was also taken into account. The soil was sorted out for earthworm's fauna and other taxa present in it. All the specimens were preserved in 95% alcohol solution in water. Earthworm species were identified under a microscope by using keys provided by Sims and Gerard (1985). Estimation of species richness, species evenness and species diversity was made by Shannon-Weiner Diversity Index. To know the effect of temperature and humidity on earthworm's fauna meteorological data were obtained from Agri-Meteorological Cell,

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Data were analyzed statistically to determine species diversity, species richness, and species evenness with Shannon-Weiner Diversity Index (H′).

To determine the impact of agrochemicals on the gene level a molecular study was also conducted. RAPD-PCR technique was applied to find out genetic similarities of different earthworm species and evaluate the effect of pesticides on genetic structure of earthworms. Prior to DNA extraction, the earthworms were starved for 48 hours to allow soil to pass through their gut. DNA was extracted by using alkaline lysis extraction method. The turbid liquid cultures were pelleted by centrifugation in micro centrifuge tubes and resuspended in 400 µl of TNE buffer. 40 µl of 10% SDS and 10 µl of 1% Proteinase-K were added and incubated at 37°C for 1hr. The 300 µl of 1M NaCl and 300µl of CTAB buffer was then added. The whole was vortexed for 15-30 sec, vigorously. Then 300 µl of Phenol: chloroform; Isoamyl alcohol (25:24:1) was added and centrifuged at 12000 rpm for 10 minutes. The DNA was precipitated using equal volume of isopropanol or ice-cold 100% ethanol and kept at -20°C for 60min. After brief centrifugation, the pellet was washed with 70% ethanol and then allowed to dry. Finally, the DNA was resuspended in 100 µl of sterile water (d3H2O). The concentration of total genomic DNA was measured by Spectrophotometer (CECIL, CE 2021. 2000 series) at 260 nm wavelength. The genomic DNA was diluted 100 times with a cuvette volume of 500µl. Quality of DNA was checked by running 5 µl DNA on 1 .0 % agarose gel prepared in 0.5X TBE buffer. The DNA samples giving smear in the gel were rejected.

For Random Amplified Polymorphic DNA analysis (Williams et al, 1990) concentration of genomic DNA. 10X PCR buffer with (NH4)SO4, MgCl2. dNTPs (dATP. dCTP. dGTP. dTTP), 10-mer random primer and Taq DNA Polymerase were optimized. The 10-base oligonucleotide primers obtained from Genelink Company were used for the amplification of the genomic DNA. PCR reaction was carried in 25 µl reaction mixture containing 3 mM MgCl2. 2.5 mM each of dATP, dCTP, dGTP, dTTP. 0.2 µM primer. 10ng of genomic DNA and 1 unit of Taq polymerase, through programmable thermal cycler (Eppendorf Mastercycler, USA). A total of 20 primers were used for the analysis. The thermal cycler was programmed for 5 minutes initial Denaturation at 95°C followed by 1-minute denaturation at 95°C. 1 minute primer annealing at 37°C and 2 minutes extension at 72°C for 40 cycles and then final extension at 72°C for 10 minutes.

The PCR products were electrophoresed at 80 V in 1.0% agarose gel for approximately 2 hours using 0.5X Tris Boric acid (TBE) buffer containing Ethidium Bromide (0.5µg/ml) along with a DNA molecular size marker.

Amplified fragments were scored by starting from top of the lane to its bottom. All visible and unambiguously scorable fragments amplified by the primers were scored under the heading of total scorable fragments. Amplification profile of six earthworm species were compared with each other and to molecular size marker and bands of DNA fragments were scored as present (1) or absent (0).

The data of the primers were used to estimate genetic similarity on the basis of number of shared amplification products (Nei and Li, 1979).

The coefficients were calculated by the following statistical equation:

$$F = 2Nxy / (Nx + Ny)$$

Where:

"F" is the similarity coefficient in which Nx and Ny are the numbers of fragments in population x and y respectively whereas Nxy is the fragment shared by the two populations.

Similarity coefficients were utilized to generate a dendrogram by using un-weighted pair group method of arithmetic means (UPGMA).

Results

All specimens of earthworms were extracted from the soil along with other soil macrofauna sampled from two types of sugarcane fields, receiving low input and high input of agrochemicals, hereafter will be called LIP and HIP fields respectively. LIP fields harboured significantly greater number of specimens. As many as 131 earthworm specimens belonging to six species were found from these fields. *P. posthuma* (57) was most abundant species followed by other species such as

Table 1: Summary of Findings in LIP and HIP lands

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
LIP	131	5	0.65	0.78	0.84	0.16
HIP	42	3	0.38	0.48	0.80	0.20

Table 2: Summary of Findings in LIP and HIP Lands Under Sub-Shadow Edges

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
Overall	86	5	0.56	0.70	0.82	0.18
LIP	65	5	0.57	0.70	0.81	0.19
HIP	21	3	0.36	0.48	0.75	0.25

P. morrisi (24) *P. suctoria* (21), *P. hawayana* (18).*P. elongata* (9) and *P. houlleti* (2) (Table 1). Diversity ($H^{/}$) of earthworm in LIP fields was found to be (0.65) whereas maximum diversity ($H^{/}$max) calculated by Shannon- Weiner index of diversity was (0.78). The evenness (J) value was (0.84) while dominance (D) was (0.16). HIP sugarcane field harboured only 42 specimens representing 3 species viz., *P. posthuma* (28). *P. suctoria* (9) and *P. hawayana* (5). HIP sugarcane field were less diversified as compared to LIP. Diversity ($H^{/}$) and maximum diversity ($H^{/}$max) was found to be (0.38) and (0.48) respectively. Evenness (J) value was (0.80) and Dominance (D) was (0.20) (Table 1).

Keeping in view the phytomorphic heterogeneity at different sites in the sugarcane fields, three sites/microhabitats were explored for earthworms. Sub-shadow edge was an elevated ridge along the crop field, separating it from adjacent fields covered by some shrub/herb/tree on the edge. An open edge elevated ridge separating the two crop fields or making the boundary of the field along the sugarcane crop and Inner side across the field.

Sub-Shadow edge

This microhabitat was found to be more suitable for the earthworms. Out of 173 earthworm specimens, 86 were collected from this site of sugarcane field throughout sampling period. These specimens were identified as belonging to five species viz*; P. posthuma* (44), *P. morrisi* (16) *P. hawayana* (14), *P. suctoria* (9), *and P. elongata* (3) (Table 2).

Diverstiy ($H^{/}$) of earthworm, along the sub-shadow was found to be 0.56 where maximum diversity ($H^{/}$ max) calculated by Shannon-Wiener index of diversity was 0.70. The evenness (J) value for sub-shadow was 0.82 while dominance (D) was 0.18.

The sub-shadow edge site of LIP sugarcane field harboured significantly greater number of specimens. As many as 65 earthworm specimens, belonging to five species was found from this site. The no. of specimens observed here was *P. posthuma* (30), *P. morrisi* (16), *P. hawayana* (12), *P. suctoria* (4) and *P. elongata* (3).

Diversity ($H^{/}$) of earthworm along the LIP sub-shadow was found to be 0.57, whereas maximum diversity ($H^{/}$max.) calculated by Shannon-Wiener index of diversity was 0.70. The evenness (J) value was (0.81) while dominance (D) was (0.19).

The sub shadow edge site of HIP sugarcane field harboured only 21 specimens representing three species viz; *P. posthuma* (14), *P. suctoria* (5), *P .hawayana* (2) (Table 2).

Diversity ($H^{/}$) and maximum diversity ($H^{/}$max) calculated by Shannon-Wiener index of diversity along HIP sub shadow were found to be 0.36 and 0.48 respectively. The evenness (J) value was (0.75) while the dominance (D) was (0.25).

Out of these 86 specimens, 17 were immature and 69 were mature. Diversity ($H^{/}$) of immature earthworm, along the sub-shadow was found to be 0.52, where maximum diversity ($H^{/}$max) calculated by Shannon-Wiener index of diversity was 0.60.

Table 3: Summary of Mature and Immature Specimens in LIP and HIP Lands Under Sub-Shadow Edges

Land	Number of Organisms	Diversity H'	H' max	Evenness	Dominance
Immature	17	0.52	0.60	0.86	0.14
Mature	69	0.43	0.70	0.62	0.38

Table 4: Summary of Findings in LIP and HIP Lands Under Open Edges

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
Overall	53	5	0.54	0.70	0.77	0.23
LIP	41	5	0.53	0.70	0.75	0.25
HIP	12	3	0.35	0.48	0.72	0.28

Table 5: Summary of Mature and Immature Specimens in LIP and HIP Lands Under Open Edges

Land	Number of Organisms	Diversity H'	H' max	Evenness	Dominance
Immature	19	0.47	0.60	0.77	0.23
Mature	34	0.54	0.70	0.77	0.23

The evenness (J) value for sub-shadow was 0.86 while Dominance (D) was 0.14 .Diversity ($H^{/}$) and maximum diversity ($H^{/}max$) calculated by Shannon-Wiener index of diversity for mature earthworm were found to be 0.43 and 0.70 respectively. The evenness (J) value was (0.62) while the dominance (D) was (0.38) (Table 3).

Open Edge

This habitat was found to be the second suitable for earthworm. A total of 53 earthworm specimens were collected from this site of round the sampling period. These specimens were identified as belonging to five species viz; *P. posthuma* (24), *P. suctoria* (18), *P. morrisi* (5), *P. hawayana* (5) and *P. houlleti* (1) (Table 4).

Diversity ($H^{/}$) of earthworm along open edge was found to be (0.54), whereas maximum diversity ($H^{/}max.$) calculated by Shannon Wiener index of diversity was (0.70) while the evenness (J) and dominance (D) was (0.77) and (0.23). Table 4 shows that the open edge of LIP sugarcane fields harboured 41 specimens belonging to five species viz; *P. suctoria* (17), *P. posthuma*. (16), *P. morrisi* (5), *P. hawayana* (2) and *P. houlleti* (1) (Table 4). Diversity ($H^{/}$) of earthworm along LIP open edge was found to be (0.53) whereas maximum diversity ($H^{/}max$) was (0.70). The evenness (J) value was (0.75) while the dominance (D) was (0.25).

The open edge of HIP sugarcane field harboured only 12 specimen belonging to three species viz; *P. posthuma* (8), *P. hawayana* (3) and *P. suctoria* (1). The diversity ($H^{/}$) and maximum diversity ($H^{/}max$) was (0.35) and (0.48) respectively. The evenness value (J) was (0.72) while dominance (D) was 0.28.

Out of these 53 specimens, 19 were immature and 34 were mature (Table 5). Diversity ($H^{/}$) of immature earthworm, along the open edge was found to be 0.47 where maximum diversity ($H^{/}max$) calculated by Shannon-Wiener index of diversity was 0.60. The evenness (J) value for sub-shadow was 0.77 while dominance (D) was 0.23. Diversity ($H^{/}$) and maximum diversity ($H^{/}max$) calculated by Shannon-wiener index of diversity for mature earthworm were found to be 0.54 and 0.70 respectively. The evenness (J) value was (0.77) while the dominance (D) was (0.23) (Table 5).

Inside Field

The microhabitat of inside field was found to be least suitable for earthworm. Only 34 specimens were found belonging to six species. The inside field was most diversified with respect to species. These specimens were identified viz; *P. posthuma* (17), *P. elongate* (6), *P. hawayana* (4). *P. morrisi* (3), *P. suctoria* (3) and *P. houlleti* (1). Diversity ($H^{/}$) and

Table 6: Summary of Findings in LIP and HIP Lands Inside Field

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
Overall	34	6	0.63	0.78	0.81	0.19
LIP	25	5	0.54	0.70	0.77	0.23
HIP	9	2	0.27	0.30	0.90	0.10

Table 7: Summary of Mature and Immature Specimens in LIP and HIP Lands Inside Field

Land	Number of Organisms	Diversity H'	H' max	Evenness	Dominance
Immature	8	0.42	0.60	0.70	0.30
Mature	26	0.57	0.70	0.82	0.18

Table 8: Summary of Age Related Immature Specimens in LIP and HIP Lands Under Sub-Shadow Edges

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
HIP	4	3	0.45	0.48	0.93	0.07
LIP	13	4	0.46	0.60	0.76	0.24

maximum diversity ($H^{/}$max) of earthworm in the inside field was found be (0.63) and (0.78) respectively. The evenness (J) value was (0.81) while Dominance (D) was (0.19) (Table 6).

The inside field of LIP field, harboured 25 specimens belonging to five species viz. *P. posthuma* (11), *P. elongata* (6), *P. hawayana* (4), *P. morrisi* (3) and *P. houlleti* (1). Diversity ($H^{/}$) and maximum diversity ($H^{/}$max) was found to be (0.54) and (0.70), respectively. The evenness (J) was 0.77 while dominance (D) was 0.23. The inside field of HIP fields of sugarcane harboured only 9 specimens, belonging to 2 species viz.; *P. posthuma* (6) and *P. suctoria* (3). Diversity ($H^{/}$) and maximum diversity ($H^{/}$max) was found to be (0.27) and (0.30) respectively. The evenness value (J) was (0.90) while Dominance (D) was (0.10) (Table 6).

Out of these 34 specimens, 8 were immature and 26 were mature. Diversity ($H^{/}$) of immature earthworm was found to be 0.42 where maximum diversity ($H^{/}$max) calculated by Shannon-Wiener index of diversity was 0.60. The evenness (J) value was 0.70 while dominance (D) was 0.30. Diversity ($H^{/}$) and maximum diversity ($H^{/}$max) calculated by Shannon-wiener index of diversity for mature earthworm were found to be 0.57 and 0.70 respectively. The evenness (J) value was (0.82) while the dominance (D) was (0.18) (Table 7).

Age Related Diversity of Earthworm

Immature

Sub Shadow

The sub shadow edge site of HIP sugarcane field harboured only 4 immature specimens representing three species viz., *P. suctoria* (2), *P. posthuma* (1) and *P. hawayana* (1) (Table 8).

Diversity ($H^{/}$) and maximum diversity ($H^{/}$max) calculated by Shannon-Weiner index of diversity was found to be (0.45) and (0.48) respectively. The evenness (J) value was (0.93) while dominance (D) was (0.07).

Out of these 34 immature specimens collected from LIP fields, 13 specimen were found from sub-shadow edge site representing four species viz., *P. posthuma* (8). *P. hawayana* (2), *P. morrisi* (2) and *P. suctoria* (1). Diversity ($H^{/}$) and maximum diversity ($H^{/}$maximum) of earthworm were (0.46) and (0.60). The evenness (J) value was (0.76) and dominance (D) was (0.24).

Open Edge

Table 9 shows the total number of immature earthworm specimens collected from HIP open edge site was only *P. posthuma* (2). Diversity ($H^{/}$) and maximum diversity ($H^{/}$max) calculated by Shannon-Weiner index was found to be 0.00, 0.00 and even-

Table 9: Summary of Age Related Immature Specimens in LIP and HIP Lands Under Open Edges

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
HIP	2	1	0.00	0.00	0.00	0.00
LIP	17	4	0.53	0.70	0.75	0.25

Table 10: Summary of Age Related Immature Specimens in LIP and HIP Lands Inside Fields

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
HIP	4	2	0.30	0.30	1	0.00
LIP	4	3	0.45	0.48	0.93	0.07

Table 11: Summary of Age Related Mature Specimens in LIP and HIP Lands Under Sub-Shadow Edges

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
HIP	17	3	0.29	0.48	0.60	0.40
LIP	52	5	0.59	0.70	0.84	0.16

Table 12: Summary of Age Related Mature Specimens in LIP and HIP Lands Under Open Edges

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
HIP	10	3	0.38	0.48	0.79	0.21
LIP	24	5	0.53	0.70	0.75	0.25

ness (J) and dominance (D) was also found to be 0.00).

The open edge of LIP sugarcane field harbored 17 specimens belonging to four species viz. *P. suctoria* (10). *P. posthuma* (4). *P. morrisi* (2) and *P. hawayana* (1). Diversity ($H^{/}$) of earthworm along LIP open edge was found to be (0.53) whereas maximum diversity ($H^{/}$max) was (0.70) the evenness value (J) was (0.75) while dominance (D) was (0.25).

Inside field

The total number of specimens collected from HIP inside field was only 4 representing two species viz., *P. posthuma* (2) and *P. suctoria* (2). Diversity ($H^{/}$) of earthworm along inside field was found to be (0.30) whereas maximum diversity ($H^{/}$max) calculated by Shannon-Weiner index of diversity was (0.30). The evenness value (J) for HIP inside field was (1) while dominance (D) was 0 (Table 10).

The inside field of LIP fields of sugarcane harbored (4) specimen belonging to three species viz., *P. posthuma* (2), *P. elongata* (1) and *P. houlleti* (1). Diversity ($H^{/}$) of earthworm along inside field was found to be (0.45) whereas maximum diversity ($H^{/}$max) calculated by Shannon-Weiner index of diversity was (0.48). The evenness value (J) for HIP inside field was (0.93) while dominance (D) was (0.07).

Mature

Sub-Shadow

The sub shadow edge site of HIP sugarcane field harboured only 17 specimens, representing three species viz., *P. posthuma* (13), *P. suctoria* (3) and *P. hawayana* (1) (Table 11).

Diversity (H') and maximum diversity ($H^{/}$maximum) calculated by Shannon-Weiner index along HIP sub shadow were found to be (0.29) and (0.48), respectively. The evenness (J) value was (0.60) while dominance (D) was (0.40).

On the other hand, from LIP sample of sugarcane field 52 specimen were captured, belonging to five species viz., *P. pasthuma* (22) *P. morrisi* (14). *P. hawayana* (10). *P. suctoria* (3) and *P. elongate* (3).

Table 13: Summary of Age Related Mature Specimens in LIP and HIP Lands Under Sub-Shadow Edges

Land	Number of Organisms	Number of Species	Diversity H'	H' max	Evenness	Dominance
HIP	5	2	0.21	0.30	0.70	0.30
LIP	21	4	0.56	0.60	0.93	0.07

Diversity ($H^{/}$) and maximum diversity ($H^{/}max$) of earthworm were (0.59) and (0.70). The evenness (J) was (0.84) and dominance (D) was (0.16).

Open Edge

The open edge of HIP sugarcane field, harboured only 10 specimens belong to three species viz., *P. posthuma* (6), *P. hawayana* (3) and *P. suctoria* (1). Diversity ($H^{/}$) of earthworm along HIP open edge was found to be (0.38) whereas maximum diversity ($H^{/}max$) was (0.48) and dominance was (0.79) and (0.21) respectively (Table 12).

24 specimens were collected along LIP open edge site, these 24 specimens belonging to five species viz., *P. posthuma* (12). *P. suctoria* (7), *P. morrisi* (3), *P. hawyana* (1) *and P. houlleti* (1). Diversity ($H^{/}$) and maximum diversity ($H^{/}max$) calculated by Shannon-Weiner index was found to be (0.53) and (0.70). While evenness (J) value and dominance was (0.75) and (0.25), respectively.

Inside Field

Minimum number (5) of specimens were found along HIP inside field of sugarcane. These specimens were representing two species viz., *P. posthuma* (4) and *P. suctoria* (1). Diversity ($H^{/}$) of species was found to be (0.21) whereas maximum diversity ($H^{/}max$) predicted by Shannon Weiner to be (0.30). Evenness (J) value (0.70) and dominance (D) (0.30) (Table 13).

Out of these 97 specimens, 21 were found in LIP field belonging to four species viz., *P. posthuma* (9). *P. elongate* (5), *P. hawayana* (4) and *P. morrisi* (3).

Diversity ($H^{/}$) and maximum diversity ($H^{/}max$) was found to be (0.56) and (0.60) whereas evenness (J) value and dominance (D) was (0.93) and (0.07) respectively. Out of 129 mature earthworm specimen collected from different micro habitats of HIP and LIP field of sugarcane.

Monthly Variation

The immature earthworm specimen diversity and abundance in sugarcane fields varied with months. August showed the more diverse soil macro fauna with large number of specimens (13). *P. posthuma* (19) was most abundant species occurring throughout the sampling period from June through November followed by others *P. suctoria* (15), *P. hawayana* (4), *P. morrisi* (4) and only one specimen of both *P. elongata* and *P. houelliti*.

In HIP field of sugarcane only three species were collected .Equal number of two specimen were collected in the months of June, October and November. No specimen were collected in the months of July and September.

In LIP field of sugarcane, (34) specimens belonging to six species were found. August (9) and Sept. (8) showed the more diverse soil fauna while Nov. (2) showed the smallest sample of specimen. (5) Specimens were collected in the months of June, July and Oct.

The mature earthworm specimen diversity and abundance in HIP and LIP sugarcane fields varied with months. In HIP field of sugarcane (32) specimens were collected throughout the sampling period *P. posthuma* (23) was most abundant species. All the months shows almost equal diversity of specimens. While no specimens were present in the month of September.

Out of 173 specimens (97) specimens were collected from LIP field of sugarcane. August showed the more diverse soil macro fauna with largest number of specimens (32). October, September, July, June and November had (15), (14), (14) (12) & (10) specimens respectively. The LIP

Table 14: RAPD Amplified and Total Number of Fragments Scored for Each Primer

Primer Code	No of Amplified Loci	Polymorphic Loci	Total Amplified Loci
A-05	7	5	46
A-09	8	7	46
A-13	10	7	66
B-02	8	4	50
B-13	9	3	76
B-16	8	7	56
B-19	10	9	42
C-09	9	8	62
C-13	8	7	32
C-18	6	5	44
D-05	10	10	52
D-07	8	4	51
D-11	8	8	29
D-12	5	2	41
D-17	8	4	43

field of sugarcane showed the more diversity as compared to HIP fields of sugarcane.

Impact of Agrochemicals on Genetic Structure of Earthworms

Polymorphism at the genetic level may be detected in a variety of ways based on DNA amplification. Among these, RAPD has gained much importance due to its simplicity and non-requirement of prier sequence of the genome. The RAPD assay also referred to as arbitrary primed PCR, was first time described by Williams et al, 1990. RAPD assay are based on the use of short random sequence primers, 9 to 10 bases are in length, which hybridize with sufficient affinity to chromosomal DNA sequence at low annealing temperature such that they can be used to initiate amplification of regions of the plant and animal genome. If two RAPD primers anneal within a few kilobases of each other in the proper orientation, a PCR product with a molecular length corresponding to the distance between two primer results. The number and location of these random primer sites vary for different earthworm species. Thus following separation of the amplification products by agarose gel electro-phoresis, a pattern of bands which in theory, is characteristics of all organisms (Welsh and McClelland, 1990; Williams et al, 1990).

The rapidcycler RAPD program can process upto 96 samples at one time and takes two hours to complete; the only limitation to processing all these samples is the availability of gel electrophoresis units and number of wells per gel. RFLP analysis, Southern blot hybridization analysis takes approximately 4 days to complete. RAPD analysis has been to be an inexpensive molecular biology procedure for the fingerprinting of *Earthworm species*. All the procedure was conducted in Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad.

DNA of nine isolates of earthworm species were amplified with 15 different primers. A total of DNA 123 Loci were amplified with an average of 8.2 Loci per primer that ranged from 5 to 10 with molecular weights range of 200 bp to 1300 bp. six primers were found non-polymorphic (C-9, C-13, A-12, A-08 and A-07), they were excluded from the study. The procedure was repeated twice to ensure the reproducibility of the results. No change and difference was observed in the no. and length of the fragments/ bands.

Dendrogram showing the clustering of pesticide (HIP sample) affected group of earthworms as genetically mutated, *P. posthuma, P. suctoria, P. hawayana*, suspected to be the tolerant at the cost of their genetic modification.

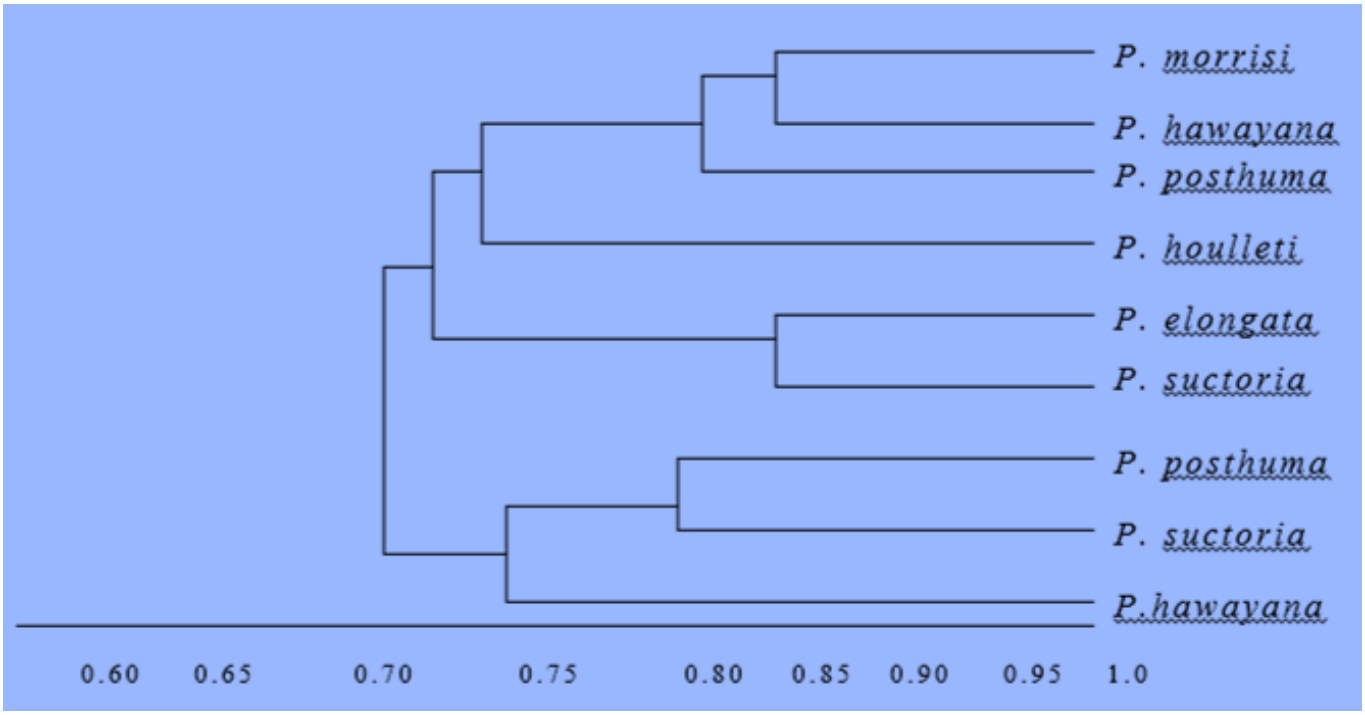

Figure 1: Phylogenetic Analysis of Species Data

The genetic similarity matrix was obtained through RAPD-PCR using 15 different primers based on Nei and Li, (1979) co-efficient of similarity. The genetic similarities were ranging from 60.0% to 87.62%. The maximum similarity was found between A and C i.e. 87.62% and minimum similarity was observed between E and I which is 60.0 %. The phylogenetic tree/ dandrogram was constructed using the RAPD data with the help of UPGMA. Two main cluster subgroups were obtained showing high diversity among the organisms. It was evident from the cluster analysis that high level of genetic similarity was observed between A & C and is closely clustered with B at 84 %. The similar genetic similarity was observed between E and F.

Discussion

Earthworms act as a barometer for soil health. Earthworm cannot flourish in habitat of crop land where synthetic fertilizers and pesticides are of paramount importance (Rudsenske, 2004). These lines correlated with this study in which earthworms were less abundant in high input (HIP) sugarcane fields than those of low input (LIP).

Earthworms were found abundantly on the sub-shadow edge sites along the edges of the pair fields of sugarcane. This finding was in line with the work of (Hanski and Gilpin, 1997), who demonstrated that size, isolation and shape of habitats patches induce changes in species abundance and biodiversity.

According to Rudsenske (2004) earthworms are quite intolerant of drought and frost, they prefer cool temperature and moist soil. Therefore, leaving crop residue on the surface serve a dual purpose. In the summer, they keep soil cool and moist adding organic matter like cover crops, also support the earthworm activity.

These investigation have shown positive effect of low input farming system on earthworm in comparison with high input farming system. If low input farming is linked with semi natural habitats, diverse community of earthworm can be conserved with higher farming and intensity. There is a higher risk that agro ecologically important species (earthworm) as well as nature conservation relevant species will be reduced.

To enhance the earthworm population in cropland, and to save these drought sensitive

macrodecomposers the edge flora should be conserved or some cover crop within the crop system or at least on the edges of the field should be covered with some vegetation. Because these more towards shady sites to avoid extreme desiccating heat of the summer as the sugarcane persists in the field during most of summer as seed crop.

A RAPD study for the characterization of earthworm species was the pioneer study of this type and no such reference was found which could depict the earlier findings of the similar study on earth-worms.

In present study maximum number of amplified loci were obtained using primer B-13 i.e. Nine times repetitively found in the entire genome.

The genetic similarity matrix of RAPD data for the nine organisms was constructed based on coefficient of similarity. The matrix was utilized for the construction of dendrogram using the UPGMA method. Dendrogram was highly branched suggestive for a genetically diverse population of two major groups. Interestingly, these two groups, were found to be based on the changes which occurred in the genetic structure of one group of the same species as those of other group but collected form sugarcane field of high input (HIP) of agrochemicals.

Identified form their morphological characters, six earthworm species, namely *P. morrisi*, *P. hawayana*, *P. posthuma*, *P. houlleti*, *P. elongata*, *P. suctoria* from LIP sugarcane fields were confirmed as different species. But, three of these species namely *P. posthuma*, *P. suctoria* and *P. hawayana* showed their changed genetic material as these were collected from agrochemical affected (HIP) fields of sugarcane. It meant that the pesticidal impact on the genetic structure of the worm could have the ability to cause mutation changing the path of evolution. Sub lethal doses of different chemicals have been found to cause varied effects on earthworm populations (Anton et al, 1990; Hans et al, 1990; Bharathi and Subba, 1984; Drewes and Callahan, 1988).

Conclusions

From the species richness and diversity comparison through Shannon-Weiner index, it was concluded that the sub shadow site was relatively more diverse with earthworms than open edge and inside field. Out of 173 specimens, 86 were collected from sub shadow site while (53) and (34) from open edge and inside field of sugarcane, respectively .A slight fluctuation in monthly collected population was attributed to the ecological conditions. The ecological conditions i.e. the monsoon season and the rapid growth of plants (Coley and Aide, 1991).

The genetic variation in all molecularly characterized earthworms species included in the present study was very high. The band reading and the results of cluster analysis should however be interpreted with caution, as genetic constitutions of these earthworms species could be altered and might cause a change in the relatedness to other species in the dendrogram .A slight alteration could result a change in the phylogeny of the species and hence result in the failure of accurate molecular characterization. Outcome of the present study can be helpful for the advanced scientific studies in molecular Zoology.

This study is the first report using RAPD analysis to understand the genetic diversity of native earthworm species of Pakistan. Based on the results, it is suggested that polymorphic markers out of RAPD method be used for identification of different species in future. Producing much polymorphic information of target genomes with more primers can compensate the shortcoming of this method. However more detailed experiments that can deduce phylogenetic configuration should follow to prove the genetic differentiation based on polymorphic markers. These results can be taken as a starting point for future researches aimed at defining the level of intra and inter species genetic diversity. For this purpose, a large number of earthworm species or any predator or pest species should be sampled and analyzed using additional primers. Furthermore, in order to detect genetic discrimination among different species discriminate bands can be cloned and sequenced. The RAPD patterns of the primers

used in the present study could be a source of additional DNA markers that may be used in the development of genome maps for earthworm species.

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