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Collection, molecular identification and study of earthworm species procured from different soil habitats of Madurai district, Tamil Nadu, India

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Abstract
Study on biodiversity of earthworm is very important since its impact on increasing soil fertility. The present study explored the earthworm species from different soil habitats of Madurai district, Tamil Nadu, India. The studied five distinct locations were cultivating land of Vadipatti, non cultivating land of Thirumangalam, grass land of Usilampatti, garden of Alagar Kovil and Sewage Soil of Melur. Based on the physicochemical conditions of the soil conditions like pH, temperature, organic carbon, organic matter and moisture, the diversity of earthworm greatly varied and morphologically distinct earthworm from each location was identified using molecular identification methodology with COI gene partial sequence. From the five different locations, four different species of earthworm Was identified *viz*., *Drawida sp., Perionyx excavatus, Lampito mauritii* and *Drawida japonica,* respectively. Among the earthworm species, *Drawida* sp. represented the dominating species with 33.33% of overall collected earthworm, followed by 25% of each *Perionyx excavatus* and *Lampito mauritii* and the least values of 16.67% was represented by *Drawida japonica*. This is the first report on diversity of earthworm species isolated from different soil habitats of Madurai district, Tamil Nadu, India.

Keywords: Earthworm species, Madurai district, physicochemical parameters, molecular identification; COI gene

1. Introduction
The silent role of earthworms in improving soil properties especially role of earthworms in promoting soil fertility, has been known since ancient times. Darwin (1881) was the first to observe and offer a scientific explanation of their true role in the ecosystem and his conclusions led to an upsurge of interest in earthworms from the late nineteenth century onwards [1]. Earthworms are widely distributed throughout the world particularly in the temperate and tropical regions and their population contributes about 80% of the total biomass of the soil [2].

Researchers have identified and named more than 4400 distinct species of earthworms worldwide [3], each with unique physical, biological and behavioural characteristics that distinguish each one of them from the other and Julka *et al*. [4] reported 590 species of earthworms from India. Earthworms are perhaps the most important soil organisms in terms of their influence on organic matter breakdown, soil structural development and nutrient cycling, especially in productive ecosystem [5]. The earthworm cast increases organic compound, cytokinin and auxin concentration in the soil [6] which is considered positive on ecosystems.

Distribution of earthworms is usually irregular [7] and the diversity vary in relation to the type of soil [8] and ecological factors especially edaphic factors (moisture and temperature) [9]. Study on biodiversity of earthworm is very important since its impact on increasing soil fertility. The present study explored the earthworm diversity from different soil habitats of Madurai district, Tamil Nadu, India using molecular identification approach and studied the physicochemical properties of soil in correlation with the species dynamics of various earthworms.

2. Materials and Methods
2.1. Sample Collection
Different soil samples were collected for the isolation and study of earthworm in and around Madurai district, Tamil Nadu, India. Top soil samples were collected at a depth of 0–20 cm using soil auger from five different areas *viz*., cultivating land of Vadipatti, non cultivating land of Thirumangalam, grass land of Usilampatti, garden soil from Alagar kovil and Sewage soil from Melur during the month of September, 2017. From each site 0.5 Kg was collected,

five sub-sites were taken for the purpose of random sampling which totally make 2.5 Kg. The samples collected from the five sub-sites in each areas were individually pooled together to obtain a composite sample. Finally, five bulk soil samples one from each stated areas were transferred in to polyethylene bags and transported to the laboratory for further analysis.

2.2. Moisture Content

Soil moisture content was determined by oven drying method [10]. 10 g of composite soil sample was taken. The samples were oven dried at 105°C for 24 hrs. Dry weight of the sample was taken till it showed its constant weight. The loss in weight corresponds to the amount of water present in the soil sample. The formula below was used to calculate the percentage of moisture content in each of the soil samples [11].

$$\text{Moisture content (MC) (\%)} = \frac{\text{Loss in weight on drying (g)}}{\text{Initial sample weight (g)}} \text{ x } 100$$

2.3. pH

The pH of the soil samples was measured in water suspension (1:2.5) as described by Jackson [10]. Air dried soil of 20 g was taken in a beaker and to this 50 ml of water was added. The mixture was stirred with glass rod for 10 min and was allowed to stand for 30 min. The pH meter (ELMETRON, CPI-501, Poland) was calibrated using standard buffer solution of pH 4.0, 7.0 and 10.0. Then electrode of the pH meter was inserted in to the supernatant solution and the pH reading was taken.

2.4. Organic carbon and organic matter

The organic carbon content of the soil samples were determined by the method of Walkey and Black [12]. 1 g finely ground soil sample was passed through 0.5 mm sieve without loss was taken into 500 ml conical flask, to which 10 ml of 1 N potassium dichromate and 20 ml conc. H_2SO_4 were added with measuring cylinder. The contents were shaken for a minute and allowed to stand for 30 min. Then 200 ml distilled water, 10 ml Ortho phosphoric acid and 1 ml diphenylamine indicator were added. The solution was titrated against 0.5 N ferrous ammonium sulfate till the colour flashes from blue-violet to green. The blank titration was carried at the beginning without soil. The results were calculated by the following formulas:

$$\text{Organic carbon \%} = \frac{N \text{ x } (V1 - V2) \text{ x } 0.39 \text{ x mcf}}{S}$$

2.4.1. Where

N = Normality of ferrous ammonium sulfate (FAS)
V1 = Volume of 0.5 N
FAS required to neutralize 10 ml of 1 N K2Cr2O7 i.e. blank reading (ml).
V2 = Volume of 0.5 N
FAS needed for titration of soil sample (ml)
S = Weight of air-dry sample (g)
0.39 = 0.003 x 100% x 1.31 (0.003 is the mill equivalent weight of carbon in g). It is assumed that only 77% of the organic matter is oxidized and a fraction of 100/77 = 1.31
Organic matter (%) = Organic carbon (%) x 1.724
1.724 = average content of carbon in soil organic matter is equal to 58%.

2.5. Temperature

Using a long iron rod or similar tool, a vertical hole was created in the soil sampling locations that were about 10 cm deep which firmly accommodate a thermometer. Then, the soil thermometer was inserted into the soil to a depth of 10 cm and the reading was noted after at least one minute.

2.6. Collection of Earthworm

Earthworms were collected from the possible different sites of all the above mentioned different five locations. To avoid the dry season when soil is hard to excavate and earthworms are more difficult to find, sampling took place wet season of September, 2017. Soil pits (20 × 20 × 20 cm) were dug using a spade and earthworms were hand-sorted in the field. The collected earthworms were then stored in plastic containers with perforated lids along with soil. They were maintained in these boxes by sprinkling water every day until further use.

2.7. Molecular identification of earthworm

2.7.1. Genomic DNA isolation

Earthworms were washed in distilled water, then tissue samples (muscular body wall) were taken from behind the clitellum (mostly the tip of the tail) and preseved in 98% ethanol. DNA extraction was performed using 0.2 gram of clitellum using the Roche Kit (Germany) according to the manufacturer's instructions

2.7.2. Primers

Universal set of the COI primers
HCO2198 - 5' - TAA ACT TCA GGG TGA CCA AAA AAT CA- 3'
LCO1490 - 5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3'

2.7.3. PCR amplification

The PCR was done on a thermal cycler (Eppendorf) with 50µl reaction mix. The reaction mix contained 10× amplification buffer (5µl), 1.5mM $MgCl_2$ (5µl), 1µl of each forward and reverse primer, 1µl dNTP and 0.25µl Taq polymerase. After an initial denaturation at 95°C for 1min, amplification was carried out with 35 cycles of 35s at 94 ◦C, 40s at 55 ◦C, 2min at 72 ◦C followed by a final extension for 8min at 72 ◦C. The PCR products were analyzed by electrophoresis using 1.2% agarose gel (Genei).

2.7.4. DNA Sequencer

The PCR product was purified using the Qiagen PCR purification kit and then sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA).

2.7.5. Phylogenetic tree reconstruction

The evolutionary distances were computed using the maximum Neighbor-Joining method [13]. The evolutionary analyse was conducted using MEGA7 software [14]. The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred.

3. Results and Discussion

3.1. Collection of soil sample and its physicochemical parameters

In Tamil Nadu, India, very limited information is available on the distribution pattern of earthworms. The data on earthworm diversity is available only for the stations like Palni Hills [15], Madras [16], Sirumalai Hills [17] and Dindigul [18]. The increasing demand of vermicompost production and the need for studying the efficiency of local wild earthworm species

for Vermicompost technology, the study on earthworm diversity is very significant.

Soil collecting sites were carefully chosen and collected from five different locations, Madurai district, Tamil Nadu, India; they were cultivating land of Vadipatti, non cultivating land of Thirumangalam, grass land of Usilampatti, garden soil from Alagar Kovil and Sewage soil from Melur. Five different physicochemical parameters were monitored from all the samples, in which pH and temperature were calculated *in situ* conditions and the other parameters like moisture content, organic carbon and organic matters were evaluated from the sample brought from the sampling locations immediately after when it reached the laboratory.

Regarding pH, more alkaline pH (8.0 - 8.3) was recorded in the sewage soil whereas in the non cultivating land, a slight acidic pH (6.8 - 7.0) was observed. Further, all the samples of cultivating land, grass land, garden soil showed slightly alkaline pH *viz*., 7.2 - 7.4, 7.1 - 7.3 and 7.3 - 7.5, respectively. Likely, among the sampling locations, sewage soil showed the highest temperature of 30.6 - 33.7ºC followed by non cultivating land with 29.6 - 32.4ºC, garden with 28.4 - 31.4ºC, grass land 29.8 - 30.9ºC and the lowest degree was recorded in Cultivating land with 28.0 - 30.6ºC.

Similarly, sewage soil was recorded for the maximum moisture content (9.462-12.021%), organic carbon (5.56 - 6.84%) and organic matter (12.8 – 16.42%) followed by garden soil contained 8.892-10.12% moisture content, 3.42 - 5.34% organic carbon and 9.21 – 12.85% organic matter, cultivating land observed with 8.884-10.076% moisture content, 3.38 - 5.02% organic carbon and 8.56 – 10.9% organic matter, grass land showed 8.724-10.043% moisture content, 2.18 - 3.98% organic carbon and 6.18 – 9.42% organic matter and the least results were observed for non cultivating land with 6.124 - 8.10% moisture content, 2.07 - 2.92% organic carbon and 4.98 – 6.82% organic matter. The detailed parameters were shown in the table 1.

3.2. Earthworm collections and molecular identification

All the above mentioned five different locations were thoroughly searched for morphologically distinct earthworm species. The collection was covered up from different sites like dry, moist, organic matter enriched sites, etc. and collected species were carefully handled for further molecular identification procedure. For convenience, the collected earthworm species were initially named by the first letter abbreviation of the name of sample collection station *viz*., SCLV (Sample of cultivating land of Vadipatti), SNCLT (Sample of non cultivating land of Thirumangalam), SGLU (Sample of grass land of Usilampatti), SGAK (Sample of garden of Alagar Kovil) and SSSM (Sample of Sewage Soil of Melur) followed by Arabic numbers which were clearly illustrated in the table 2 and 3.

Table 1: Physicochemical properties of the soil samples used for the examination of earthworm study

Soil sample	Physicochemical parameters				
	Moisture	pH	Temperature	Organic carbon	Organic matter
Cultivating land	8.884-10.076%	7.2 - 7.4	28.0 - 30.6ºC	3.38 - 5.02%	8.56 – 10.9%
Non cultivating land	6.124-8.10%	6.8 - 7.0	29.6 - 32.4ºC	2.07 - 2.92%	4.98 – 6.82%
Grass land	8.724-10.043%	7.1 - 7.3	29.8 - 30.9ºC	2.18 - 3.98%	6.18 – 9.42%
Garden	8.892-10.12%	7.3 - 7.5	28.4 - 31.4ºC	3.42 - 5.34%	9.21 – 12.85%
Sewage soil	9.462-12.021%	8.0 - 8.3	30.6 - 33.7ºC	5.56 - 6.84%	12.8 – 16.42%

Table 2: Abbreviated ID of sample collection sites

Samples	Collection site	Abbreviated ID
Sample –I	Cultivating Land of Vadipatti	SCLV
Sample –II	Non Cultivating Land of Thirumangalam	SNCLT
Sample –III	Grass Land of Usilampatti	SGLU
Sample –IV	Garden of Alagar Kovil	SGAK
Sample –V	Sewage Soil of Melur	SSSM

There were four different species of earthworm was found from the above mentioned five selected locations. Based on the molecular identification methods, these four strains were identified using molecular methods with the help of Cytochrome c oxidase subunit 1 (COI) gene partial sequencing methodology. The phylogenetic position of these strains was determined by amplifying the COI gene region and sequences were examined by BLAST analysis. Based on the BLASTN homology of the COI gene sequence of the strains against the nucleotide sequence collection of the NCBI Genbank sequence database, they were identified as *Drawida sp., Perionyx excavatus, Lampito mauritii* and *Drawida japonica*, respectively.

Among the samples, cultivating land of Vadipatti showed only one species of earthworm *viz*., *Drawida sp.,* likely, the non cultivating land of Thirumangalam showed three different earthworm, they were *Drawida sp., Perionyx excavatus and Lampito mauritii*, further, grass land of Usilampatti showed all the four earthworm species identified in this study *viz*., *Drawida sp., Perionyx excavatus and Lampito mauritii* and *Drawida japonica*, the garden soil from Alagar kovil evidenced two different earthworm, *Drawida sp.* and *Drawida japonica* and the sewage soil from Melur evidenced *Perionyx excavatus* and *Lampito mauritii* (Table 4).

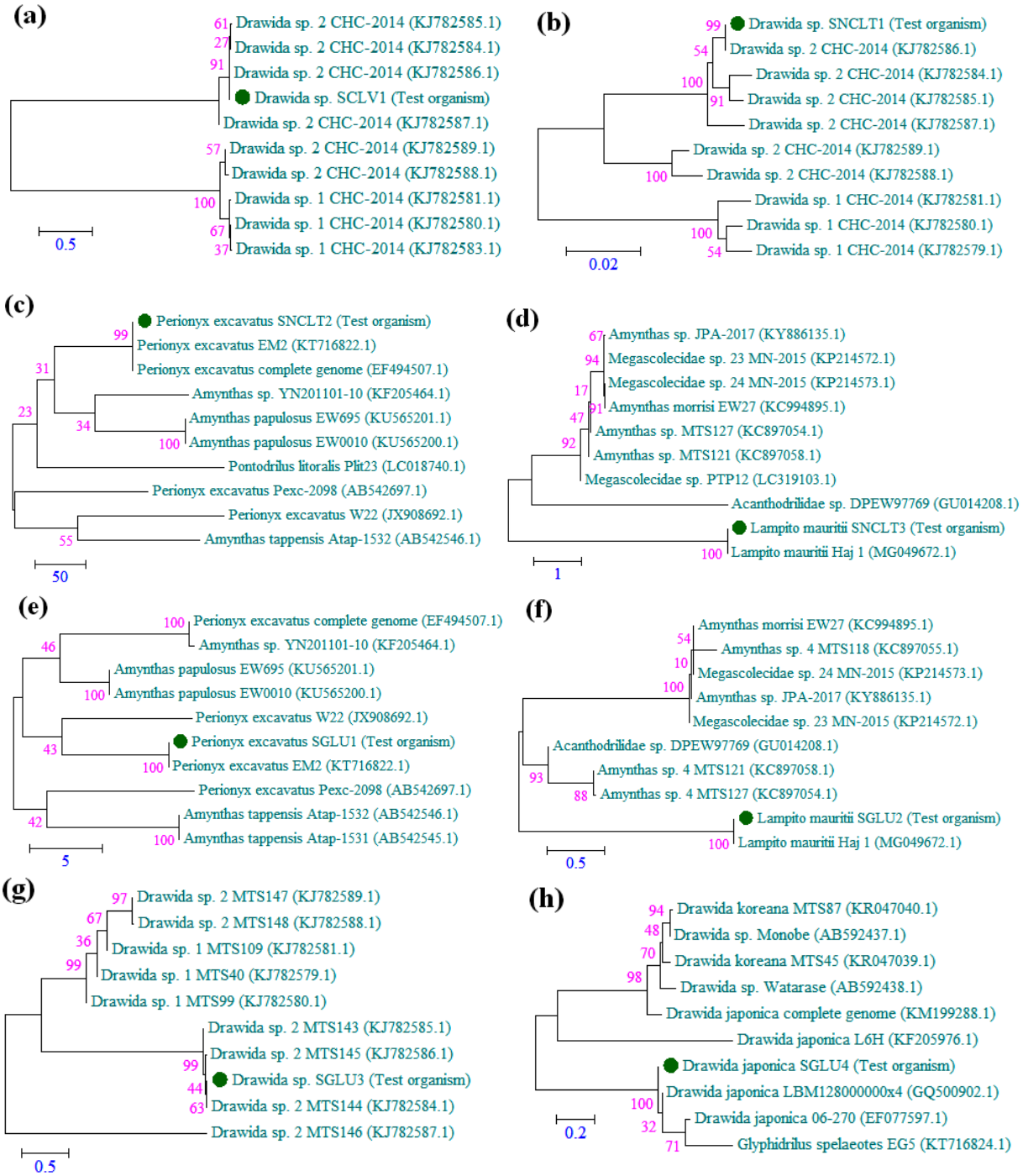
Table 3: Earthworm abbreviated ID and molecular identification details

Earthworm Abbreviated ID	Molecular Identification Name
SCLV1	*Drawida sp.*
SNCLT1	*Drawida sp.*
SNCLT2	*Perionyx excavatus*
SNCLT3	*Lampito mauritii*
SGLU1	*Perionyx excavatus*
SGLU2	*Lampito mauritii*
SGLU3	*Drawida sp.*
SGLU4	*Drawida japonica*
SGAK1	*Drawida sp.*
SGAK2	*Drawida japonica*
SSSM1	*Perionyx excavatus*
SSSM2	*Lampito mauritii*

Based on the evolutionary relationships, the phylogenetic analysis for these twelve earthworm species was analysed with reference to closest NCBI (BLASTn) species based on COI gene sequence and the phylogenetic tree was plotted based on maximum Neighbor-Joining (NJ) method. All the twelve strains *viz*., *Drawida sp.* SCLV1 (Fig. 1a), *Drawida sp.* SNCLT1 (Fig. 1b), *Perionyx excavatus* SNCLT2 (Fig. 1c), *Lampito mauritii* SNCLT3 (Fig. 1d), *Perionyx excavatus* SGLU1 (Fig. 1e), *Lampito mauritii* SGLU2 (Fig. 1f), *Drawida sp* SGLU3 (Fig. 1g), *Drawida japonica* SGLU4 (Fig. 1h), *Drawida sp.* SGAK1 (Fig. 1i), *Drawida japonica* SGAK2 (Fig. 1j), *Perionyx excavatus* SSSM1 (Fig. 1k) and *Lampito mauritii* SSSM2 (Fig. 1l) were individually studied using the maximum NJ phylogenetic tree and all the 12 sequences have showed 100% similarity with the available sequence in NCBI Genebank in which the identification of the species was confirmed from it.

Table 4: Earthworm collection and molecular identification results

Samples	Type	Place	Name of the earth worm
Sample –I	Cultivating land	Vadipatti	*Drawida sp.*
Sample –II	Non cultivating land	Thirumangalam	*Drawida sp., Perionyx excavatus, Lampito mauritii*
Sample –III	Grass land	Usilampatti	*Perionyx excavatus, Lampito mauritii, Drawida sp., Drawida japonica*
Sample –IV	Garden	Alagar kovil	*Drawida sp., Drawida japonica*
Sample –V	Sewage soil	Melur	*Perionyx excavatus, Lampito mauritii*



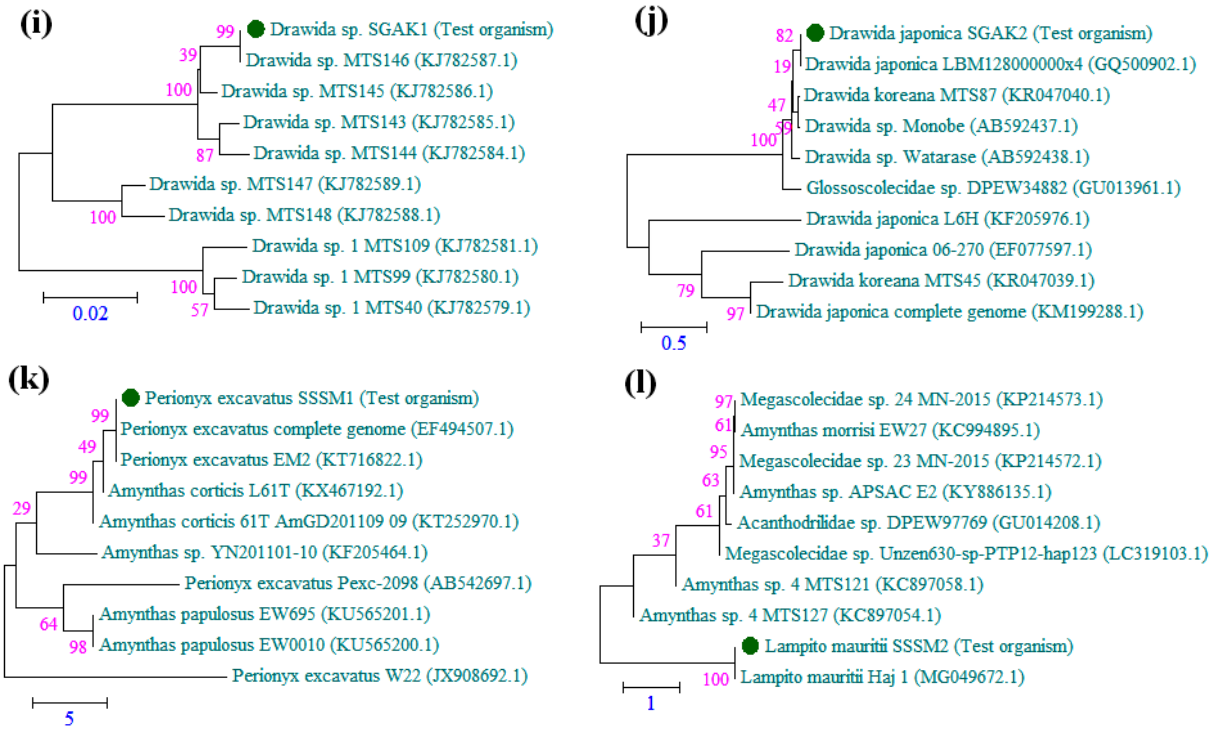


Fig 1: NJ phylogenetic tree of (a) *Drawida sp.* SCLV1, (b) *Drawida sp.* SNCLT1, (c) *Perionyx excavatus* SNCLT2, (d) *Lampito mauritii* SNCLT3, (e) *Perionyx excavatus* SGLU1, (f) *Lampito mauritii* SGLU2, (g) *Drawida sp* SGLU3, (h) *Drawida japonica* SGLU4, (i) *Drawida sp.* SGAK1, (j) *Drawida japonica* SGAK2, (k) *Perionyx excavatus* SSSM1 and (l) *Lampito mauritii* SSSM2

3.3. Physicochemical properties of the soil in correlation with the species dynamics of various earthworms

Among the identified earthworm species in the five selected sampling locations, *Drawida* sp. constituted the maximum species, counted of 33.33% of the overall identified morphologically distinct species and was found in all the samples except the sewage soil which may be due to the sensitivity of the species to the sewage harsh conditions recorded. Similarly, *Drawida japonica* was not found in sewage soil as well as non cultivating land whereas found in both grass land and garden soil which constituted 16.67% of the overall population. Further, both the sewage soil and non cultivating land showed distinct as well as abnormal parameters than the rest of the collection spots. Moreover, both the *Perionyx excavatus* and *Lampito mauritii* constituted each 25% of the total earthworm species which were found in sewage soil, non cultivating land and grass land, further, grass land was evidenced with all the four different species of earthworm identified in this study (Table 5). From this observation, it was clear that both the *Perionyx excavatus* and *Lampito mauritii* were resistant for many environmental conditions for their survival.

Table 5: Biodiversity of earthworm in the five different collected samples

Samples	*Drawida sp.*	*Perionyx excavatus*	*Lampito mauritii*	*Drawida japonica*
Cultivating land of Vadipatti	+	-	-	-
Non cultivating land of Thirumangalam	+	+	+	-
Grass land of Usilampatti	+	+	+	+
Garden of Alagar kovil	+	-	-	+
Sewage soil of Melur	_	+	+	-

Symbols: '-' and '+' denotes presence and absence of specimen

Similar to the present investigation, study on diversity of earthworms was carried out in different soil habitat conditions of Assam, north-east India. Relative occurrence of earthworm species varied according to soil habitat conditions. *Amynthas diffringens, Perionyx excavates, Glyphidrilus gangeticus* and *Lampito mauritii* were dominant species under agricultural land use system while *Metaphire posthuma* and *Dichogaster saliens* were the dominant species under open grass land and mixed forest system [19].

4. Conclusion

From this study, the diversity of earthworm species across different soil habitats of Madurai district has been explored. Further, the species dynamic in relation to physicochemical parameters of soil has also been evidenced using standard methods. Moreover, the present investigation is the first report on diversity of earthworm species collected from different soil habitats of Madurai district, Tamil Nadu, India.

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