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Molecular Diversity of *Lampito mauritii* and *Perionyx excavatus* Across North Eastern Ghats of Odisha Using RAPD Markers

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Abstract

RAPD-PCR technique using 15 RAPD random primers was used to analyze genetic variation of Lampito mauritti and Perionyx excavatus adult worms collected from the agroclimatic zone of North Eastern Ghats of Odisha which includes four different districts. Studies on molecular characterization of L. mauritti and P. excavatus from different geographical locations indicated variation in the molecular profiling. Analysis of genetic diversity was performed using DNA marker systems with RAPD to define genetic relationships among 24 isolates of earthworm. RAPD-PCR data showed significant differentiation among population. The two species analysed in the present study showed high effective population sizes so that within each sampling site a high genetic diversity was observed. RAPD markers were found to be the most effective among the various marker systems used in the present days for the genetic characterization of earthworm individuals.

Keywords: *Lampito mauritti, Perionyx excavatus, RAPD-PCR, genetic variation*

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INTRODUCTION

Earthworms constitute up to 90% of the soil invertebrate biomass and are important ecosystem engineers [1, 2]. According to Julka [3, 4], the subcontinent of India has got a massive fauna of *Oligochaete* (earthworms) which are represented by 509 species and 67 genera. India has a very high percentage of endemic population, together at genus and species level; about 71% of genera and 89% of earthworm species are native.

The studies on growth, reproduction and life cycle of the widespread Indian megascolicid worm, *Lampito mauritti*—one among the four endemic species—are very limited. It can withstand wide range of temperature, soil moisture and various other physical factors [5]; also with wide choice of habitats and food preferences it has the highest frequency of distribution [6]. In the same manner, *Perionyx excavatus* is an earthworm found commonly over a large area of tropical Asia [7, 8]; although it has been transported to Europe and North America. This is an epigeic species which lives in organic wastes, and high moisture contents and adequate amounts of suitable organic material are required for populations to become fully established and for them to process organic wastes efficiently.

Identification of earthworm species on the basis of morphology is difficult, the reason being the lack of the stable and easily scorable diagnostic morphometric characters [9]. A number of molecular markers such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), and Simple Sequence (SSR) are used for fingerprinting [10] which have insightful use in genetic diversity assessment [11]. The genetic diversity and closeness in any species along with earthworms are assessed by RAPD-PCR techniques that are extremely helpful and potent. The use of RAPD and PCR-based markers are beneficial which does not require sequence information of a gene to be amplified [32]. RAPD is a PCR- based assay that has been shown to be useful in generating molecular markers for species or strain identification in a variety of organisms [12].

Kautenburger [13] studied the genetic resemblance between *Lumbricus terrestris* population which were collected from two maize fields using RAPD-PCR technique.

Meenatchi *et al.* [14] used RAPD-PCR technique to examine genetic variations in six strains of *Eudrilus eugeniae* (Kinberg) sampled from six sites in three different states of South India by making use of 20 random primers. Biruntha *et al.* [15] used RAPD in *Perionyx excavatus* sampled from four different sites to analyze the extent of genetic variation and polymorphism. Studies on *Eisenia fetida* carried out from diverse locations of Himachal Pradesh, exhibited greater inter- and intra population variation by molecular markers. Cluster analysis clearly discriminated each earthworm isolates according to their location. With the help of RAPD analysis, genetic variation has been identified in a similar species, it would help to identify the taxonomical nature of the earthworms [16].

For molecular analysis, the effectiveness of RAPD markers was investigated to identify polymorphism among 24 isolates of earthworms (*E. fetida, E. eugeniae*). A total of 10 RAPD markers were used for characterization, which showed 95.7% polymorphism [17].

The present study attempted to use RAPD–PCR technique to amplify genomic DNA by using random primers to quantify the extent of genetic variation within *Lampito mauritti* and *P. excavatus* strains collected from southern parts of the state Odisha and to study the possible phylogenetic relationships among these populations.

MATERIALS AND METHODS

Collection of Sample

The adult worms were collected from agroclimatic zone of North Eastern Ghats of Odisha, India which includes four different districts—Rayagada, Gajapati, Ganjam and Koraput—lies between 19° 10' N–83° 24' E, 19° 23' N–85° 3' E, 18° 51' N–82° 44' E and 19° 11' N–84° 11' E latitudes, respectively. The samples were collected during the months of July to October 2017. The soil is brown in colour and the texture varies from sandy to clay loam. Earthworms with adjacent soil samples were collected from the study sites by random sampling, particularly near the site of surface casting. Three random samples (25 cm × 25 cm × 30 cm) located at least 5 m apart were taken at monthly intervals following Anderson & Ingram (1993) [33]. The earthworms were collected by the hand sorting and wet sieving method. These earthworms were maintained in a laboratory and fed with dried cow dung and waste material of four different sampling sites. These areas were chosen to represent variety of habitat conditions for earthworms.

Preparation of Sample

For gut clearance, the earthworms were kept in distilled water overnight. After that the earthworms were desensitized by 70% alcohol and dissected using sharp surgical blade to get the clitellum part.

Analysis of Morphological Variation

All samples were obtained from different locations for better morphological studies. Earthworm individuals of each sample collected from same location and morphologically similar were examined. Morphological characterization by length (mm), number of segments, position of clitellum, position of spermathecae, and position of male and female pores was performed by visual examination and microscopy. Species identification was carried out by the Zoological Survey of India, Kolkata, India.

Genomic DNA Extraction and PCR Amplification

The earthworm samples were kept in water to remove soil before proceeding to DNA extraction. For isolation of DNA, modified Cetyltrimethyl ammonium bromide (CTAB) method, described by Murray and Thompson (1980) [34] was used. Earthworms were desensitized in 70% ethanol and the clitellum part which was reported to be a good part for genomic DNA extraction [18] was dissected by using sharp laser blade. Earthworm tissues of different species up to 1 cm were taken in clean and dry eppendorf tube. To the tissue 200 µl of 1xTE buffer was added, mixed properly followed by 500 µl CTAB buffer and mixed properly. To the solution, 3–5 µl Proteinase K was added and vigorously vortexed. About 2 µl of RNase was added and incubated at -20 °C for 1 h or overnight or incubated at 55 °C for 1 h and mixed intermittently. To the tubes equal volume of Chloroform: Isoamyl alcohol (24:1)

was added and centrifuged at 11000 RPM for 7 min. The upper layered aqueous phase was transferred carefully to fresh centrifuged tubes and above step was repeated twice for more purified DNA. To the tubes 35 µl of chilled 7.5 M ammonium acetate, 200 µl isopropanol was added and DNA was left to settle down for 20 min. The tubes were centrifuged at 11000 RPM for 10 min and the supernatant was discarded. To the tubes 500 µl of 70% ethanol was added and centrifuged at 11000 RPM for 3 min and supernatants were discarded. Equal volume of the 100% ethanol was added to the tube and centrifuged at 11000 RPM for 3 min and supernatants were discarded. The tubes were kept for drying at room temperature. Then the dry pellet or DNA was dissolved in 30 µl of 1X TE (10 mM Tris.HCl and 1 mM EDTA) buffer and incubated for 2 h at room temperature. Lastly the samples were stored at -20 °C for further use (Table 1).

Quantification of DNA by Agarose Gel Electrophoresis

1X TE buffer was used for 30 min at 110v for electrophoresis. About 2 µl of 6X loading dye with 3 µl of the isolated genomic DNA samples was loaded on 1% agarose gel. Ethidium bromide was added to it to check the quality of DNA. The gels were photographed on gel documentation system (protein simple Fluor chem. M).

Table 1: *RAPD-PCR Analysis: RAPD-PCR was Conducted Using 15 Random Primers.*

Sl No	Primer Id	Sequence (5′-3′)
1	OPA 1	CAGGCCCTTC
2	OPA 3	AGTCAGCCAC
3	OPA 5	AGGGGTCTTG
4	OPA 6	GGTCCCTGAC
5	OPA 7	GAAACGGGTG
6	OPA 8	GTGACGTAGG
7	OPA 9	GGGTAACGCC
8	OPA 10	GTGATCGCAG
9	OPA 11	CAATCGCCGT
10	OPA 12	TCGGCGATAG
11	OPA 13	CAGCACCCAC
12	OPA 14	TCTGTGCTGG
13	OPA 15	TTCCGAACCC
14	OPA 16	AGCCAGCGAA
15	OPA 17	GACCGCTTGT

PCR reactions were carried out in a total reaction volume of 20 µl comprising of 2 µl (20 ng) genomic DNA, 2 µl PCR buffer (10 X) containing $MgCl_2$ (20 mM), 2 µl (20 mM) dNTP (Qiagen Pvt. Ltd), 0.33 units of Taq DNA polymerase, and 2 µl (15ng) each of oligonucleotide primer. The isolated DNA samples were checked for quality by running in 1.3% agarose gel. Only intact DNA samples were used for further analysis. The concentration was calculated on the basis of OD reading at 260 nm. Amplifications were carried out in an Eppendorf master gradient cycler (Eppendorf, AC, Germany). Amplification conditions for RAPD analysis includes 1 min initial denaturation at 94 °C followed by 35 cycles of 1 min at 94 °C for denaturation, 1 min at 36 °C for primer annealing, 2 min at 72 °C for primer extension and 10 min for the final primer extension at 72 °C. Amplified products were electrophoresed in 1.4% agarose gel electrophoresis containing ethidium bromide, DNA staining dye and the photograph was taken under transilluminator emitting UV light.

Data Scoring and Statistical Analysis

The clear, reproducible, and scorable allele classes were scored by binary data matrices on the basis of their presence (1) or absence (0) in the gel. All the alleles were scored to avoid over/under estimate the diversity. Diffused alleles or alleles revealing ambiguity in scoring were considered as missing data and designated as '999'. Genetic similarity between pairs was calculated according to Jaccard's similarity coefficient (UPGMA), and a dendrogram was generated by using NTSYS-pc version 2.1 software.

RESULTS

Morphological Variability Among Earthworm Isolates

Samples collected from different locations of four districts were identified as *L. mauritii* and *P. excavatus* based on key morphological features such as length, number of segments, position of clitellum, position of spermathacae, and position of male and female pores on the segments (Table 2). The *L. mauritii* sampled from Gajapati district was found as the longest (160 mm) species and *P. excavatus* collected from Koraput district was found to be the smallest (76 mm) in body length. *L. mauritii* had more number of segments (124) as

compared to other species. *P. excavatus* collected from Ganjam district was found to have fewer number of segments (92). The position of clitellum in *L. mauritii* and *P. excavatus* varied from 22 to 30 and 16 to 21 number of segments, respectively.

Genetic Variations in *Perionyx excavatus* and *Lampito mauritii* Collected from Various Sites

The number of polymorphic bands generated by RAPD primers along with monomorphic bands is given in Table 3. Maximum number of bands i.e., 11 bands were scored with primer OPA 9 followed by 10 bands with primer OPA 10. Minimum number of bands i.e., four bands was scored with primer OPA 12. Analysis of four strains each of *P. excavatus* and *L. mauritii* with 15 primers showed repeatable amplification with a total of 111 bands produced with an average of 7.4 per primer. Analysis also showed that 56 bands were monomorphic with an average of 3.73 per primer and 55 bands were polymorphic with an average of 3.66 per primer. The total percentage of polymorphism was found to be an average of 51.57% per primer (Table 4) with a maximum polymorphism shown by OPA 5 (71.42%).

Table 2: *Morphological Variability Among Earthworms Collected from Four Different Districts.*

District	Name of species	No. of samples	Body length (mm)	Number of segments	Position of clitellum	Average body weight (g)
Rayagada	*Lampito mauritii*	3	145	103	22–30	1.27
Rayagada	*Perionyx excavatus*	3	112	97	16–21	0.84
Ganjam	*Lampito mauritii*	3	115	116	22–30	0.91
Ganjam	*Perionyx excavatus*	3	88	92	16–21	0.73
Gajapati	*Lampito mauritii*	3	160	124	22–30	1.35
Gajapati	*Perionyx excavatus*	3	80	88	16–21	0.62
Koraput	*Lampito mauritii*	3	125	113	22–30	1.17
Koraput	*Perionyx excavatus*	3	76	93	16–21	0.68

Table 3: *Polymorphic Bands Generated by RAPD Primers.*

Sl No	Primer Id	Sequence (5′-3′)	Total band	No. of monomer-phic bands	No. of polymor-phic bands	Polymorphism (%)
1	OPA 1	CAGGCCCTTC	6	2	4	66.66
2	OPA 3	AGTCAGCCAC	8	3	5	62.5
3	OPA 5	AGGGGTCTTG	7	2	5	71.42
4	OPA 6	GGTCCCTGAC	5	2	3	60
5	OPA 7	GAAACGGGTG	9	7	2	22.22
6	OPA 8	GTGACGTAGG	7	3	4	57.14
7	OPA 9	GGGTAACGCC	11	5	6	54.54
8	OPA 10	GTGATCGCAG	10	7	3	30
9	OPA 11	CAATCGCCGT	9	5	4	44.44
10	OPA 12	TCGGCGATAG	4	1	3	75
11	OPA 13	CAGCACCCAC	9	5	4	44.45
12	OPA 14	TCTGTGCTGG	8	4	4	50
13	OPA 15	TTCCGAACCC	5	3	2	40
14	OPA 16	AGCCAGCGAA	6	2	4	66.66
15	OPA 17	GACCGCTTGT	7	5	2	28.57
		Total	111	56	55	51.54%

Table 4: *Summary of Statistics of RAPD Analysis of Perionyx excavatus and Lampito mauritii.*

Sl. No.	Particulars	Counts
1	Total number of bands	111
2	Total number of polymorphic bands	55
3	Maximum number of bands produced per primer	11
4	Minimum number of bands produced per primer	4
5	Average polymorphic percentage per primer	51.57

The RAPD technique was used to find out the extent of genetic diversity in *P. excavatus* and *L. mauritii* collected from four different districts of North Eastern Ghats of Odisha, India and primers OPA (1–17) were used for amplification. The amplification profile of *L. mauritii* is presented in Figure 1 with primer OPA 5, OPA 6, OPA 7 and the amplification profile of *P. excavatus* is presented in Figure 2 with primer OPA 6, OPA 9, OPA 10, and OPA 12.

Genetic Relationship Between Earthworm Isolates

The dendrogram representing genetic relationships among *P. excavatus* and *L. mauritii* of four different districts based on Jaccard's similarity coefficients was constructed which resulted in two major clusters. The first cluster comprised of individuals from *Lampito* GP1, *Perionyx* K2, *Lampito* G3, *Lampito* GP3, *Perionyx* G2, *Perionyx* R3 and the second cluster comprised of *Lampito* K1, *Lampito* R3, *Perionyx* G3,

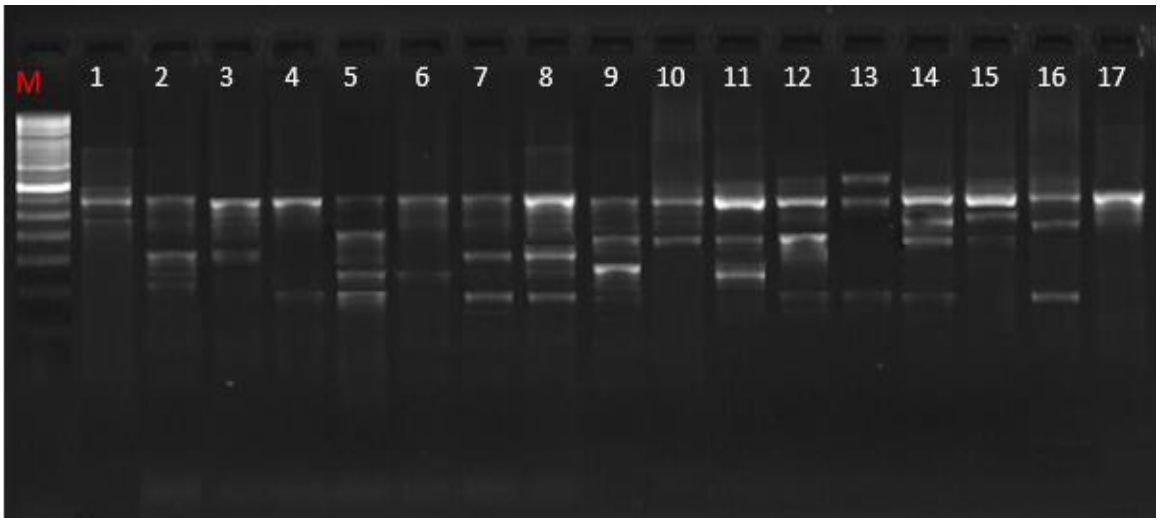

Fig. 1: *RAPD PCR Amplification of DNA Isolated from Lampito mauritii Collected from Four Districts of North Eastern Ghats of Odisha (Lane M: marker).*

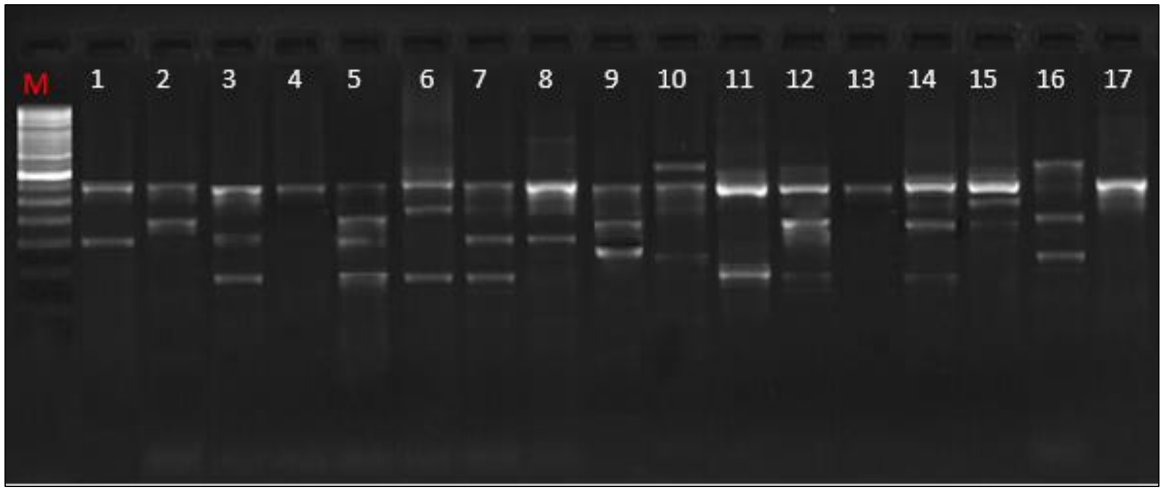

Fig. 2: *RAPD PCR Amplification of DNA Isolated from Perionyx excavatus Collected from Four Districts of North Eastern Ghats of Odisha (Lane M: marker).*

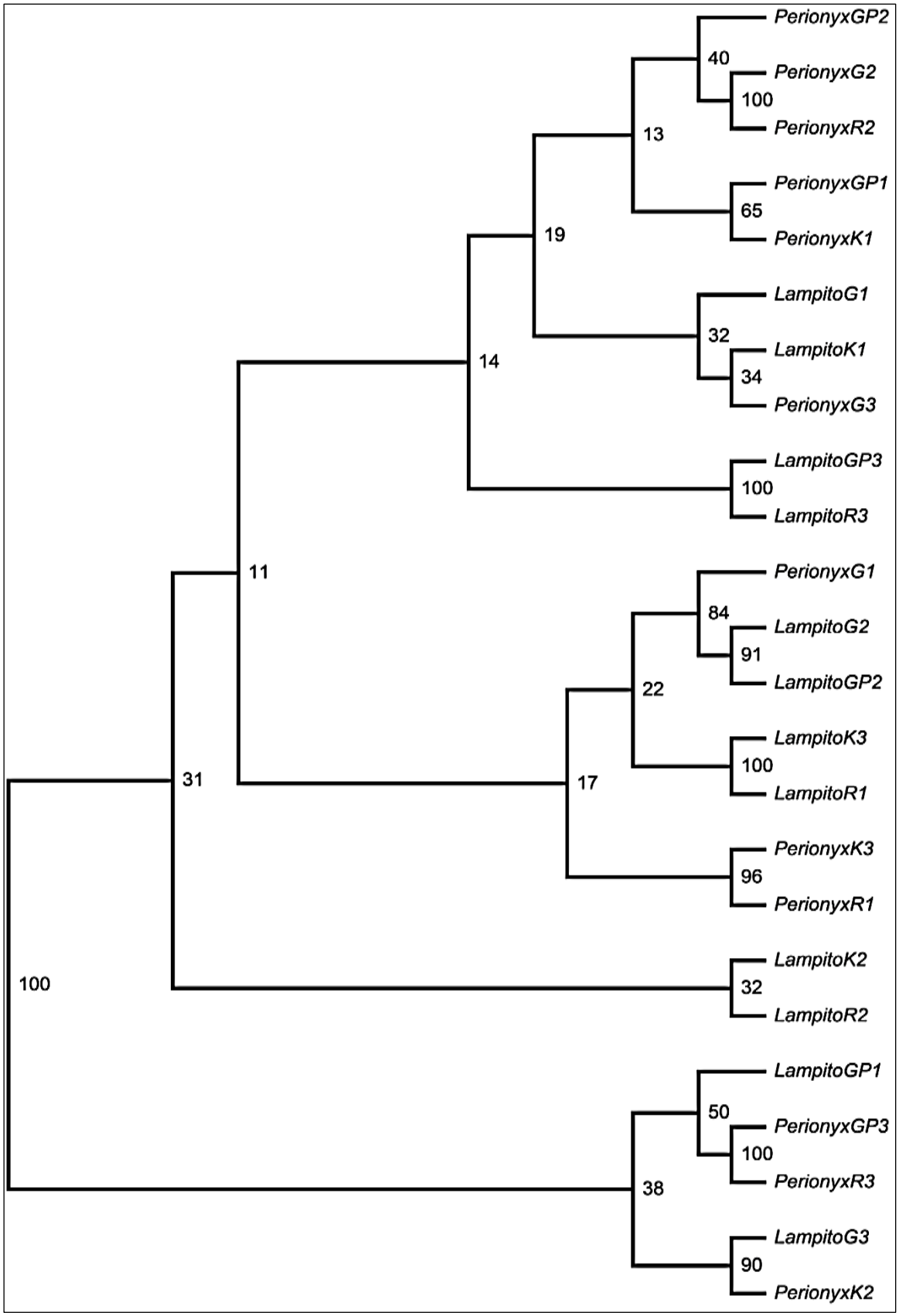

Fig. 3: *UPGMA-Based Dendrogram Representing Genetic Relationships among Perionyx excavatus and Lampito mauritii of Four Different Districts based on Jaccard's Similarity Coefficients (G: Ganjam district, GP: Gajapati district, R: Rayagada district, K: Koraput district).*

Lampito G1, *Perionyx* GP1, *Perionyx* K1, *Lampito* R1, *Lampito* K3, *Perionyx* GP2, *Perionyx* R2, *Perionyx* G1, *Lampito* G2, *Lampito* GP2, *Lampito* K2, *Lampito* R2, *Perionyx* K3, *Perionyx* R1. Similarity coefficients for combined data of all the markers were calculated using Jaccard`s similarity coefficients (Figure 3) which were further used for the construction of dendrogram. UPGMA-based clustering for all the markers of RAPD is depicted. The pooled matrix showed maximum genetic similarity of 1.0 between *Lampito* R1 and *Lampito* K3, between *Perionyx* GP3, *Perionyx* R3 followed by 0.90 between *Lampito* GP3 and *Lampito* R3, between *Perionyx* G2 and *Perionyx* R2 follwed by 0.80 between *Perionyx* G1 and *Lampito* G2, between *Perionyx* K3 and *Perionyx* R1. Minimum genetic similarity was observed between *Perionyx* K1 and *Perionyx* R1 strains with genetic similarity value of 0.20.

DISSCUSSION

The knowledge on the earthworm fauna of India has been enriched by Julka and Senapati [19, 20], Senapati *et al.* (1990) [35], Julka and Paliwal [21] and Paliwal and Julka [22]. However, a close review of literatures on the study of earthworms in Odisha indicates that a lot of research work has been done on earthworm biodiversity in past several years from 1976 to 2016 [23–30]; all of which are basically at morphological level. Molecular methods have become powerful and precise tools for identification and analysis of genetic diversity of earthworm species. Therefore an attempt has been made by this research work to find out polymorphism among 24 earthworm strains at genetic level with the help of RAPD markers. The present work, therefore, is a humble attempt in this direction and contributes to update our contemporary knowledge on the biodiversity of earthworm's resources in the study area at molecular level.

The indigenous earthworms (*L. mauritii* and *P. excavatus*) which are commonly found in Indian soils, has appeared as an efficient tool for organic waste management. Based on key morphological features, all the individuals were classified into two earthworm species, namely *P. excavatus* and *L. mauritii*. Molecular markers that are considered most robust can be effectively used in characterization of genetic variability in various species. In the present study, analysis of genetic diversity was performed using DNA marker systems with RAPD to define genetic relationships among 24 isolates of earthworm. RAPD-PCR data showed significant differentiation among population.

RAPD markers were found to be the most effective among the marker systems used in the present investigation for the genetic characterization of earthworm individuals. RAPD can be a powerful tool for initial assessment of genetic diversity among different isolates [31] including earthworms.

The two species analysed in the present study showed high effective population sizes so that within each sampling site a high genetic diversity can be observed. However, the loss of genetic variability due to intensive agricultural land use can significantly undermine the viability of populations, particularly for some long-lived species with lower reproductive rates. This study, with strictly controlled experimental conditions, suggests that RAPDs can be used to measure the degree of genetic similarity between individuals, populations and species of *L. mauritii* and *P. excavatus* earthworms in Odisha, India.

CONCLUSION

The present study indicated that *L. mauritii* and *P. excavatus* strains across the locations spread in state of Odisha, India though appeared similar morphologically but exhibited considerable amount of genetic variations. This may be due to different climatic conditions and niche availability, which could have strong bearing on their evolution and hence might be sharing more nucleotides in genome level, creating variation within the strains of the earthworms. The present study on molecular characterization of earthworms is the first report from Odisha, India. Various earthworm species could be validated at molecular level by screening different markers, which could provide valuable information for ecological genetic studies in earthworm species, which can be utilized for identification, characterization and conservation of earthworm fauna across the state at various ecological habitats.

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