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ISOLATION AND MOLECULAR IDENTIFICATION OF MAJOR BACTERIAL SPECIES OF RED SOIL AND VERMICOMPOST MADE FROM *Eleusine coracana* BIOMASS

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ABSTRACT

The amount of plant growth-promoting microbial population is a direct indicator of soil health. Biochemical isolation and molecular identification study was conducted in Singapura village of Bengaluru district in 2024 using 16S rRNA technique of major bacterial species present in red soil and vermicompost made from *Eleusine coracana* biomass using the earthworm species *Eudrilus eugeniae*. There were six major bacterial species identified from red soil belonging to genera *Pseudomonas* and *Stutzerimonas.* The most abundant species among them was *Pseudomonas oligotrophica,* and other species having similar genome were *Stutzerimonas stutzeri, Pseudomonas phenolilytica*, *Pseudomonas songnenensis*, *Stutzerimonas nitrititolerans,* and *Stutzerimonas chloritidismutans.* These were mostly chemoorganotrophs and were able to survive in low-nutrient soils. No bacteria were identified to be beneficial for plant growth. On the other hand, there were ten major bacterial species identified from vermicompost made from *Eleusine coracana* biomass belonging to genera *Enterobacter* and *Atlantibacter.* The most abundant species among them was *Enterobacter hormaechei,* and other genetically similar species were *Enterobacter quasihormaechei, Enterobacter bugandensis, Enterobacter chuandaensis, Atlantibacter hermannii, Enterobacter cancerogenus, Enterobacter mori, Enterobacter sichuanensis, Enterobacter quasiroggenkampii* and *Enterobacter ludwigii.* Out of ten species, six were plant growth-promoters, one species was a candidate for bioremediation, one species was useful in biomedical applications, and two species were human pathogens. These results showed that vermicompost made from *Eleusine coracana* biomass contained considerable populations of bacteria which were beneficial for plant growth. With availability of huge amount of *Eleusine coracana* biomass, its conversion into vermicompost can be a sustainable solution for improving soil health.

(Key words: *Pseudomonas*, *Enterobacter*, *Eleusine coracana*, sustainable, vermicompost)

INTRODUCTION

The role of soil organic matter in maintaining soil quality is well known. The soil organic matter has been defined as the organic fraction of soil including plant, animal and microbial residues. Increasing concerns of soil degradation and CO_2 emission-induced global warming have forced us to focus our research on understanding the nature and function of soil organic matter (Katyal *et al.,* 2016). Soil microbial biomass has a direct relationship with the amount of soil organic matter formation and stabilization. It is also well correlated with many soil health indices. Good agricultural practices improve microbial biomass which in turn promote rapid turnover of soil organic matter (Rao *et al.,* 2019). A healthy soil has physical, chemical and biological processes functioning efficiently subject to the limits imposed by a given ecosystem. Thus, the soil health is considered as a more sensitive and dynamic way to document its condition, response to management changes and stress tolerance imposed by natural forces or human uses (Bhaduri *et al.,* 2014). Vermicomposting is a sustainable and environment-friendly option for recovering nutrients from waste biomass with the help of earthworms (Soobhany, 2019). It provides organic carbon to the soil which results in higher seed yield and nutrient uptake. It also improves soil ecosystem by increasing biological nitrogen fixation and promotes growth of mineral-mobilizing bacteria like phosphate-solubilizers, zinc-solubilizers and nitrogen-fixers (Kaur *et al.,* 2023). The composition of microbial community at any instant during vermicomposting process depends on the rate of degradation of organic matter. These changes are due to microbial succession (Aira *et al.,* 2019). The first microbial colonizers during composting process are mainly heterotrophs which are replaced by other populations as the composting process ensues. These changes are due to the rate of organic carbon derived from the initial substrate. The different microbial communities involved in the various stages of vermicomposting are less known (Cai *et al.,* 2018).

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A study on vermicomposting of a leguminous shrub scotch broom (*Cystisus scoparius*) with the earthworm species *Eisenia andrei* revealed increase in taxonomic and functional diversity of bacterial community including nitrification efficiency, metabolic capacity and streptomycin and salicylic acid synthesis (Dominguez *et al.,* 2019).

For this study, soil samples were obtained from the Singapura village, Bengaluru Urban district (latitude 13.0747356° N, longitude 77.5376929° E). It is characterized by red and laterite soils. The soils are shallow to very deep, well-drained loam to clay with moderate to moderately rapid permeability. The drainage area of the district is prominently influenced by the South-West monsoon (Anonymous, 1990). Finger millet (*Eleusine coracana*) or Ragi is an important crop grown in this soil. Crop residues are a potent source of organic matter and nutrient substitution through composting (Rao *et al.,* 2019). In the present study, vermicompost was prepared from the residues of *Eleusine coracana* and samples from red soil and vermicompost were analyzed biochemically to isolate the major colonies of microbes. The most abundant colonies in both samples were further analyzed using 16S rRNA technique to determine the major species of microbes.

MATERIALS AND METHODS

Preparation of vermicompost

Approximately 10 kg of fresh and dry *E. coracana* straw was obtained from a local farmer in July 2024 from Singapura Village, Bengaluru Urban District. It was shredded and allowed for partial decomposition in cement tanks for a period of 8 weeks after adding thin slurry of cow dung and maintained at 75% to 80% moisture by sprinkling water regularly. After 8 weeks of partial decomposition, adult earthworm species *Eudrilus eugeniae* were introduced at the rate of 10 g into 1 kg feedstock material (Karmegam and Daniel, 2000). The entire feed stock material was converted into vermicompost in approximately 4 weeks.

Preparation of bacterial suspension

A 5g of sample each from vermicompost and red soil was mixed with 90 ml of 0.85% (w/v) physiological saline solution and incubated at 30°C in a shaker incubator at 120 rpm for 2 hours. The suspensions obtained were filtered through Whatman no.1 filter paper under sterile conditions and used for further analysis (Kumar *et al.,* 2016).

Isolation of bacteria

A quantity of 1 ml was taken from the prepared bacterial suspension and serially diluted in the range of 10^{-1} to 10^{-9}. Each dilution was plated in five replications on nutrient agar solid medium and incubated at 37°C for 48 hours. The colonies were counted and their average number was calculated. Individual colonies of bacteria showing different morphological characters on Petri plates were selected and purified on the same media by streaking 3-4 times in the fresh media. The bacterial isolates were kept on slants at 4°C and sub-cultured every 4 weeks (Filali *et al.,* 2000).

Colony morphology and biochemical characterization of bacterial isolates

The isolated bacterial colonies were grown on nutrient agar medium. The morphology of the colonies was examined under microscope. They were examined for Gram staining, shape, margin, elevation, surface texture, opacity, and colour. Biochemical analysis including catalase test, oxidase test, Voges-Proskauer test, methyl red test, indole test, starch hydrolysis test, citrate utilization test, glucose utilization test and lactose utilization test were done according to the standard protocols (Nasfi *et al.,* 2018).

Microbial identification using 16S rRNA-based molecular method

For 16S rRNA-based molecular method, DNA was isolated from the bacterial culture and amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward and reverse primers using BDT v3.1 Cycle sequencing kit in ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software, which was used to carry out BLAST with the NCBI GenBank database. Based on maximum identity score, first 10 sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7 (Kimura, 1980; Felsenstein, 1985; Kumar *et al.,* 2015). The gene sequence was submitted to GenBank Database.

RESULTS AND DISCUSSION

Colony morphology

There were six different colonies isolated from red soil which were coded as RSC1 (Red soil colony 1), RSC2, RSC3, RSC4, RSC5 and RSC6. Among these colonies, RSC1 and RSC3 had irregular shape and others had circular shape. RSC1, RSC4 and RSC6 had smooth margins, RSC2 had lobated margin, RSC3 had serrated margin, and RSC5 had irregular margin. RSC1, RSC3, RSC4 and RSC6 had flat elevation but RSC2 and RSC5 had raised elevation. RSC2 had rough surface, but remaining colonies had smooth surface. RSC4 was transparent but other colonies were opaque. Among the opaque colonies, RSC1, RSC2 and RSC6 were white in colour but RSC3 and RSC5 were yellow in colour. From the *E. coracana* biomass vermicompost, there were seven colonies isolated which were coded as RVC1 (Ragi vermicompost colony 1), RVC2, RVC3, RVC4, RVC5, RVC6 and RVC7. Among these colonies, RVC2, RVC3 and RVC7 had irregular shape but RVC1, RVC4, RVC5 and RVC6 had circular shape. RVC1, RVC4 and RVC6 had smooth margins, RVC3 and RVC7 had serrated margins, RVC2 had lobated margin and RVC5 had irregular margin. RVC3, RVC4, RVC6 and RVC7 had flat elevation but RVC1, RVC2 and RVC5 had raised elevation. The surface of RVC1 was rough but other six colonies had smooth surface. RVC4 was transparent but other six colonies were opaque. Among the opaque colonies, RVC2 and RVC6 were white in colour but remaining four colonies were yellow in colour (Table 1).

Colony count

From the red soil samples, there were six different types of colonies counted at dilution of 10^5. The count of colony 1 was 42.60 ± 1.89, colony 2 was 7.00 ± 0.71, colony 3 was 6.40 ± 0.51, colony 4 was 5.20 ± 0.37, colony 5 was 5.00 ± 0.45 and colony 6 was 3.00 ± 0.45. The total count of all colonies was 68.10 ± 6.65 (Table 2). From the *E. coracana* biomass vermicompost, there were seven different types of colonies counted at dilution of 10^5. The count of colony 1 was 3020 ± 333.77, colony 2 was 418 ± 47.58, colony 3 was 46.20 ± 6.53, colony 4 was 37.20 ± 3.81, colony 5 was 40.80 ± 6.22, colony 6 was 43.60 ± 3.08 and colony 7 was 24.60 ± 3.28. The total count of all colonies was 3600 ± 236.14 (Table 3).

Biochemical analysis

The biochemical tests of bacterial isolates from red soil showed that out of six colonies, five were Gram-positive and one was Gram-negative. Catalase test was positive in four colonies and negative in two colonies. Oxidase test was also positive in four colonies and negative in two colonies. Voges-Proskauer test was positive in three colonies and negative in three colonies. Methyl red test was positive in four colonies and negative in two colonies. Indole test was negative in all the six colonies. Starch hydrolysis test was positive in five colonies and negative in one colony. Citrate utilization, glucose utilization and lactose utilization tests were positive in all the six colonies (Table 4). The biochemical tests of bacterial isolates from *E. coracana* biomass vermicompost showed that out of seven colonies, four were Gram-positive and three were Gram-negative. Catalase test was positive in four colonies and negative in three colonies. Oxidase test was also positive in four colonies and negative in three colonies. Voges-Proskauer test was positive in five colonies and negative in two colonies. Methyl red test was positive in six colonies and negative in one colony. Indole test was positive in four colonies and negative in three colonies. Starch hydrolysis test was positive in all the seven colonies. Citrate utilization test, glucose utilization test and lactose utilization test were also positive in all the seven colonies (Table 5).

Molecular identification

The most abundant colony from the red soil was further analyzed using 16S rRNA technique to construct the phylogenetic tree (Figure 1). Based on nucleotide homology and phylogenetic analysis, the red soil had the most abundant bacterial species as *Pseudomonas oligotrophica* (Accession number PV750902) which showed close genomic similarities with the following major bacterial species: *Stutzerimonas stutzeri, Pseudomonas phenolilytica*, *Pseudomonas songnenensis*, *Stutzerimonas nitrititolerans,* and *Stutzerimonas chloritidismutans. Pseudomonas oligotrophica* has been found to have excellent capability for denitrification under the low C/N condition. It is a bacterium that lives in low-nutrient soils. Its genomic analysis has revealed many functional genes such as *napA, narG, nirS, norB* and *nosZ* which encode a number of enzymes required for complete denitrification (Zhang *et al.,* 2022). *Stutzerimonas stutzeri* is closely related to *Pseudomonas* genus although this specie is represented on a clad of its own. This specie has recently been found to be a promising candidate for degradation of N-acetyl-para-aminophenol (Paracetamol) which is one of the most popular antipyretic and analgesic used worldwide (Vargas-Ordonez *et al.*, 2023). *Pseudomonas phenolilytica* is recognized as a novel phenol-degrading bacteria (Kujur and Das, 2022). *Pseudomonas songnenensis* was first isolated from the saline and alkaline soil of Songnen plain, north-east of China. It has also been recognized as a potential denitrifier (Zhang *et al.*, 2015; Liu *et al.*, 2019). *Stutzerimonas nitrititolerans* has been found to be multidrug-resistant including cefazolin, chloramphenicol, clindamycin, streptomycin, sulfamethoxazole, tetracycline and vancomycin (Shi *et al.*, 2024). *Stutzerimonas chloritidismutans* is a facultative aerobic, strictly respiratory bacteria that is able to grow anaerobically with nitrate or chlorate. It is catalase-positive chemoorganotroph that lives in soil, wastewater sludge, marine sediment, rhizosphere and clinical specimens (Gomila *et al.*, 2022).

The second query sequence from vermicompost made from *E. coracana* biomass was also analyzed using bioinformatics tools and phylogenetic tree was constructed (Figure 2). The phylogenetic analysis revealed the closest similarity of the query sequence to *Enterobacter hormaechei* (Accession number PV752219). Thus, the *E. coracana* biomass vermicompost showed the most abundant bacterial species as *Enterobacter hormaechei* which showed close genomic similarities with the following major bacterial species: *Enterobacter quasihormaechei, Enterobacter bugandensis, Enterobacter chuandaensis, Atlantibacter hermannii, Enterobacter cancerogenus, Enterobacter mori, Enterobacter sichuanensis, Enterobacter quasiroggenkampii* and *Enterobacter ludwigii. Enterobacter hormaechei* has been shown to enhance plant growth by synthesizing indole acetic acid, ammonia, fixing free nitrogen, solubilizing potassium and phosphate. It has been identified as a potential green solution to chemical fertilizers as it can increase crop productivity by converting insoluble forms of potassium and phosphorus to available form (Ranawat *et al.,* 2021). *Enterobacter quasihormaechei* has been recognized for its ability to produce rhamnolipids biosurfactants and a potential candidate for biosurfactant-mediated oil-spill cleanup in environmental remediation. *Enterobacter bugandensis* has been isolated from rhizosphere of wheat plants (*Triticum aestivum* L.) under drought conditions and has shown different plant growth-promoting properties including nitrogen fixation, siderophore production, phosphate solubilization, and indole acetic acid and gibberellic acid production. Its inoculation to wheat plants improved plant growth in terms of root and shoot growth and chlorophyll content. It also showed drought stress ameliorating properties including increased osmolyte content, relative water content, superoxide dismutase and catalase activity and decrease in peroxidation of lipids compared to non-inoculated plants (Arora and Jha, 2023). *Enterobacter chuandaensis* has been found to produce bacterial cellulose through the polymerization of glucose monomers obtained from fruit extracts. Bacterial cellulose has potential for use in biomedical applications (Al-Hasabe *et al.*, 2024). *Atlantibacter hermannii*, previously known as *Escherichia hermannii*, is a Gram-negative, nonspore-forming motile bacteria found in a diverse range of habitats. It is a rare causative agent of human infections, especially in immunosuppressed patients undergoing hemodialysis (Hristova *et al.,* 2023). *Enterobacter cancerogenus* has been shown to promote growth of rice under salt stress with significantly increased plant biomass, plant height and root length. Its genomic analysis has revealed many plant-growth-promoting genes such as indole acetic acid production, siderophore, nutrient uptake, adaptation to harsh environment including antioxidant enzyme, exopolysaccharide, osmoprotectant accumulation, and ion homeostasis (Peng *et al.,* 2024). *Enterobacter mori* has been shown to have numerous genes that are involved in phosphate solubilization, iron transport, hormone regulation, nitrogen fixation, and resistance to environmental stress such as drought, osmotic and oxidative stress. It also produces organic compounds that can improve soil health and plant growth (Fadiji *et al.,* 2023). *Enterobacter sichuanensis* is also a plant growth-promoting rhizobacteria isolated from soybeans (*Glycine max*) which contains genes involved in providing nitrogen to plants and solubilization of phosphates (Ajibade *et al.,* 2023). *Enterobacter quasiroggenkampii* has been found to have ability to ferment inositol, D-sorbitol and melibiose but not potassium gluconate, L-fucose and methyl mannopyranoside. It is a pathogen of humans that can cause nosocomial infections (Wu *et al.,* 2020). *Enterobacter ludwigii* is a component of gut microbiota of insect herbivores that influence plant growth in nature. Its application to the germinated tomato seeds showed faster root, shoot and hypocotyl growth and produced more fruits and seeds than untreated control plants (Pan *et al.,* 2019).

From the study, it is inferred that the red soil sample was found to have six identifiable colonies, in which the most abundant colony was of *Pseudomonas oligotrophica* which is able to survive in low-nutrient soil. Its genome showed close similarity with five other bacterial species, out of which two belong to genus *Pseudomonas* and three belong to genus *Stutzerimonas*. Among the six species of bacteria, two were found to be potential denitrifiers, one was found to be paracetamol-degrader, one was phenol-degrader, one was multidrug-resistant and one was a chemoorganotroph. The *E. coracana* biomass vermicompost showed seven identifiable colonies, in which the most abundant colony was of *Enterobacter hormaechei* which had close genomic similarity with nine other bacteria, out of which eight belong to genus *Enterobacter* and one belongs to genus *Atlantibacter*. Out of a total of ten major bacterial species in the vermicompost, six species were found to be having plant growth-promoting abilities including production of hormones, resistant to environmental stress and improvement of soil health. One species is a candidate for environmental bioremediation, one species has potential use in biomedical applications and two species cause human infections. Thus, we can conclude that the vermicompost generated from *E. coracana* biomass consisted of more number of bacterial species which are useful for plant growth as well as bioremediation of pollutants.

Table 1. Colony morphology of bacterial isolates from red soil and *E. coracana* biomass vermicompost

Sources	Coded Isolates	Characters					
		Shape	Margin	Elevation	Surface	Opacity	Colour
Red soil colonies	**RSC1**	Irregular	Smooth	Flat	Smooth	Opaque	White
	RSC2	Circular	Lobated	Raised	Rough	Opaque	White
	RSC3	Irregular	Serrated	Flat	Smooth	Opaque	Yellow
	RSC4	Circular	Smooth	Flat	Smooth	Transparent	Transparent
	RSC5	Circular	Irregular	Raised	Smooth	Opaque	Yellow
	RSC6	Circular	Smooth	Flat	Smooth	Opaque	White
Ragi vermicompost colonies	**RVC1**	Circular	Smooth	Raised	Rough	Opaque	Yellow
	RVC2	Irregular	Lobated	Raised	Smooth	Opaque	White
	RVC3	Irregular	Serrated	Flat	Smooth	Opaque	Yellow
	RVC4	Circular	Smooth	Flat	Smooth	Transparent	Transparent
	RVC5	Circular	Irregular	Raised	Smooth	Opaque	Yellow
	RVC6	Circular	Smooth	Flat	Smooth	Opaque	White
	RVC7	Irregular	Serrated	Flat	Smooth	Opaque	Yellow

Note: All values are the mean and standard error of five replicates.

Table 2. Colony counts (CFU g^{-1}) of bacterial isolates from red soil

	Red soil colonies					
	RSC1	**RSC2**	**RSC3**	**RSC4**	**RSC5**	**RSC6**
Dilution	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}
No. of colonies	42.60 ± 1.89	7.00 ± 0.71	6.40 ± 0.51	5.20 ± 0.37	5.00 ± 0.45	3.00 ± 0.45
Total colonies	68.10 ± 6.65					

Note: All values are the mean and standard error of five replicates

Table 3. Colony counts (CFU g^{-1}) of bacterial isolates from *E. coracana* biomass vermicompost

	Ragi vermicompost colonies						
	RVC1	**RVC2**	**RVC3**	**RVC4**	**RVC5**	**RVC6**	**RVC7**
Dilution	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}
No. of colonies	3020 ± 333.77	418 ± 47.58	46.20 ± 6.53	37.20 ± 3.81	40.80 ± 6.22	43.60 ± 3.08	24.60 ± 3.28
Total colonies	3600 ± 236.14						

Note: All values are the mean and standard error of five replicates

Table 4. Biochemical tests of bacterial isolates from red soil

	Red soil colonies					
Tests	**RSC1**	**RSC2**	**RSC3**	**RSC4**	**RSC5**	**RSC6**
Gram staining	-	+	+	+	+	+
Catalase test	+	+	-	-	+	+
Oxidase test	+	+	+	-	+	-
Voges-Proskauer test	-	+	-	-	+	+
Methyl red test	-	-	+	+	+	+
Indole test	-	-	-	-	-	-
Starch hydrolysis test	-	+	+	+	+	+
Citrate utilization test	+	+	+	+	+	+
Glucose utilization test	+	+	+	+	+	+
Lactose utilization test	+	+	+	+	+	+

Note: All values are the mean and standard error of five replicates

Table 5. Biochemical tests of bacterial isolates from *E. coracana* biomass vermicompost

	Ragi vermicompost colonies						
Tests	**RVC1**	**RVC2**	**RVC3**	**RVC4**	**RVC5**	**RVC6**	**RVC7**
Gram staining	+	+	-	-	+	+	-
Catalase test	+	+	-	+	-	+	-
Oxidase test	-	+	+	-	+	-	+
Voges-Proskauer test	+	+	+	-	-	+	+
Methyl red test	+	-	+	+	+	+	+
Indole test	-	-	+	-	+	+	+
Starch hydrolysis test	+	+	+	+	+	+	+
Citrate utilization test	+	+	+	+	+	+	+
Glucose utilization test	+	+	+	+	+	+	+
Lactose utilization test	+	+	+	+	+	+	+

Note: All values are the mean and standard error of five replicates.

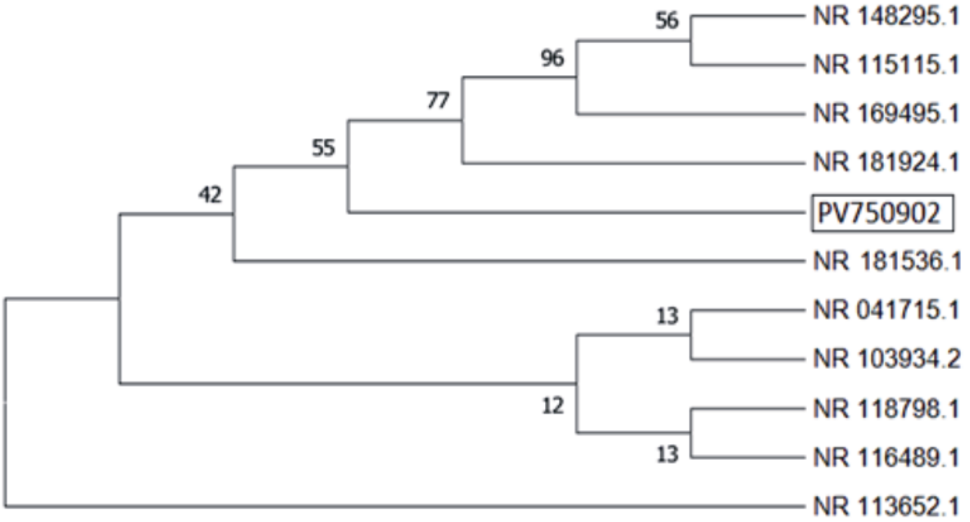

Figure 1. Molecular phylogenetic analysis of the query sequence (PV750902) of red soil

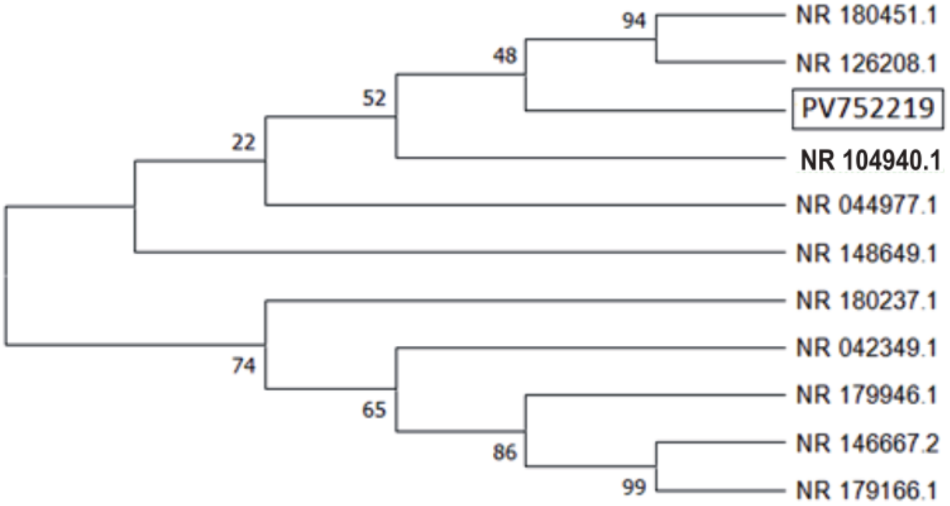

Figure 2. Molecular phylogenetic analysis of the query sequence (PV752219) of *E. coracana* vermicompost

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