DOI: 10.5846/stxb201606211204

Authors: Xianghua Yuan, Zhou Yanling, Song Qingzi, Tuo Hongmei, Ma Qinqin, Wang Yiding

Date: 2017-03-08T00:00:00+00:00

Abstract

To investigate the variation characteristics of soil physicochemical properties and actinomycete diversity during the earthworm ingestion process, earthworm habitat soil, gut contents, and earthworm casts were regarded as three special habitat soils representing the pre-, mid-, and post-ingestion stages. Actinomycetes from these three stages were isolated and purified using the pure culture method; actinomycete diversity was analyzed using the clone library method; basic physicochemical properties were determined using national standard methods; and correlations between soil physicochemical properties and actinomycete diversity were analyzed using principal component analysis and correlation analysis. The results showed that 27, 15, and 17 actinomycete strains were obtained from habitat soil, gut contents, and earthworm casts, respectively. Based on morphological and cultural characteristics and 16S rDNA sequence identification, actinomycetes from habitat soil were classified into the genera Streptomyces, Nocardiopsis, and Actinosynnema, while those from gut contents and earthworm casts all belonged to the genus Streptomyces. Actinomycete diversity decreased sequentially from habitat soil to earthworm casts to gut contents. The habitat soil library contained 40 OTUs, divided into 11 families, with unknown bacteria accounting for 24%, and Nocardioidaceae being the dominant flora; the gut content library contained 20 OTUs, divided into 6 families, with unknown bacteria accounting for 3.3%, and Microbacteriaceae being the dominant flora; and the earthworm cast library contained 30 OTUs, divided into 6 families, with unknown bacteria accounting for 11.7%, and Streptomycetaceae being the dominant flora. There was no significant difference in total phosphorus content among the three soils, while all other physicochemical parameters were lowest in habitat soil. Gut contents had the highest available nitrogen content, while earthworm casts had the highest contents of organic matter, total nitrogen, potassium, and available phosphorus and potassium. Principal component analysis and correlation analysis revealed that during the earthworm ingestion process, soil available phosphorus, total nitrogen, total potassium, available potassium, and organic matter contents exerted substantial effects on

actinomycete diversity, with total nitrogen and available phosphorus showing significant negative correlations with actinomycete diversity (correlation coefficients of -0.998 and -1, respectively), thereby providing a theoretical basis for clarifying the interrelationships among earthworms, soil, and actinomycetes.

Full Text

DOI: 10.5846/stxb201606211204

Yuan Xianghua, Zhou Yanling, Song Qingzi, Tuo Hongmei, Ma Qinqin, Wang Yiding. Changes in soil physicochemical properties and actinomycete diversity during earthworm ingestion. *Acta Ecologica Sinica*, 2017, 37(4): 1199-1210.
Yuan X H, Zhou Y L, Song Q Z, Tuo H M, Ma Q Q, Wang Y D. Variations of soil physical-chemical properties and the diversity of actinomycetes during the process of swallowing of earthworms. *Acta Ecologica Sinica*, 2017, 37(4): 1199-1210.

Changes in Soil Physicochemical Properties and Actinomycete Diversity During Earthworm Ingestion

Yuan Xianghua, Zhou Yanling, Song Qingzi, Tuo Hongmei, Ma Qinqin, Wang Yiding*

College of Life Sciences, Sichuan Normal University, Chengdu 610101

Abstract: To clarify the changes in soil physicochemical properties and actinomycete diversity during earthworm ingestion, earthworm living soil, intestinal contents, and feces were regarded as three special habitat soils representing the pre-, mid-, and post-ingestion stages of earthworms. Actinomycetes from the three stages were isolated and purified by the pure-culture method; actinomycete diversity in the three stages was analyzed by the clone-library method; the basic physicochemical properties of soils before, during, and after ingestion were determined using national standard methods; and the correlations between soil physicochemical properties and actinomycete diversity were analyzed using principal component analysis and correlation analysis. The results showed that 27, 15, and 17 strains of actinomycetes were obtained from living soil, intestinal contents, and feces, respectively. Based on morphology, culture characteristics, and 16S rDNA sequence identification, actinomycetes from living soil were classified as *Actinosynnema*, *Nocardiopsis*, and *Streptomyces*, whereas actinomycetes from both intestinal contents and feces belonged to *Streptomyces*. Actinomycete diversity decreased in the order living soil, feces, and intestinal contents. The living-soil library contained 40 OTUs, belonging to 11 families, with unknown bacteria accounting for 24%, and Nocardiaceae was the dominant bacterial group; the intestinal-content library contained 20 OTUs, belonging to 6 families, with unknown bacteria accounting for 3.3%, and Microbacteriaceae was

the dominant bacterial group; the feces library contained 30 OTUs, belonging to 6 families, with unknown bacteria accounting for 11.7%, and Streptomycetaceae was the dominant bacterial group. There was no significant difference in total phosphorus content among the three soils; living soil had the lowest contents of the other physicochemical indicators, intestinal contents had the highest content of available nitrogen, and feces had the highest contents of organic matter, total nitrogen, potassium, available phosphorus, and potassium. Principal component analysis and correlation analysis showed that, during earthworm ingestion, soil available phosphorus, total nitrogen, total potassium, available potassium, and organic matter contents had relatively strong effects on actinomycete diversity; among them, total nitrogen and available phosphorus were significantly negatively correlated with actinomycete diversity, with correlation coefficients of −0.998 and −1, respectively. These findings provide a theoretical basis for clarifying the interactions among earthworms, soil, and actinomycetes.

Keywords: actinomycetes; diversity; earthworms; soil physicochemical properties; pure culture; 16S rDNA clone library; principal component analysis; correlation

Variations of soil physical-chemical properties and the diversity of actinomycetes during the process of swallowing of earthworms

YUAN Xianghua, ZHOU Yanling, SONG Qingzi, TUO Hongmei, MA Qinqin, WANG Yiding*

College of Life Science, Sichuan Normal University, Chengdu 610101, China

Abstract: The aim of the present study is to explore the dynamics of the diversity of actinomycetes and physical and chemical properties of soil during the process of swallowing of earthworms. Surrounding living soil of earthworms, intestinal contents, and earthworm feces acted as the special habitat soils of prophase, metaphase, and anaphase during the process. The pure culture method was used to separate and purify actinomycetes from the three phases, and a clone library was constructed to analyze the diversity of actinomycetes. The soil physical-chemical properties in three phases were determined according to the national standard. The correlation of physical-chemical properties of soil and the diversity of actinomycete was analyzed by using the principal component analysis and the correlation analysis. Results showed that 27, 15, and 17 actinomycetes were separated from living soil, intestinal contents, and earthworm feces, respectively. Morphological analysis and 16S rDNA sequencing showed that actinomycetes from living soil belonged to *Actinosynnema*, *Streptomyces*, and *Nocardiopsis*, whereas those separated from the intestinal contents and feces belonged to *Streptomyces*. The diversity of actinomycetes declined in relation to living soil, feces, and intestinal contents. Clone libraries of living soil, intestinal

Funding: Sichuan Provincial Department of Education provincial-level college student innovation and entrepreneurship training program project (201510636068); Sichuan provincial university key laboratory open project (SCYZ201408)

Received: 2016-06-21; **Revised:** 2016-11-10

* Corresponding author. E-mail: lemonlyty@sohu.com

http://www.ecologica.cn

contents, and feces had 40, 20, and 30 operational taxonomic units (OTUs) divided into 11, 6, and 6 families; 24%, 3.3%, and 11.7% were unknown bacterium. The dominant actinomycetes were Nocardioidaceae, Microbacteriaceae, and Streptomycetaceae, respectively. There was no significant difference of total P content between the three phases. The physical and chemical properties of living soil were the lowest. The intestinal contents had the most contents of available N, whereas feces had the most contents of organic matter, total N and K, and available P. The principal component analysis and the correlation analysis showed that available P, total N, total P, available P, and organic matter content of the soil had a great influence on the diversity of actinomycetes. There was a negative relationship between the content of available P, total N, and actinomycete diversity in the process of swallowing of earthworms, whereas the coefficient was −0.998 and −1, respectively. The present study provided a theoretical basis for further research on the relationship among earthworms, soil, and actinomycetes.

Key Words: actinomycetes; diversity; earthworm; physical-chemical properties of soil; cultivation; library of 16S rDNA clone; principal components analysis; correlation

With the intensification of environmental degradation, people' s awareness of protecting the soil ecological environment has been increasing, and the study of interactions between soil and organisms has become especially important. Earthworms are a major animal group in soil. Through ingestion, they break up soil and use active enzymes in the intestinal tract to decompose soil organic matter, thereby increasing soil fertility[^1-^2]. As major producers of biologically active substances among microorganisms[^3], actinomycetes provide active substances for their hosts and are mainly responsible for the formation of humic acid during the process of earthworm ingestion of soil[^4], which is of great importance for improving soil fertility and quality.

Improvement of soil fertility by earthworms is accomplished mainly through ingestion. Soil containing large numbers of microorganisms enters the intestinal tract after being ingested by earthworms and is then excreted in the form of casts; thus, intestinal contents and casts may be regarded as soil under a special habitat[^5]. Soil physicochemical properties are important indicators

characterizing the quality of soil fertility[^6]. Reports have indicated that, during earthworm ingestion, both soil physicochemical properties and the number of soil actinomycetes change[^7-^9], but the characteristics of changes in actinomycete diversity and the correlation between the two have not been reported. Clarifying the characteristics of changes in soil actinomycete diversity and soil physicochemical properties during earthworm ingestion, as well as their correlations, can reveal the dynamic relationship between changes in actinomycete diversity and soil physicochemical properties during this process, providing a theoretical basis for biological restoration of soil. In this study, earthworm-inhabited soil, intestinal contents, and casts were regarded as the pre-, mid-, and post-ingestion stages of earthworm feeding, respectively. Actinomycetes in the three stages were isolated and purified, clone-library methods were used to analyze actinomycete diversity in the three stages, and the characteristics of changes in soil physicochemical properties and actinomycete diversity were investigated. The study provides a theoretical basis for elucidating the correlations among earthworms, soil physicochemical properties, and actinomycete diversity, and has important application value for environmental protection and maintenance of soil ecology.

1 Materials and Methods

1.1 Sample collection and treatment

The study site was located on a cultivated field in Datong Town, Qingbaijiang District, Chengdu City, Sichuan Province (30°41$ ‘33’ ‘$ N, 104°13$’ 37’’ $ E). The area has a subtropical monsoon humid climate. The soil type is paddy soil; the water–upland rotation system is adopted, and the crops planted include rice, rapeseed, *Brassica juncea*, etc.

Using the S-shaped sampling method[^{10}], plow-layer soil was collected at 0 −20 cm. This soil was regarded as earthworm-inhabited soil, and earthworms were also collected. After identification, the earthworm species was *Pheretima californica*. The soil was mixed thoroughly and divided into three portions, which were used, respectively, for determining soil physicochemical properties, extracting total soil DNA, and collecting fresh casts. Fresh casts were collected by the method of Haynes et al.[^{11}]. After all samples were collected, they were rapidly transported back to the laboratory for experiments.

After mud on the surface of the earthworm body was removed, the body surface was sterilized with 75% ethanol and rinsed three times with sterile water. The earthworms were dissected to obtain intestinal contents, which were then mixed thoroughly.

1.2 Determination of basic physicochemical properties

Impurities in the earthworm-inhabited soil, intestinal contents, and casts were removed. After natural air-drying, the samples were ground and passed through sieves with diameters of 2 mm and 0.15 mm. The basic physicochemical properties of the samples were determined according to national standard methods[^{10}]. Each index was measured in triplicate, and SPSS 18.0 was used to test the significance of differences in the basic physicochemical properties.

1.3 Isolation, identification, and phylogenetic analysis of culturable actinomycetes

1.3.1 Isolation and identification of culturable actinomycetes

Aqueous suspensions of the three types of samples were prepared and shaken at 40°C and 140 r/min for 30 min. Three parallel replicates were prepared for each sample. Actinomycetes were isolated and purified with reference to the optimized method of Shi Xuequn[^{12}], and identical strains were identified and merged according to the method of Ruan Jisheng[^{13}].

1.3.2 Determination of 16S rDNA sequences of culturable actinomycetes

Total bacterial DNA was extracted enzymatically. The universal bacterial 16S rRNA gene primers 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1540R

(5′-AAGGAGGTGATCCAACCGCA-3′) [14] for 16S rDNA PCR amplification. The amplification system was: 12.5 μL 2×PCR Mix; 1 μL of each primer; 0.5 μL DNA; 10 μL double-distilled water. The reaction program was: predenaturation at 94°C for 30 s; denaturation at 94°C for 10 s; annealing at 55°C for 1 min; extension at 72°C for 1 min; 30 cycles; final extension at 72°C for 10 min [15]. After electrophoretic detection, PCR products were sent to Shanghai Majorbio Bio-Pharm Technology Co., Ltd. for sequencing.

1.3.3 Phylogenetic analysis of culturable actinomycetes

The gene sequences of model strains similar to the obtained 16S rDNA sequences were selected using the BLAST program, and MEGA 5.0 was used to construct a phylogenetic tree by the N-J method, with a bootstrap value of 1000 [16]. *Acidimicrobium ferrooxidans* (U75647.1) was used as the outgroup.

1.4 Construction of an actinomycete 16S rDNA clone library

1.4.1 Amplification and purification of actinomycete 16S rDNA

Total DNA was extracted with a SoilGen DNA Kit and used as the template. Actinomycete 16S rRNA gene-specific primers F243 (5′-

GGATGAGCCCGCGGCCTA-3′) and R513 (5′-CGGCCGCGGCTGCTGGCACGTA-3′) [17] were used to amplify actinomycete 16S rDNA from the three samples. The PCR system and program followed the method of Han Jun [18], and the products were purified again after electrophoretic detection.

1.4.2 Construction of the actinomycete 16S rDNA clone library and screening of positive clones

16S rDNA was ligated into the pMD19-T vector (TaKaRa). The ligation system was: 1.0 μL pMD19-T Vector, 5.0 μL Solution I, and 4.0 μL Template. The ligation products were transformed into competent *E. coli* DH5α. After blue-white screening [19], positive clones were randomly selected and sent to Chengdu Qingke Zixi Biotechnology Co., Ltd. for sequencing.

1.5 Phylogenetic analysis of uncultured actinomycetes

Vector sequences in 16S rDNA were removed online (http://www.ncbi.nlm.nih.gov/tools/vecscreen/), chimeras (Chimeria) were removed (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl), sequences were aligned with Clustal-X (multiple alignment) [21], and the similarity of the alignment results was analyzed with BioEdit 7.0 [22]. Sequences with similarity $ $97.0% were assigned to the same operational taxonomic unit (Operational taxonomic unit, OTU) [23]. Non-actinomycete OTUs were removed, and one sequence from each OTU was selected to construct the phylogenetic tree according to the method described above.

1.6 Analysis of actinomycete diversity and of the correlation between diversity and physicochemical properties

SPADE (Species prediction and diversity estimation) (http://chao.stat.nthu.edu.tw/softwareCE.html.) was used to assess actinomycete diversity in the clone libraries by the Estimated sample coverage, Species richness, Shannon Index, and Simpson Index. Analytic Rarefaction version (http://strata.uga.edu/software/index.html) was used to plot rarefaction curves [24]. Office Excel 2003 was used for statistical analysis and data processing, and SPSS 18.0 was used for principal component analysis of physicochemical properties and for analysis of the correlation between physicochemical properties and actinomycete diversity.

2 Results and Analysis

2.1 Analysis of basic physicochemical properties

As shown in Table 1, the pH values of the three samples were all close to neutral, with the pH of intestinal contents being slightly lower. The contents of total nitrogen, potassium, and available potassium in intestinal contents and feces did not differ significantly, but all were significantly higher than those in the living soil. The available potassium contents in intestinal contents and feces

Fig. 1 The phylogenetic tree of culturable actinobacteria from living soil

Figure 1: Fig. 1 The phylogenetic tree of culturable actinobacteria from living soil

were particularly high, at 0.52 g/kg and 0.53 g/kg, respectively, 1.4 times that in the living soil. The contents of organic matter and available phosphorus increased successively in the living soil, intestinal contents, and feces; among them, the available phosphorus contents in intestinal contents and feces were 3.6 and 1.7 times that in the living soil, respectively. There was no significant difference in total phosphorus content among the three samples.

Table 1 Basic physical-chemical properties of 3 kinds of sample

Samples	pH (H_2O)	Total organic C (g/kg)	Total N (g/kg)	Available N (g/kg)	Total P (g/kg)	Available P (g/kg)	Total K (g/kg)	Available K (g/kg)
Living soil	6.84	50.6160$±2.4912^c	0.9890±0.0022^b	0.0652±0.0061^c	1.1800±0.0191^a	0.0270±0.0006^c	11.7850±0	

Data are mean ± standard deviation ($n = 3$). Different letters after data within the same column indicate significant differences ($P < 0.05$).

different; the available nitrogen content decreased successively in the gut contents, worm casts, and living soil.

2.2 Phylogenetic analysis of culturable actinomycete 16S rDNA

The phylogenetic tree of culturable actinomycete 16S rDNA showed that the 27 actinomycete strains in the earthworm living soil (Fig. 1) were distributed among three genera: *Streptomyces* (25 strains), *Nocardiopsis* (1 strain), and *Actinosynnema* (1 strain). From the gut contents

Fig. 1 The phylogenetic tree of culturable actinobacteria from living soil

and 15 and 17 actinomycete strains were obtained from soil and feces, respectively (Fig. 2, Fig. 3), all belonging to *Streptomyces*. Among them, T23, T26, and T28 were strains common to living soil and feces; S4, S5, and S10 were strains common to living soil and intestinal contents; and F5 and F25 were strains common to intestinal contents and feces. In summary, earthworm ingestion reduced the species and number of culturable actinomycetes; *Streptomyces* was the dominant genus in all three stages of earthworm-ingested soil. *Streptomyces* is the most widely distributed and most abundant actinomycete in soil[25].

Fig. 2 The phylogenetic tree of culturable actinobacteria from intestinal soil

Figure 2: Fig. 2 The phylogenetic tree of culturable actinobacteria from intestinal soil

2.3 Construction of the clone library

The fragment amplified with actinomycete 16S rRNA gene-specific primers was approximately 270 bp, consistent with the expected fragment size. After ligation, transformation, and verification, the clones were stored in plates.

Fig. 2 The phylogenetic tree of culturable actinobacteria from intestinal soil

2.4 Analysis of operational taxonomic units in the library

No chimeras (Chimeria) were detected among the 281 positive clone sequences after verification. The earthworm living soil, intestinal contents, and feces clone libraries contained 40, 20, and 30 OTUs, respectively (Table 2).

2.5 Diversity analysis of actinomycetes in the library

The results of actinomycete diversity analysis are shown in Table 2: the coverage (C) of the clone libraries from earthworm living soil, intestinal contents, and feces was 20.0%, 80.3%, and 66.7%, respectively; species richness was 256.0, 44.0, and 44.5, respectively; the Shannon index was 5.279, 2.658, and 3.789, respectively; and the Simpson index was 0.02815, 0.18785, and 0.05037, respectively.

Table 2 The number of 16S rDNA clones and diversity Index in the 3 samples

Sample	Total Clones	OTUs	Coverage/%	Species richness	Shannon index	Simpson index
Living soil	45	40	20	256	5.279	0.02815
Intestinal contents	61	20	80.3	44.0	2.658	0.18785
Feces	51	30	66.7	44.5	3.789	0.05037

The intestinal-content clone library had the highest coverage, encompassing 80.3% of the actinobacteria in the intestinal contents; the living-soil and earthworm-feces clone libraries contained 20.0% and 66.7%, respectively, of the actinobacteria in the corresponding environments. Species richness is an important indicator for evaluating the number of actinobacterial species in an

Fig. 3 The phylogenetic tree of culturable actinobacteria from earthworm feces

Figure 3: Fig. 3 The phylogenetic tree of culturable actinobacteria from earthworm feces

environment. Comparison of the richness indices showed that actinobacterial species were most numerous in the living soil, at nearly 5.8 times the number in the intestinal contents and feces. A larger Shannon index indicates higher actinobacterial diversity. The consistent results for species richness and the Shannon index together indicate that actinobacterial diversity was highest in the living soil, intermediate in the earthworm feces, and lowest in the intestinal contents. A larger Simpson index indicates higher evenness; thus, the evenness of actinobacteria in the intestinal contents was the highest, followed by that in the earthworm feces, and the evenness of actinobacteria in the living soil was the lowest.

Fig. 3 The phylogenetic tree of culturable actinobacteria from earthworm feces

The rarefaction curves show (Fig. 4) that the curve of the intestinal-content clone library had already become flat, the curve of the earthworm-feces clone library tended to become flat, whereas the curve of the living-soil clone library was almost linear. According to the theory of Kemp et al. [26], the intestinal-content clone library basically covered all actinobacterial species in the earthworm intestinal contents; the earthworm-feces clone library covered most actinobacterial species in the feces; but the living-soil clone library covered only a small number of actinobacterial species in the earthworm living soil. This result confirms that ingestion by earthworms reduced soil actinobacterial diversity: actinobacterial diversity decreased successively from living soil to feces to intestinal contents, and the living soil contained more abundant actinobacterial resources.

2.6 Phylogenetic analysis of uncultured actinobacteria based on 16S rDNA

The phylogenetic tree of uncultured actinobacteria showed that the earthworm living-soil clone library contained 40 OTUs (45 clones), of which 11 OTUs (11 clones) belonged to unknown actinobacteria; 1 OTU (1 clone) belonged to the family Acidimicrobiaceae in the order Acidimicrobiales; and 28 OTUs (33 clones) were distributed among 10 families of the order Actinomycetales (Fig. 5), namely Pseudonocardiaceae, Nocardioidaceae, Nocardiaceae, Microbacteriaceae, Actinomycetaceae, Micromonosporaceae, Mycobacteriaceae, Streptomycetaceae, Tsukamurellaceae, and Kineococcaceae. Nocardiaceae contained 7 clones.

(15.6%), and was the dominant actinomycete group in the living soil. Nocardioidaceae and Streptomycetaceae contained 6 (13%) and 5 clones (11%), respectively, and were groups of intermediate abundance; Microbacteriaceae, Geoder-

Fig. 4 Rarefaction curve of unculturable actinobacteria at 3 soil samples

Figure 4: Fig. 4 Rarefaction curve of unculturable actinobacteria at 3 soil samples

matophilaceae, and Kineosporiaceae each contained 1 clone (2%).

The intestinal-content clone library contained 20 OTUs (61 clones), among which 2 OTUs belonged to unknown actinomycetes (2 clones), and 18 OTUs (59 clones) were distributed among 6 families of Actinomycetales (Fig. 6), namely Nocardioidaceae, Streptomycetaceae, Nocardiaceae, Microbacteriaceae, Mycobacteriaceae, and Micromonosporaceae. Microbacteriaceae contained 48 clones (79%) and was the dominant actinomycete group in the intestinal contents. Nocardiaceae contained 4 clones (6.6%) and was a group of intermediate abundance; Mycobacteriaceae contained 1 clone (1.6%), and each of the remaining 3 families contained 2 clones (3.3%).

Fig. 4 Rarefaction curve of unculturable actinobacteria at 3 soil samples

The cast clone library contained 30 OTUs (51 clones), among which 5 OTUs belonged to unknown actinomycetes (6 clones), and 25 OTUs were distributed among 6 families of Actinomycetales (Fig. 7), namely Nocardioidaceae, Streptomycetaceae, Nocardiaceae, Microbacteriaceae, Mycobacteriaceae, and Glycomycetaceae. Streptomycetaceae contained 15 clones (29%) and was the dominant actinomycete group in casts; Microbacteriaceae contained 13 clones (25.5%) and was a group of intermediate abundance; Glycomycetaceae contained only 1 clone (2%).

In summary, in the earthworm living soil (before ingestion), actinomycete diversity was highest and the number of unknown actinomycetes was greatest. After the soil entered the earthworm intestine through ingestion (during ingestion), actinomycete diversity decreased to the lowest level and the number of unknown actinomycetes sharply declined. After the soil was excreted in the form of casts (after ingestion), actinomycete diversity rebounded and the number of unknown actinomycetes increased markedly. In addition, the dominant groups also fluctuated markedly across the three stages: Nocardioidaceae, Microbacteriaceae, and Streptomycetaceae were the dominant groups before, during, and after ingestion, respectively; moreover, Nocardioidaceae was widely distributed in all three stages.

2.7 Principal component analysis of basic soil physicochemical factors

During earthworm ingestion, the various soil physicochemical properties jointly affected actinomycete diversity. Therefore, principal component analysis was selected for multivariate statistical analysis, and the main influencing factors were identified using dimensionality-reduction thinking. According to the principle of

Fig. 5 The phylogenetic tree structured by 16S rDNA clone library from living soil

Figure 5: Fig. 5 The phylogenetic tree structured by 16S rDNA clone library from living soil

principal component analysis, when the cumulative variance contribution rate is greater than 85%, it can basically reflect the variation information of the system. Principal component analysis was performed on 8 basic soil physicochemical factors, and the results (Table 3) showed that the 8 basic soil physicochemical factors could be divided into 2 components.

Table 3 Factor loading, eigenvalue and cumulative percentage of principal component analysis

Factors	Component 1	Component 2	Factors	Component 1	Component 2
Total N	1.000	0.009	Available P	0.999	−0.049
Available K	0.997	−0.077	Total K	0.997	−0.080
Total organic C	0.932	−0.362	pH	−0.817	−0.577
Available N	0.709	0.705	Total P	−0.508	0.862
Eigenvalue	6.283	1.717	Percent/%	78.535	21.465
Cumulative/%	78.535	100.000			

The eigenvalues of both components were greater than 1. In principal component 1, the loadings of total N, available P, total K, available K, and total organic matter all exceeded 0.900, indicating that principal component 1 was a composite factor of total N, available P, K elements, and organic matter, among which the loadings of total N and available P were the largest in principal component 1. In principal component 2, total P, available N, and pH had relatively large loadings, among which the loading of total P was the largest. The contribution rate of principal component 1 was 78.835%, and that of principal component 2 was 21.465%. Principal component 1 had the largest contribution rate, indicating that total N, available P, total K, available K, and organic matter played a major role in changes in actinomycete diversity during earthworm ingestion.

Fig. 5 The phylogenetic tree structured by 16S rDNA clone library from living soil

effect.

Fig. 6. The phylogenetic tree structured by 16S rDNA clone library from intestinal soil

Figure 6: Fig. 6. The phylogenetic tree structured by 16S rDNA clone library from intestinal soil

2.8 Correlation analysis between soil physicochemical properties and actinomycete diversity

According to the results of principal component analysis, the correlations between physicochemical properties and diversity were analyzed. The results are shown in Table 4. It can be seen that the species richness of actinomycetes was correlated with soil

Fig. 6 The phylogenetic tree structured by 16S rDNA clone library from intestinal soil

Soil available phosphorus content showed a significant negative correlation, with a correlation coefficient of −0.998; it showed an extremely significant negative correlation with soil total nitrogen content, with a correlation coefficient of −1. This indicates that increases in soil available phosphorus and total nitrogen contents reduced the number of soil actinomycete species. In addition, pH, organic matter, available nitrogen, available potassium, and total potassium contents all had profound correlations with actinomycete diversity, indicating that during earthworm ingestion of soil, soil physicochemical properties and actinomycete diversity influenced one another.

3 Discussion

3.1 Changes in soil physicochemical properties

This study found that the contents of various physicochemical indicators in the earthworm-inhabited soil (before ingestion) were all relatively low; the available nitrogen content was highest in the intestinal contents (during ingestion); and the contents of organic matter, total nitrogen, total potassium, available phosphorus, and available potassium were all highest in the casts (after ingestion). This indicates that earthworm ingestion of soil markedly increased the effective components of the soil, could significantly enhance soil fertility, and suggests that earthworm casts can be used as a high-quality organic fertilizer for soil improvement, fertility cultivation, and crop yield enhancement[^{27}]. Principal component analysis of soil physicochemical properties and their correlations with actinomycete diversity showed that, during the process of earthworm ingestion of soil, soil total nitrogen and available phosphorus contents may be related to actinomycete diversity.

Fig. 7 The phylogenetic tree structured by 16S rDNA clone library from earthworm feces

The similarity was closely related. It can be seen that the increase in soil fertility,

Phylogenetic tree structured by 16S rDNA clone library from earthworm feces

Figure 7: Phylogenetic tree structured by 16S rDNA clone library from earthworm feces

in addition to being affected by the physical crushing in the earthworm stomach and by intestinal mineralization, is also influenced by the types of actinomycetes released from the intestine[^{28}-30].

Table 4 Correlation analysis between the diversity indexes and soil physical-chemical properties of 3 samples

Item	pH	Total organic C	Total N	Available N	Total P	Available K	Available P	Total K
Coverage	−0.925	0.828	0.977	0.849	−0.302	0.954	0.963	0.954
Species richness	0.823	−0.928	−1.000**	−0.717	0.498	−0.996	−0.998*	−0.996
Shannon index	0.987	−0.68	−0.903	−0.947	0.079	−0.862	−0.876	−0.861
Simpson index	−0.952	0.27	0.607	0.989	0.385	0.536	0.56	0.534

* Correlation is significant at the (P<0.05) level; ** correlation is extremely significant at the (P<0.01) level.

3.2 Changes in actinomycete diversity

Both the pure-culture method and the culture-independent method used in this study confirmed that actinomycete diversity decreased successively in living soil (before ingestion), casts (after ingestion), and gut contents (during ingestion). This may be because, after soil enters the anoxic environment, actinomycete diversity is reduced [31]; at the same time, earthworms depend on ingestion of soil particles rich in microorganisms to maintain survival [32], and cellulase, protease, and phosphatase in the gut during ingestion may reduce some sensitive microorganisms in the soil [33-34]. In addition, the clone library method showed that Microbacteriaceae, present in small amounts in living soil, were absolutely dominant actinomycetes in the gut contents, whereas Streptomycetaceae, present in small amounts in living soil and gut contents, became dominant actinomycetes in the feces. It is inferred that some actinomycetes in the soil proliferated substantially in the gut, leading to a decrease in actinomycete

diversity in the gut contents and casts. Actinomycete diversity in casts was slightly higher than that in gut contents, possibly because the aerobic environment in fresh casts allowed actinomycete diversity to recover. The trend in actinomycete diversity observed in this study is consistent with the results obtained by Furlong et al. [9] using the clone library method. The results obtained by Parthasarati et al. using a culture method are inconsistent with those of this study [4], possibly because of differences in earthworm species and culture substrates [35].

Principal component analysis showed that soil total N, available P, total K, available K, and organic matter had relatively large effects on changes in actinomycete diversity during earthworm ingestion. Correlation analysis between soil physicochemical properties and actinomycete diversity showed that increases in soil available P and total N contents led to a significant decrease in actinomycete diversity. Both analyses indicated that, during earthworm ingestion, actinomycete diversity was affected by soil physicochemical properties.

The clone library method showed that actinomycetes in earthworm living soil (before ingestion), gut contents (during ingestion), and casts (after ingestion) were mainly distributed in the order Actinomycetales, consistent with the findings of Knapp et al. [35]. In addition, the dominant bacterial groups at the three stages of earthworm ingestion fluctuated markedly. Nocardiaceae was the dominant bacterial group before ingestion; Microbacterium accounted for only 2% before ingestion, increased sharply to 79% in the middle stage, becoming the dominant group, and then decreased to 25.5% in the later stage. This is consistent with the report by Huang [36], suggesting that actinomycetes of the genus Microbacterium may contribute to decomposition of plant-derived food. Streptomycetaceae were rarely distributed in the middle stage of ingestion, but increased markedly in the later stage. The gut environment of the earthworm may be favorable for the reproduction of Microbacteriaceae actinomycetes and may have an obvious inhibitory effect on the survival and reproduction of Streptomycetaceae actinomycetes.

The pure-culture method is a necessary means of obtaining functional actinomycetes. In this study, a total of 59 actinomycete strains were obtained, of which Streptomyces accounted for 96.6%. The Streptomyces obtained from the two special habitats of the earthworm gut and casts may possess stronger activity and function. Studies have reported that *Streptomyces flavogriseus* in Fig. 2 can produce antimycins [37]; thus, its closely related strains S2, S4, and S6 are very likely to have the ability to produce antimycins, which requires further study.

In summary, using the culture method and clone library method, this study clarified the characteristics of changes in soil actinomycete diversity and soil physicochemical properties before, during, and after earthworm ingestion. Principal component analysis and correlation analysis showed that total N and available P contents significantly affected actinomycete diversity. These findings further improve understanding of the interactions among earthworms, soil, and actino-

mycetes, and provide a theoretical basis for integrated animal-microbial remediation of the environment and protection of the soil ecological environment.

References

[1] Shan J, Liu J, Wang Y F, Yan X Y, Guo H Y, Li X Z, Ji R. Digestion and residue stabilization of bacterial and fungal cells, protein, peptidoglycan, and chitin by the geophagous earthworm *Metaphire guillelmi*. Soil Biology and Biochemistry, 2013, 64: 9-17.

[2] Bernard L, Chapuis-Lardy L, Razafimbelo T, Razafindrakoto M, Pablo A L, Legname E, Poulain J, Brüls T, O' Donohue M, Brauman A, Chotte J L, Blanchart E. Endogeic earthworms shape bacterial functional communities and affect organic matter mineralization in a tropical soil. The ISME Journal, 2012, 6(1): 213-222.

[3] Jiang Yi, Tang Shukun, Zhang Yuqin, Jiang Kai, Xu Lihua. Bioactive substances produced by actinomycetes. *Microbiology China*, 2007, 34(1): 188-190.

[4] Parthasarathi K, Ranganathan L S, Anandi V, Zeyer J. Diversity of microflora in the gut and casts of tropical composting earthworms reared on different substrates. Journal of Environmental Biology, 2007, 28(1): 87-97.

[5] Egert M, Marhan S, Wagner B, Scheu S, Friedrich M W. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). FEMS Microbiology Ecology, 2004, 48(2): 187-197.

[6] Sparling G P. Ratio of microbial biomass carbon to soil organic matter carbon as a sensitive indicator of changes in soil organic matter. Australian Journal of Soil Research, 1992, 30(2): 195-207.

[7] Decaëns T, Mariani L, Lavelle P. Soil surface macrofaunal communities associated with earthworm casts in grasslands of the Eastern Plains of Colombia. Applied Soil Ecology, 1999, 13(1): 87-100.

[8] Zhang Lihong, Xu Guanghui. Study on the synergistic effect of microorganisms and earthworms on soil fertility. Acta Ecologica Sinica, 1990, 10(2): 116-120.

[9] Furlong M A, Singleton D R, Coleman D C, Whitman W B. Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*. Applied and Environmental Microbiology, 2002, 68(3): 1265-1279.

[10] Liu Guangsong. Soil Physical and Chemical Analysis and Profile Description. Beijing: China Standards Press, 1996.

[11] Haynes R J, Fraser P M. A comparison of aggregate stability and biological activity in earthworm casts and uningested soil as affected by amendment with wheat or lucerne straw. European Journal of Soil Science, 1998, 49(4): 629-636.

[12] Shi Xuexiang, Song Haichao, Liu Zhuang. Preliminary exploration of methods for isolating actinomycetes from soils contaminated by nuclear radiation in Hainan Province. Chinese Agricultural Science Bulletin, 2006, 22(10): 431-435.

[13] Ruan Jisheng. Rapid Identification and Systematic Classification of Actinomycetes. Beijing: Science Press, 2011.

[14] Jiang Guobiao, Ma Qinlin, Fang Zhixuan, Yong Bin, Liu Xinlin, Wang Yiding. Screening and identification of endogenous bacteria in a high-efficiency phosphorus-dissolving wheat strain. Journal of Sichuan Normal University: Natural Science Edition, 2012, 35(1): 122-126.

[15] Song Qingzi, Wang Yiding, Zhou Yanling, Ma Qinlin, Yuan Xianghua. Diversity of culturable actinomycetes in earthworm intestines in cultivated soil. Journal of Sichuan Normal University: Natural Science Edition, 2016, 39(2): 284-288.

[16] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 2011, 28(10): 2731-2739.

[17] Wu J T, Wang L, Ma F, Yang J X, Li S Y, Li Z. Effects of vegetative-periodic-induced rhizosphere variation on the uptake and translocation of metals in *Phragmites australis* (Cav.) Trin ex. Steudel growing in the Sun Island Wetland. Ecotoxicology, 2013, 22(4): 608-618.

[18] Han Jun, Liu Hui-Jun, Yuan Zhimin, Yao Jun. Analysis of genetic diversity of culturable actinomycetes in petroleum-contaminated soil. Environmental Science & Technology, 2014, 37(3): 32-36.

[19] Feng Ge, Xu Shanna, Wang Chao, Yan Lingen, Zhang Xuan, Li Jing. Diversity of uncultured actinomycetes in the Yellow River Delta coastal wetland. Microbiology China, 2013, 40(9): 1570-1579.

[20] Huber T, Faulkner G, Hugenholtz P. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics, 2004, 20(14): 2317-2319.

[21] Thompson J D, Gibson T J, Plewniak F, Jeanmougin F, Higgins D G. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research, 1997, 25(24): 4876-4882.

[22] Hall T A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 1999, 41: 95-98.

[23] Stach J E M, Maldonado L A, Masson D G, Ward A C, Goodfellow M, Bull A T. Statistical approaches for estimating actinobacterial diversity in marine sediments. Applied and Environmental Microbiology, 2003, 69(10): 6189-6200.

[24] Xie Qingyi, Qu Zhi, Ma Hai-Xia, Hong Kui. Diversity of actinomycetes in soils of mangrove forests from the Wenchang area. Chinese Journal of Tropical Crops, 2012, 32(8): 1466–1470.

[25] Xu Lihua, Yang Yirong, Jiang Chenglin. Study on the ecological distribution of actinomycetes in Yunnan soils. Acta Microbiologica Sinica, 1996, 36(3): 220–226.

[26] Kemp P F, Aller J Y. Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. FEMS Microbiology Ecology, 2004, 47(2): 161–177.

[27] Cui Yuzhen, Niu Mingfen. Effects of earthworms on soil composting and on yield and quality of *Herba Cistanches*. Chinese Journal of Soil Science, 1998, 29(4): 156–157.

[28] Elvira C, Sampedro L, Benítez E, Nogales R. Vermicomposting of sludges from paper mill and dairy industries with *Eisenia andrei*: a pilot-scale study. Bioresource Technology, 1998, 63(3): 205–211.

[29] Parthasarathi K, Ranganathan L S. Longevity of microbial and enzyme activity and their influence on NPK content in pressmud vermicasts. European Journal of Soil Biology, 1999, 35(3): 107–113.

[30] Carpenter D, Hodson M E, Eggleton P, Kirk C. Earthworm induced mineral weathering: preliminary results. European Journal of Soil Biology, 2007, 43(S1): S176–S183.

[31] Krištůfek V, Tajovský K, Pizl V. Ultrastructural analysis of the intestinal content of earthworm *Lumbricus rubellus* Hoffm. (Annelida, Lumbricidae). Acta Microbiologica et Immunologica Hungarica, 1994, 41(3): 283–290.

[32] Bohlen P J, Scheu S, Hale C M, McLean M A, Migge S, Groffman P M, Parkinson D. Non-native invasive earthworms as agents of change in northern temperate forests. Frontiers in Ecology and the Environment, 2004, 2(8): 427–435.

[33] Tiunov A V, Scheu S. Microfungal communities in soil, litter and casts of *Lumbricus terrestris* L. (Lumbricidae): a laboratory experiment. Applied Soil Ecology, 2000, 14(1): 17–26.

[34] Scheu S, Schlitt N, Tiunov A V, Newington J E, Jones H T. Effects of the presence and community composition of earthworms on microbial community functioning. Oecologia, 2002, 133(2): 254–260.

[35] Knapp B A, Podmirseg S M, Seeber J, Meyer E, Insam H. Diet-related composition of the gut microbiota of *Lumbricus rubellus* as revealed by a molecular fingerprinting technique and cloning. Soil Biology and Biochemistry, 2009, 41(11): 2299–2307.

[36] Huang K, Li F S, Wei Y F, Fu X Y, Chen X M. Effects of earthworms on physicochemical properties and microbial profiles during vermicomposting

of fresh fruit and vegetable wastes. Bioresource Technology, 2014, 170: 45-52.

[37] Carlson S, Tanouye U, Omarsdottir S, Murphy B T. Phylum-specific regulation of resistomycin production in a *Streptomyces* sp. via microbial coculture. Journal of Natural Products, 2015, 78(3): 381-387.

Note: Figure translations are in progress. See original paper for figures.