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A novel investigation of microbiome from vermicomposting liquid produced by Thai earthworm, *Perionyx* sp. 1

Kraisittipanit, R.[1,2], Tancho, A.[2,3], Aumtong, S.[3] and Charerntantanakul, W.[1*]

[1]Program of Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand; [2]Natural Farming Research and Development Center, Maejo University, Chiang Mai, Thailand; [3]Faculty of Agricultural Production, Maejo University, Thailand.

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Abstract The whole microbiota structure in vermicomposting liquid derived from Thai earthworm, *Perionyx* sp. 1 was estimated. It showed high richness microbial species and belongs to 127 species, separated in 3 fungal phyla (Ascomycota, Basidiomycota, Mucoromycota), 1 Actinomycetes and 16 bacterial phyla (Acidobacteria, Armatimonadetes, Bacteroidetes, Balneolaeota, Candidatus, Chloroflexi, Deinococcus, Fibrobacteres, Firmicutes, Gemmatimonadates, Ignavibacteriae, Nitrospirae, Planctomycetes, Proteobacteria, Tenericutes and Verrucomicrobia). The OTUs data analysis revealed the highest taxonomic abundant ratio in bacteria and fungi belong to Proteobacteria (70.20 %) and Ascomycota (5.96 %). The result confirmed that *Perionyx* sp. 1 VCL was high microbiota product which suitable for using for organic agriculture. Consequently, it is firstly reported in analysing the Thai-VCL microbiota by NGS technology. We successfully build up the microbial genetic database for Thai VCL for further application improvement.

Keywords: Earthworm, Metagenomic, NGS technology, *Perionyx* sp. 1, Vermicomposting liquid

Introduction

Perionyx sp. 1, is a Thai commercial terrestrial earthworm and identified by earthworm specialist under Natural Agriculture Research and Development Center. It has also been cultured and studied for a long time since Earthworm and Development Information Center (old name of our unit). It was to produce vermicompost and vermicomposting liquid. This strain has been widely used in Thailand and nearby countries. In our research, all earthworm has been collected from famers from dairy farms in Sankampeang district, Chiang Mai. Previously, Tancho (2013) found that this strain is highly efficient to convert

[*]**Corresponding Author:** Charerntantanakul, W.; **Email:** wasin@mju.ac.th

the organic wastes from residuals (markets) to compost (Vermicompost) via the gut associated processing (GAP) by microorganisms. Interestingly, this earthworm is suitable for culture in Thailand more than other species. Due to the fact that, it can adjust to tropical climate change as well as it has the eating rate from animal waste, organic vegetables. All of which can efficiently produce organic fertilizer while providing more minerals, vitamins and plant hormones. Besides, it can also produce the brown liquid by microbial degradation activities through the alimentary tract processing called that vermicomposting liquid and it consists of many substances i.e., plant hormones, PGPB etc. Hence, it is a premium earthworm liquid production for plant growth and soil improvement.

The vermicomposting liquid (VCL) is the commercial liquid bio-fertilizer produced by *Perionyx* sp. 1. It has variety special properties, for instance, it is the rich in minerals and plant growth promoting hormones (Tancho, 2013). Interestingly, it is odourless and environment friendly to use in organic farming in Thailand and another country. Previously, this product was used for testing in many crops for a long time, and it was confirmed that it can stimulate plant growth and inhibit some of pathogenesis in plants via stimulating plant immunization in addition to resisting plant disease i.e., *Phyhium*, *Rhizoctonia*, *Verticillium*, *Phomposis* (Edwards and Arancon, 2004). Moreover, this brand has special complex bacteria microbiota, especially *Enterobacter hormaechei*, *Enterobacter cloacae*, *Aeromonas punctate*, *Aeromonas sanarellii*, *Aeromonas enteropelogenes*, *Aeromonas media* and *Bacillus aryabhatta.* All of which can produce high quantity concentration indole-3-acetic acid as 131.39 µg/ml, 119.83 µg/ml, 80.44 µg/ml, 72.80 µg/ml, 65.28 µg/ml, 51.74 µg/ml and 32.45 µg/ml, respectively (Arraktham *et al*., 2016). However, it lacks of the data from another microbe colonized in *Perionyx* sp. 1 and its product.

Hence, the high-throughput sequencing technology or next generation sequencing (NGS) (Behjati and Tarpey, 2013) was used as the main tool for this study. For the reason that, this technique is used to sequent all metagenomics in a short time and wildly used with many research fields, especially the study of the whole genetic structure of individual organism, or metagenome level, or environmental contamination. According to a study, Wang *et al.* (2019) found that Actinobacteria, Firmicuts and Proteobacteria, these are the three dominant groups of bacteria in arsenic contaminated soil, which is absorbed throughout the gut of *Metaphire sieboldi*. In another case, this technology was used to examine the antibiotic resistant gene expression which transmits in the gut of *Metaphire guillelmi* earthworm (Choa *et al.*, 2019). Moreover, it is studied all taxonomic abundant microorganisms in

central gulf of Thailand passed metagenomics analysis (Sripan, 2016). Furthermore, Koo *et al*. (2018) demonstrated that metagenomics study and gene expression could apply for studying gene expression level from microbiome in different cold ecosystem. From the above mentioned, it can take into account that the whole microbial structure from *Perionyx* sp. 1 which is Thai commercial earthworm under the Natural Agriculture Research and Development Center. Consequently, the main objective of this study was to apply the NGS technology for estimating total microorganisms within microbiome in *Perionyx* sp. 1 via vermicomposting liquid (VCL) product.

Materials and methods

Collection of VCL and metagenomic extraction

One hundred ml of vermicomposting liquid (VCL) was collected from the Natural Farming Research and Development Center, Maejo University, Chiang Mai, and 600 µl of which was extracted for metagenome DNA using Stool Microbiome DNA Kit (Invitrogen). Quality and quantity of isolated DNA were determined by Nanodrop (Allsheng) and 1% agarose gel electrophoresis. DNA was stored at -20^{o}C.

Library preparation

The metagenomics samples were digested by transposase enzyme (Macrogen) into reads (DNA fragments), and were duplicated using different adapters. For prokaryote, its function is to build amplicon. All amplicon would be joined with P5 adapter (5’ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3’) and P7 adapter (5’ GTCTCGTGGGCTCGGAGATGGATATAAGAGACAG 3’). For fungal species, P5 adapter (5’ TTGGTCATTTAGAGGAAGTAA 3’) and P7 adapter (5’ CGTTCTTCATCGATGC 3’) were applied to fungal metagenome adapters before amplifying targeted DNA segments by PCR to the sequence reads. (Table 1)

Table 1. Amplicon primer (forward and reverse) sequence of 16s rRNA and ITS1

	Amplicon primer sequence for 16s rRNA	**Amplicon primer sequence for ITS1**
F	5’ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3’	5’ TTGGTCATTTAGAGGAAGTAA 3’
R	5’ GTCTCGTGGGCTCGGAGATGGATATAAGAGACAG 3’	5’ CGTTCTTCATCGATGC 3’

* F = forward primer, R = reverse primer

Operational taxonomic unit (OTU) analysis and statistical taxonomic analysis

The DNA fragments were completed to contig and analysed for the nucleotide sequence using illuminar Miseq (Macrogen) by quality control at Q30 (99.9 %). The read alignment was run by Fast Length Adjustment of short reads (FLASH). Raw data were used to analyse the taxonomic clustering by Pyton program before running the OTUs output data by rDnaTool (Pyton) program. Raw data and nucleotide sequences were blasted for the taxonomic diversity and species richness using Greengene data base.

Results

The metagenomics from VCL (*Perionyx* sp.1) was amplified using V3-V4 region primer to build amplicon. 118,070 reads count and 90,564 reads count were created and managed by FLASH program to contig. All contiqs were estimated and passed at Q30 as 81.95% and 98.35%. For prokaryote and fungal metagenomics, respectively. The total bases for this study showed 35,539,070 for prokaryote and 27,207,145 for fungal metagenomics (Table 2).

Table 2. ***F***ast ***L***ength ***A***djustment of ***SH***ort reads (FLASH) program analysis showed the pared score (Q20, Q30) and read count in vermicoposting liquid metagenomics based on 16s/ITS1 region gene for prokaryote and fungi, respectively

Sample name	Total Bases	Read Count	Q20 (%)	Q30 (%)
VCL for 16s rRNA	35,539,070	118,070	91.67	81.95
VCL for ITS1	27,207,145	90,564	99.61	98.35

However, the diversity identification was performed through illuminar Miseq and the statistic diversity by alpha_diversity.py program. It estimated to be five statistical parameters (Table 3).

Table 3. The statistics analysis using alpha_diversity.py program for diversity richness and alpha diversity of 16s rRNA (bacteria and actinomycetes) and ITS1 (fungi)

Sample name	OTUs	Choa1	Shannon	Inverse Simpson	Good's Coverage
VCL for bacteria and actinomycetes	174	174	4.9694022	0.915572934	1
VCL for fungi	86	86	2.3183891	0.533239795	1

The Prokaryote taxonomic richness were identified as 17 Phyla by 16s rRNA as Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Balneolaeota, Candidatus, Chloroflexi, Deinococcus, Fibrobacteres, Firmicutes, Gemmatimonadates, Ignavibacteriae, Nitrospirae, Planctomycetes, Proteobacteria, Tenericutes and Verrucomicrobia, all of phyla separated in 34 classes 97 genera and 104 species were blasted (Table 4). Nevertheless, the *Zobellella taiwanensis*, belongs to proteobacteria, is the highest species abundant as 26.37%.

Table 4. Metagenomics analysis profiling of Prokaryote from Vermicomposting liquid which analysed by 16s rRNA

classes	genera	species	Species abundant
Acidobacteriia	*Paludibaculum*	*Paludibaculum fermentans*	0.59%
Vicinamibacteria	*Luteitalea*	*Luteitalea pratensis*	0.10%
Acidimicrobiia	*Aciditerrimonas*	*Aciditerrimonas ferrireducens*	0.05%
	Blastococcus	*Blastococcus saxobsidens*	0.12%
	Angustibacter	*Angustibacter aerolatus*	0.05%
Actinobacteria	*Micromonospora*	*Micromonospora polyrhachis*	0.23%
	Egicoccus	*Egicoccus halophilus*	0.10%
	Euzebya	*Euzebya tangerina*	0.35%
Nitriliruptoria	*Nitriliruptor*	*Nitriliruptor alkaliphilus*	2.96%
	Fimbriimonas	*Fimbriimonas ginsengisoli*	0.15%
Fimbriimonadia	*Rhodothermus*	*Rhodothermus marinus*	0.10%
Bacteroidia	*Lentimicrobium*	*Lentimicrobium saccharophilum*	0.05%
	Sediminibacterium	*Sediminibacterium roseum*	0.05%
	Taibaiella	*Taibaiella koreensis*	0.05%
Chitinophagia	*Terrimonas*	*Terrimonas lutea*	0.18%
Cytophagia	*Ohtaekwangia*	*Ohtaekwangia koreensis*	0.33%
	Wandonia	*Wandonia haliotis*	0.20%
Flavobacteriia	*Planktosalinus*	*Planktosalinus lacus*	0.38%
Saprospiria	*Lewinella*	*Lewinella nigricans*	0.07%
Sphingobacteriia	*Solitalea*	*Solitalea koreensis*	0.20%
		Balneola alkaliphila	0.28%
	Balneola	*Balneola vulgaris*	0.10%
	Gracilimonas	*Gracilimonas mengyeensis*	0.12%
	Rhodohalobacter	*Rhodohalobacter halophilus*	0.67%
Balneolia	*Vampirovibrio*	*Vampirovibrio chlorellavorus*	0.05%
Anaerolineae	*Thermomarinilinea*	*Thermomarinilinea lacunifontana*	3.80%
	Caldilinea	*Caldilinea aerophila*	0.12%
	Caldilinea	*Caldilinea tarbellica*	0.05%
Caldilineae	*Litorilinea*	*Litorilinea aerophila*	0.62%
Dehalococcoidia	*Dehalogenimonas*	*Dehalogenimonas lykanthroporepellens*	0.28%
Thermoflexia	*Thermoflexus*	*Thermoflexus hugenholtzii*	0.35%
	Truepera	*Truepera radiovictrix*	0.07%
Deinococci	*Meiothermus*	*Meiothermus luteus*	0.07%
Fibrobacteria	*Fibrobacter*	*Fibrobacter succinogenes*	0.12%
	Gracilibacter	*Gracilibacter thermotolerans*	0.05%
	Symbiobacterium	*Symbiobacterium terraclitae*	0.05%
	Natranaerobaculum	*Natranaerobaculum magadiense*	0.10%
Clostridia	*Thermodesulfobium*	*Thermodesulfobium acidiphilum*	0.20%
Limnochordia	*Limnochorda*	*Limnochorda pilosa*	0.52%
Gemmatimonadetes	*Roseisolibacter*	*Roseisolibacter agri*	0.07%
Longimicrobia	*Longimicrobium*	*Longimicrobium terrae*	0.37%
Ignavibacteria	*Ignavibacterium*	*Ignavibacterium album*	0.37%

Table 4 (Con.)

classes	genera	species	species abundant
Nitrospira	*Nitrospira*	*Nitrospira japonica*	0.35%
	Algisphaera	*Algisphaera agarilytica*	1.19%
	Phycisphaera	*Phycisphaera mikurensis*	0.10%
Phycisphaerae	*Tepidisphaera*	*Tepidisphaera mucosa*	0.05%
	Gimesia	*Gimesia maris*	0.05%
Planctomycetia	*Pirellula*	*Pirellula staleyi*	0.05%
	Bauldia	*Bauldia consociata*	0.05%
	Methyloceanibacter	*Methyloceanibacter caenitepidi*	2.54%
	Methyloligella	*Methyloligella solikamskensis*	2.31%
	Mongoliimonas	*Mongoliimonas terrestris*	0.12%
	Blastochloris	*Blastochloris sulfoviridis*	0.05%
	Hyphomicrobium	*Hyphomicrobium aestuarii*	0.47%
	Rhodoplanes	*Rhodoplanes pokkaliisoli*	0.05%
	Rhodoplanes	*Rhodoplanes roseus*	0.05%
	Rhodoplanes	*Rhodoplanes tepidicaeni*	0.20%
	Methylopila	*Methylopila capsulata*	0.05%
	Methylosinus	*Methylosinus trichosporium*	1.05%
	Chelativorans	*Chelativorans composti*	0.05%
	Ensifer	*Ensifer shofinae*	0.23%
	Liberibacter	*Liberibacter crescens*	0.05%
	Sinorhizobium	*Sinorhizobium saheli*	0.05%
	Parvibaculum	*Parvibaculum hydrocarboniclasticum*	0.05%
	Pyruvatibacter	*Pyruvatibacter mobilis*	0.20%
	Rhodoligotrophos	*Rhodoligotrophos jinshengii*	6.09%
	Pseudolabrys	*Pseudolabrys taiwanensis*	0.10%
	Hyphobacterium	*Hyphobacterium vulgare*	0.12%
	Defluviimonas	*Defluviimonas nitratireducens*	0.05%
	Paracoccus	*Paracoccus pantotrophus*	0.10%
	Aliidongia	*Aliidongia dinghuensis*	0.05%
	Azospirillum	*Azospirillum brasilense*	3.25%
	Haematospirillum	*Haematospirillum jordaniae*	0.44%
	Inquilinus	*Inquilinus ginsengisoli*	5.40%
	Lacibacterium	*Lacibacterium aquatile*	0.05%
	Marinibaculum	*Marinibaculum pumilum*	0.20%
	Nitrospirillum	*Nitrospirillum amazonense*	0.20%
	Niveispirillum	*Niveispirillum irakense*	0.05%
	Lyticum	*Lyticum flagellatum*	0.05%
	Sandaracinobacter	*Sandaracinobacter sibiricus*	0.05%
Alphaproteobacteria	*Sphingomonas*	*Sphingomonas vulcanisoli*	0.15%
	Azohydromonas	*Azohydromonas riparia*	0.05%
	Nitrosomonas	*Nitrosomonas halophila*	0.62%
	Dechloromonas	*Dechloromonas agitata*	0.10%
Betaproteobacteria	*Azoarcus*	*Azoarcus olearius*	0.07%
Deltaproteobacteria	*Geobacter*	*Geobacter pickeringii*	0.05%
	Pseudohongiella	*Pseudohongiella spirulinae*	0.20%
	Oceanimonas	*Oceanimonas smirnovii*	0.05%
	Zobellella	*Zobellella denitrificans*	0.35%
	Zobellella	*Zobellella taiwanensis*	26.37%
	Mangrovitalea	*Mangrovitalea sediminis*	2.91%
	Haliea	*Haliea atlantica*	0.10%
	Thiohalobacter	*Thiohalobacter thiocyanaticus*	0.05%
	Thioalbus	*Thioalbus denitrificans*	8.77%
	Thioalkalivibrio	*Thioalkalivibrio sulfidiphilus*	0.10%
	Thioprofundum	*Thioprofundum hispidum*	4.84%
	Thioprofundum	*Thioprofundum lithotrophicum*	0.45%
	Kangiella	*Kangiella profundi*	0.07%
	Cysteiniphilum	*Cysteiniphilum litorale*	0.89%
Gammaproteobacteria	*Vibrio*	*Vibrio mediterranei*	0.22%

Table 4 (Con.)

classes	genera	species	species abundant
	Acholeplasma	*Acholeplasma parvum*	0.57%
Mollicutes	*Acholeplasma*	*Acholeplasma vituli*	0.05%
Opitutae	*Oleiharenicola*	*Oleiharenicola alkalitolerans*	0.05%
Verrucomicrobiae			
	Limisphaera	*imisphaera ngatamarikiensis*	0.10%

On the other hand, the fungi were found in three phyla (Ascomycota, Basdiomycota and Mucoromycota) and *Nigrospora oryzae* was the dominant species belongs to Ascomycota, *Malassezia restricta* is the dominant species of Basidiomycota and only one species, *Lichtheimia corymbifera*, was the dominant species found in Mucoromycota phylum. The detail information is shown in Table 5, 6 and 7.

Table 5. Metagenomics analysis profiling of Phylum Ascomycota from Vermicomposting liquid was analysed by *ITS1* gene region

classes	genera	species	species abundant
		Cladosporium tenuissimum	0.76%
Dothideomycetes	*Cladosporium*		
	Pseudocercospora	*Pseudocercospora* sp.	0.01%
	Stagonosporopsis	*Stagonosporopsis* sp.	0.20%
Eurotiomycetes	*Aspergillus*	*Aspergillus flavus*	0.04%
		Aspergillus penicillioides	0.10%
Saccharomycetes	*Candida*	*Candida tropicalis*	0.52%
	Debaryomyces	*Debaryomyces coudertii*	0.02%
	Clavispora	*Clavispora akabenensis*	0.01%
	Kazachstania	*Kazachstania humilis*	0.02%
Sordariomycetes	*Colletotrichum*	*Colletotrichum nymphaeae*	0.47%
	Fusarium	*Fusarium equiseti*	0.28%
	Nigrospora	*Nigrospora oryzae*	3.43%
	Eutypella	*Eutypella* sp.	0.08%

Table 6. Metagenomics analysis profiling of Phylum Basidiomycota from Vermicomposting liquid was analyzed by *ITS1* gene region

classes	genera	species	species abundant
Agaricomycetes	*Coprinopsis*	*Coprinopsis cinerea*	0.17%
	Auricularia	*Auricularia cornea*	0.02%
	Earliella	*Earliella scabros*	0.16%
		Sterigmatomyces halophilus	0.13%
	Sterigmatomyces		
Malasseziomycetes	*Malassezia*	*Malassezia globosa*	0.09%
		Malassezia restricta	0.25%
Tremellomycetes	*Tremelloles*	*Tremelloles* sp.	0.14%
	Hannaella	*Hannaella sinensis*	0.02%
		Hannaella sp.	0.02%

Table 7. Metagenomics analysis profiling of Phylum Mucoromycota from Vermicomposting liquid was analysed by *ITS1* gene region

class	genus	species	species abundant
Mucoromycetes	*Lichtheimia*	*Lichtheimia corymbifera*	0.01%

Discussion

In general, the taxonomic microbial abundance in different earthworm gut reared on various species and various feed or cultured site. This study was the first report that examine the microbial population in microbiome of the VCL which is produced by *Perionyx* sp. 1 earthworm. It belongs to the Megascolecidae family and could also be found in Thailand and several countries in Asia (Gate, 1939). Using a modern high throughput sequencing technology (Next Generation Sequencing), we found that it is a powerful tool for estimating the complete structure of the microbial population.

It is found that the variable region gene of V3-V4 is the highest bacterial classification region. This gene can amplify the DNA segments in metagenome from *Perionyx* sp. 1 VCL in 118,070 reads. Each of the reads (DNA fragments) can be tested against the pared score at Q30 (81.95 %). The result showed quality read passed 99.9 % at excellent accuracy. The FLASH program could also order all nucleotide and revealed the raw data through the test in 4 statistics (OTUs, Choa1, Shannon, Inverse Simpson). The OTUs result estimated the bacteria and actinomycete taxonomic abundance were high, 17 prokaryotic phyla were found (Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Balneolaeota, Candidatus, Chloroflexi, Deinococcus, Fibrobacteres, Firmicutes, Gemmatimonadates, Ignavibacteriae, Nitrospirae, Planctomycetes, Proteobacteria, Tenericutes and Verrucomicrobia) and 34 classes, 97 genera and 104 species were classified.

In the taxonomic abundance analysis, the alpha diversity expressed that the phylum Proteobacteria had the highest relative abundance ratio in this study which determined at 70.2%. The data from VLC metagenomic level confirmed the data report of Arraktham *et al*. (2016) that it is estimated that as Proteobacteria is the most dominant bacterial group. Furthermore, this phylum has been found in general agricultural soil and important to bio-fertilizer according to Koo *et al*. (2018) who supported our result that type of earthworm eat in various organic matters from agricultural soil and vegetables from markets, all of which are important food sources. This phylum was found in many classes, for instance, Alpha proteobacteria, Beta proteobacteria, Delta proteobacteria and Gamma proteobacteria. In addition, *Zobellella* sp. which is belongs to Gamma proteobacteria, with the highest frequency at 26.37%.

Moreover, some studies indicated that Proteobacteria and Firmicutes were the two phyla and found in toxic soil as also reported by Wang *et al*. (2019).

This research is expected to be an important source supporting our study when look at the early fermentation process at the low nutrient phase, but these bacteria can also be good for colonizing and converting little nutrients to high amounts of minerals. Hence, this product that is supposed to be full of soil bacterial community that necessary for plant nutrients.

Actinomycetes were determined and found on three species such as *Blastococcus saxobsidens***,** *Angustibacter aerolatus* and *Micromonospora polyrhachis*. However, the use of 16s rRNA gene can amplify and confirm the actinomycete amplicon DNA from metagenome. Therefore, the research study is firstly reported for the diversity richness and relative taxonomic abundance of prokaryote from commercial VCL genomic analysis in Thailand. The result gave the data same with Koskey *et al.* (2020) which reported that the use of 16s rRNA gene could also categorize the bacteria OTUs as 159.

Moreover, fungal community was detected by NGS technology as it can also be identified in three majoring phyla i.e., Ascomycota, Basidiomycota and Mucoromycota from 90,564 reads by Q30 at 98.35% in addition to the OTUs which showed that Ascomycota is the most relative abundance in VCL. However, ITS1 spacer region gene is a region of general fungal gene and sometimes can amplify other eukaryotic microorganisms. For instance, an arthropod in this report was screened in specimen at 0.1 % which be hard to protect them from some insect DNA contamination such as house fly (order Diptera). However, it was successfully done in reaching the goal by using NGS technology for Mycobiome screening fungi community from VCL. Therefore, it is the first study showing the genetic database of fungi from *Perionyx* sp. 1 VCL.

However, most of the bacteria, actinomycetes and fungi in VCL were not growing in cultural media (PDA, NA or SDA). It is inconvenient to study them from cultural conventional methods. Consequently, this study can confirm that NGS is the clearest technology to detect the microbiome in VCL. It is definitely useful for DNA data base.

Hence, we summarized a total of microbial in microbiome from VCL was produced by Thai *Perionyx* sp. 1. The collected data are very important for genetic data base to guarantee in Thai VCL band produced by Natural Farming Research and Development Center, Maejo University.

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