Genome announcement of Steinernema khuongi and its associated symbiont from Florida

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Abstract

Citrus root weevil (Diaprepes abbreviatus) causes significant yield loss in citrus, especially in Florida. A promising source of control for this pest is biological control agents, namely, native entomopathogenic nematodes (EPNs) within the genus Steinernema. Two species of endemic EPN in Florida are S. diaparepesis, abundant within the central ridge, and S. khuongi, dominating the flatwood regions of the state. These citrus-growing regions differ significantly in their soil habitats, which impacts the potential success of biological control measures. Although the genome sequence of S. diaparepesis is currently available, the genome sequence of S. khuongi and identity of the symbiotic bacteria is still unknown. Understanding the genomic differences between these two nematodes and their favored habitats can inform successful biological control practices. Here, MiSeq libraries were used to simultaneously sequence and assemble the draft genome of S. khuongi and its associated symbionts. The final draft genome for S. khuongi has 8,794 contigs with a total length of ~82 Mb, a largest contig of 428,226 bp, and N50 of 46 kb; its BUSCO scores indicate that it is > 86% complete. An associated bacterial genome was assembled with a total length of ~3.5 Mb, a largest contig at 116,532 bp, and N50 of 17,487 bp. The bacterial genome encoded 3,721 genes, similar to other Xenorhabdus genomes. Comparative genomics identified the symbiotic bacteria of S. khuongi as Xenorhabdus poinarii. These new draft genomes of a host and symbiont can be used as a valuable tool for comparative genomics with other EPNs and their symbionts to understand host range and habitat suitability.

Keywords: genome; nematode; endosymbiont; entomopathogenic

Introduction

Entomopathogenic nematodes (EPNs) are a group of nematodes that are cosmopolitan in distribution and cause disease in insects. Commercially used as biological control agents for soil insects in the field and the greenhouse systems (Georgis et al. 2006; Denno et al. 2008, Lacey and Georgis 2012, Nicolopoulos-Stamati et al. 2016), these nematodes are broadly classified into two families, Steinemernatidae and Heterorhabditidae (Kaya and Gaugler 1993). Each family is symbiotically associated with a specific genus of entomopathogenic bacteria Xenorhabdus and Photorhabdus, for Steinemernatidae and Heterorhabditidae, respectively (Adams et al. 2006). This close and specific association between EPN and bacteria form an insecticidal complex that is effective against a wide range of insect hosts (Stock and Blair 2008).

Citrus is one of the major crops of Florida, totaling over 56% of the total citrus produced in the United States (Cowell et al. 2018). Among different pests of citrus, the citrus root weevil, Diaprepes abbreviatus (Linnaeus) is one of the most damaging pests (Nigg et al. 2001; Campos-Herrera et al. 2019). This insect is polyphagous and can damage other crops and ornamental plants. It was first detected in Florida during the mid-1960s (Beavers and Selhime 1975) and is spreading to other states including Southern Texas and Southern California (Lapointe 2008). In Florida, over 100,000 acres of citrus are infested causing 72 million dollars in losses (Stuart et al. 2008). Citrus root weevil can result in the rapid decline of citrus trees and overwhelm the entire groove within a short time. The severity of the infection and increasing infestation of this pest has prompted the use of diverse management strategies including chemical and biological control. Within known biological control strategies, EPNs are one of the most well-studied and widely used for citrus root weevil in Florida (Campos-Herrera et al. 2013; Duncan et al. 2013). Different species of commercially available nematodes within the genus Steinernema and Heterorhabditis have been tested in laboratory and field settings (McCoy et al. 2000; Shapiro and McCoy 2000; Duncan et al. 2013). Originally, Steinernema carpocapsae and Heterorhabditis bacteriophora were used but then replaced by S. riobrave and H. indica due to a shown greater efficacy against D. abbreviatus, based on insects mortality (Stuart et al. 2008). However, field efficacy of nonnative EPN to control the weevil was variable and some applications failed to control insect populations. Major factors responsible for the variability of the applied treatments are a regional variation of soil habitat (Duncan et al. 2001; McCoy et al. 2002) and diverse community structures of native EPN within different ecological regions (Duncan et al. 2003, 2007). In Florida, citrus is grown in two distinct climates, flatwood and central ridge. The infection rates of native EPN to citrus root weevil larvae varied between these ecoregions with higher population...
diversity and lower insect damage among central ridges and vice versa among the flatwood region (Duncan et al. 2003; Futch et al. 2005). Within the different species of the native EPN, S. diaprepsi was isolated from the larvae of the citrus root weevil and was found to be highly effective in controlling the pest. However, the population of this nematode is more frequent within citrus orchards in the central ridge (Nguyen and Duncan 2002; El-Borai et al. 2007b; El-Borai et al. 2007a). In 2009 a novel population of a Steinernema species was recovered during the field study along the flatwood region of Florida (El-Borai et al. 2009). Initially, it was coined as “SxArc” as s member of S. glaseri. The occurrence of this species was frequent in the subsequent survey of nematodes (Nguyen and Buss 2011; Campos-Herrera et al. 2013; Campos-Herrera et al. 2016). Later it was identified as a new species S. khuongi based on morphological differences and DNA sequence analyses (Stock et al. 2019).

Despite the potential as a biological control agent, the lack of efficacy in the field has hindered the wider adaptation of EPN. Some effort has been made through artificial selection of desired traits such as increased tolerance to environmental conditions, desiccation, and ultraviolet light, as well as improved host-seeking ability, virulence, and resistance to nematicides. Most of these traits are unstable, have reduced overall fitness, or result in inbreeding depression resulting in poor efficacy (Burnell 2002; Glazer 2015; Lu et al. 2016). Native nematodes usually provide better insect control compared to commercially available nematodes as they are better adapted in the local environmental condition (Gaugler 1988; Gaugler et al. 1997; Duncan et al. 2003; Hiltpold 2015). Crucial traits determining the efficacy of EPN as a biological control agent are infectivity, persistence, and storage stability. Identification of new species, availability of genomic sequences, and comparative genomics can help to address which genetic features contribute to the virulence and adaptability of one species to certain environmental conditions. This study aimed to simultaneously sequence the nematode S. khuongi and its bacterial symbiont native to the Florida flatwood region. Our goal was to identify the bacterial associate and characterize different genomic features of both nematode and bacteria to aim in informing novel EPN biological control agent development.

Materials and methods
Nematode culture
EPN infective juveniles (IJs) of S. khuongi “webber” strain were originally isolated from Florida citrus groves, and then cultivated in Galleria mellonella (waxworm) larvae at Citrus Research and Education Center (CREC), Lake Alfred, FL. Some strains were received from CREC and reinoculated into the wax worm, after emerging IJs were collected using modified White traps (Kaya and Stock 1997) at the Department of Entomology and Nematology, Gainesville, FL. Nematodes were stored in a tissue culture flask for subsequent identification, genomic DNA extraction, and to maintain the culture (Stock and Goodrich-Blair 2012).

DNA isolation
Approximately 10,000 IJs were washed 10 times with a 0.8% NaCl solution. Surface sterilization of nematode was done by using 4 mM Hyamine 1622 solution (Sigma-Aldrich, USA) for 30 minutes and again washed with 0.8% NaCl for 3 times (Lu et al. 2017). The sterilized nematode were flash-frozen and thawed immediately twice for DNA extraction. High molecular weight genomic DNA was extracted using a phenol-chloroform method (Donn et al. 2008). The DNA pellet was further resuspended in 100 μl Tris-EDTA buffer. University of Florida’s campus-wide Interdisciplinary Center for Biotechnology Research (ICBR) NextGen DNA Sequencing Core Facility (Gainesville, FL) performed library preparation and sequencing using MiSeq Illumina sequencing platform with 2X300v3 format.

Genome assembly
A total of ~24 million 300-nt reads were generated from the sequencing. The sequence quality of the raw reads was analyzed using FastQC (Andrews 2010). Quality trimming, read filtering, and removing adapter contamination were performed using Trimmomatic/0.36 (Bolger et al. 2014). Clean reads were subjected to de novo assembly using the SPAdes/3.13.0 assembler (Bankevich et al. 2012) with Kmer size of 21, 33, 55, 77, 99, and 127. Assembly obtained from kmer 127 was used for downstream evaluation based on the best N50 value. The preliminary genome assembly of the nematode was likely contaminated with the symbiotic bacteria and other contaminants. To remove the possible contaminant from the assembly all contigs were queried against the NCBI nucleotide database using megablast (with E-value cutoff < 1e-05) and taxonomy was assigned to each contig (McGinnis and Madden 2004). Each contig was mapped and indexed to the raw read using Bowtie2 (Langmead and Salzberg 2012). Diagnostic visualization of each contig based on GC%, coverage, and taxonomic annotation was done using Blobtools v1.0 (Kumar and Blaxter 2011; Laetsch and Blaxter 2017). Based on the taxonomic annotation of each contig in Blobplot (Figure 1) each contigs sets belonging to nematode and proteobacteria were separated. After removing all the contaminants from the nematode set of contigs, the completeness of the genome was assessed using BUSCO V3.02 (Simão et al. 2015) and the nematode BUSCO profile (https://busco-archive.ezlab.org/v3/datasets/nematoda_odb9.tar.gz). Nematode BUSCO genes are expected to be the core genes and most probably present in every newly sequenced genome. A higher number of BUSCO genes in the assembly is indicative of completeness of the assembly. The quality of the assembly and the genome statistics were determined using Quast 2.3 (Gurevich et al. 2013).

Reassembly of bacteria, annotation, and comparative genomics
Each Steinernema species are symbiotically associated with only one specific species of the genus Xenorhabdus (Enterobacteriaceae, which belongs to the gamma subdivision of the Proteobacteria) (Stock and Blair 2008). To assemble the Xenorhabdus spp. of the S. khuongi First we retained all contigs classified as Proteobacteria in the preliminary nematode assembly. We then filtered all the contigs belonging to other proteobacterial species with a high level of identity. We extracted all the read mapped to the contigs to generate the Xenorhabdus-enriched raw sequence dataset. These putative Xenorhabdus raw reads were reassembled using Spades/3.13.0 assembler using optimized settings for bacterial genome assembly (Bankevich et al. 2012). The quality of the genome assembly was determined as described above. Annotation of the bacterial genome was done by using Pathosystems Resource Integration Center (PATRIC) RAST-enabled Genome Annotation Service (Brettin et al. 2015) and shown in Figure 2. Known homologs to antibiotic-resistance genes, drug targets, transporter, and virulence factor genes were identified using PATRIC by known homology of known sequences in the following databases: Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al. 2013), DrugBank 4.0 (Law et al. 2014), Therapeutic Target Database (TTD) (Zhu et al. 2010),
Transporter Classification Database (TCDB) (Saier et al. 2016), PATRIC_VF (Mao et al. 2015), Virulence Factor (VFDB) (Chen et al. 2016), and the National Database of Antibiotic-Resistant Organisms (NDARO). A k-mer-based AMR gene detection method was used to annotate antimicrobial resistance (AMR) genes, which utilizes PATRIC’s curated collection of representative AMR gene sequence variants (Wattam et al. 2017). To characterize the closest relative of the newly sequenced bacterial genome we generated the phylogenetic tree using a codon tree in PATRIC (Wattam et al. 2017). Genomes 28 previously sequenced Xenorhabdus species (Table 1) were used to generate the tree. The codon tree used cross-genus families (PGFams) as homology groups. 100 single-copy genes were selected from each genome. The alignment of each protein sequence was done using MUSCLE (Edgar 2004), and their corresponding nucleotide were aligned using the codonalign function of Biopython (Cock et al. 2009) within PATRIC (Davis et al. 2020). Concatenated trees of proteins and nucleotide were generated from 100 rounds of bootstrapping in RAxML analysis (Stamatakis et al. 2008; Stamatakis 2014). Klebsiella pneumoniae subsp. pneumoniae KPN119 was used as an outgroup. The trees were visualized using FigTree software version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and shown

**Figure 1** Blobplot (Taxon annotated GC-coverage scatter plot) of the contigs from the genome assembly. Each contig is plotted respective of their GC content and the depth of coverage. Each dot plot represents the contigs for the BLAST annotation with significant matches that are colored by putative taxon of origin. (A) Contigs from the preliminary assembly before removing the contaminants and the endosymbionts. (B) All contigs from the final assembly of the nematode draft genome. (C) All Putative proteobacterial contigs from preliminary assembly. (D) Contigs of the reassembled endosymbionts genome.
We assessed the synteny of newly assembled bacteria and X. poinarii str G6 using a web-based program called D-GENIES (Cabanettes and Klopp 2018), and using minimap2 for alignment, shown in Figure 4. To confirm the identity of the bacteria, the genome sequence was compared with other previously reported genomes from other Xenorhabdus spp. using Pyani version 0.2.7 (Pritchard et al. 2016). Pyani is a Python 3 module that can calculate Average Nucleotide Identity (ANI) using MUMmer and blast. Pyani was also used to calculate tetra-nucleotide frequencies (TETRA) correlation indexes. In this study, 26 complete genomes of different species/strains of Xenorhabdus and one species of Photorhabdus were analyzed (Figure 5).
Results

de novo assembly of nematode genome sequence

We sequenced the *S. khuongi* genome using the MiSeq Platform generating ~24 million raw reads, equivalent to 7 Gb (~70x). The genomic DNA extraction for sequencing was done from the nematode without removing endosymbionts (proteobacteria), so it is inevitable that the preliminary assembly is contaminated with endosymbionts and other contamination. The preliminary assembly consists of 65,846 contigs with total length of 151,364,689 bp. The megablast of the preliminary contigs with E-value cutoff < 1e–05 and visualization of contigs in Blobplot divided the contigs into 8 different taxon groups (Nematoda ~33%, Proteobacteria ~13%, No distinct hit on database ~22%, Chordata ~12%, Arthropoda ~10%, Bacteroidetes and Actinobacteria ~1%, and Other ~5%; Figure 1). All the contigs that were assigned for Proteobacteria, Bacteriodes, and Actinobacteria were removed from the preliminary nematode contigs. The final assembly of the nematode was obtained with mean contigs coverage of ~41x.

The final draft genome has 8,794 contigs with a total length of 81,789,282 bp (~82 Mb) with a largest contig of 428,226 bp. The average GC content of the genome is 47.88% with N50 of 46 kb. The BUSCO assessment of the draft genome against the Nematoda database indicated 86.6% (850) completeness. A total of 982 nematode genes were searched. Among those genes, 81.3% (798) are complete and single copy, 5.3% (52) complete and duplicated genes, 6.5% (64) fragmented genes, and 6.9% (68) missing genes. Comparisons of Steinnema genomes can be found in Table 2.

Reassembly of bacteria, annotation, and comparative genomics

Approximately 2.5 million quality trimmed raw read was mapped to putative proteobacterial contigs from the first preliminary assembly of the genome. Raw read mapped to proteobacterial contigs contribute approximately 10% of the initial filtered read sets. Proteobacterial contigs belonging to the genus *Xenorhabdus* were filtered and its corresponding raw reads used for optimized
bacterial genome reassembly resulted in 468 contigs with a total length of 3,484,021 bp (~3.5 Mb). The longest contig of the genome is 116,532 bp long, a contig N50 of 17,487 bp, and an average GC content of 45%. Based on the annotation statistics and a comparison to other genomes in PATRIC within this same species, this genome appears to be of good quality. Annotation of the genome resulted in 3,721 protein-coding sequences (CDS), 29 transfer RNA (tRNA) genes, and 3 ribosomal RNA (rRNA) genes (Figure 2). The genome annotation included 940 are hypothetical proteins and the remaining 2781 proteins with functional assignments. Protein with the functional assignment included 906 proteins with Enzyme Commission (EC) numbers, 735 with Gene Ontology (GO) assignments, and 639 proteins that were mapped to KEGG pathways.

The taxonomic identification of symbiotic bacteria associated with S. khuongi is still unknown. To validate the identity and place the genome in the bacteria we constructed the phylogenetic tree applying a multigene approach using the whole genome sequences. Multigene phylogeny provides robust phylogeny compared to using single genes, and these resolved trees are essential to study the co-evolution of bacteria with their nematode hosts and the information can be used for the classification of strain and new isolate within the same genera (Tailliez et al. 2010). The phylogenetic tree built in the PATRIC server was similar to the phylogenetic tree derived from the distance analysis of four concatenated protein-coding sequences as described by Tailliez et al. 2010. Single-copy genes from 30 different strains/isolates of bacteria were used to construct the tree with the RAxML algorithm (Table 1). A total of 45,907 amino acids and 137,721 nucleotides were aligned to construct the trees (Figure 3). In the codon tree, bacterial sequences isolated from bacteria associated with S. khuongi (Xenorhabdus_sp) formed a cluster close to Xenorhabdus poinarii str G6 (GenBank Accession: FO704551.1) isolated from S. glaseri (Ogier et al. 2014) within clade C_X-I among the Xenorhabdus genus.

Endosymbiont X. poinarii is known to be associated with different species of EPNs for example S. glaseri (Akhurst 1986)
S. cubanum (Fischer-Le Saux et al. 1999). These two already identified nematode species that harbor X. poinarii are phylogenetically related, and belong to the same clade within the Steinernema genus (Nadler et al. 2006; Stock et al. 2019). Phylogenetic classification of S. khuongi based on the large subunit region (28S) clustered S. cubanum, S. glaseri, and S. khuongi very close to each other into the same clade also known as ”glaseri-group” of Steinernema spp. (Stock et al. 2019).

The genome assembly of Xenorhabdus poinarii str. G6 (GenBank Accession: FO704551.1) was also annotated in PATRIC to compare the genome characteristics of the bacteria. The genomes of two strains have almost the same G+C content but some differences in the number of tRNA and rRNA genes. Sequencing, assembly, and annotation information of Xenorhabdus sp. sk and Xenorhabdus poinarii str. G6 is shown in Table 3.

The D-GENIES genome-wide comparison of Xenorhabdus sk to the reference X. poinarii str G6 shows that these genomes are highly collinear at the genome level. The symbiotically associated bacteria of S. khuongi seem to be very close to X. poinarii (Figure 4).

Because of this close relationship, it may be difficult to specify based on phenotypic characteristics. Speciation using the DNA-DNA hybridization (DDH) technique provides better insight into genomic interrelationship and offer a reliable answer to differentiating species (Rossello-Mora and Amann 2001). The bioinformatics method that mirrors the DDH is the average nucleotide identity (ANI) between genomes (Richter and Rossello-Mora 2009). Average nucleotide identity values (ANI %) of the whole-genome assembly were calculated between S. khuongi bacterial symbionts (Xenorhabdus sp. sk), 26 Xenorhabdus genome assemblies available in GenBank, with Photorhabdus asymbiotica genome as an outgroup. An ANI calculation performed with MUMmer indicated

**Figure 5** Heatmap of average nucleotide identity based on MUMmer (ANIm) based on whole genome alignment of 26 Xenorhabdus species 1 Photorhabdus asymbiotica and Xenorhabdus sp from S. khuongi. The left heatmap shows ANIm percentage identity. Dendrogram shows similarity according to ANIm identity scores. The right heat map shows the Tetra nucleotide frequency correlation coefficient of the above-mentioned species genome alignment.

**Table 2** Features of Steinernema spp. draft genomes

| Steinernema        | Carpocapsae | Scapterisci | Feltiae | Glaseri | Monticolum | Feltiae (NW) | Diaperesi | Khuongi |
|--------------------|-------------|-------------|---------|---------|------------|--------------|-----------|---------|
| Estimated genome size (Mb) | 84.5        | 79.4        | 82.4    | 92.9    | 89.3       | 121.6        | 118       | 82      |
| N50 (bp)            | 7,362,381   | 90,783      | 47,472  | 37,444  | 11,556     | 60,433       | 11,474    | 42,000  |
| Number of scaffolds/contigs | 16          | 2,877       | 5,839   | 7,515   | 14,331     | 4,678        | 35,545    | 8,794   |
| GC content (%)      | 45.7        | 47.98       | 46.99   | 47.63   | 42.01      | NA           | 45.01     | 47.88   |
| N content (Mb)      | NA          | 0.76        | 2.76    | 3.37    | 4.34       | 0            | 0         | 0       |
| N content (%)       | 0.54        | 0.96        | 3.36    | 3.64    | 4.87       | 0            | 0         | 0       |
| Maximum scaffold size (bp) | 20,922,283 | 1,149,164  | 1,470,990| 339,094| 110,081    | 1,315,981    | 1,706,490| 428,226 |
| Predicted genes     | 91,957      | 31,378      | 33,459  | 34,143  | 36,007     | 32,304       | NA        | NA      |
| Complete BUSCO      | 87%         | 84.5%       | 84.32%  | 59.4%   | 69.2%      | 87.27%       | 85%       | 86.6% (850) |
| Single-copy BUSCOs  | NA          | 79.3%       | 80.55%  | 57.8%   | 65.4%      | 76.8%        | 79.6%     | 81.3% (798) |
| Duplicated BUSCOs   | NA          | 5.2%        | 3.77%   | 1.6%    | 3.8%       | 10.59%       | 5.40%     | 5.3% (52) |
| Fragmented BUSCOs   | 7%          | 8.1%        | 8.35%   | 12.9%   | 12.5%      | 7.33%        | 7.10%     | 6.5% (64) |
| Missing BUSCOs      | 6%          | 7.4%        | 7.54%   | 27.7%   | 18.3%      | 5.40%        | 7.90%     | 6.9% (68) |
| Reference           | Serra et al. (2019) | Dillman et al. (2015) | Fu et al. (2020) | Baniya et al. (2020) | This study |
that the genome Xenorhabdus sp. from S. khuongi had 97% sequence similarity with that of X. poinarii str. G6. Similarly, tetranucleotide correlation between these two species was 99.8%. Average nucleotide identity (ANIb) using BlastN at NCBI was also calculated whose result indicated that these two bacteria share 96.5% sequence identity. The average nucleotide identity (ANI) of 94 to 96% is generally used as the cutoff value to separate bacterial species based on their genome sequences (Konstantinidis and Tiedje 2005; Richter and Rossello-Mora 2009). Not only does our study suggest that the genome reported here is similar to those within its genus. Because our genome has nearly 9,000 gaps, to improve the quality of the genome it is recommended to combine short reads like ours with additional sequencing of long-insert DNA libraries from other technologies. However, even in their current state, these genomes should be valuable tools for comparative and functional genomics. Rather than sequence members of a symbiotic association we sequenced, assembled, and analyzed the genome of both symbionts and host of the EPN in one sequencing. This method of experiment and analyzing the data saves the time and cost of sequencing, but also retrieves valuable information that is hard to recover using more conventional methods.

### Discussion

The symbiotic association of specific bacteria within the gut of EPNs makes the genome sequencing of only one partner difficult. Due to their close association, it is nearly impossible to get clean DNA of only one species. Working with the newly identified nematode species makes it more complex because of the unknown association of co-bionts. The use of a blob-plot allows each contig from the assembly to be separated based on the taxonomy and GC-coverage and can easily discern the nematode, bacterial symbionts, and other contamination if any present, in the assembly (Figure 1). This approach proves to be a robust tool that reduces the cost of the sequencing and cuts the complexity of the host-symbiont system as a simple metagenomic project and provides better insight to differentiate the unique genomes captured during sequencing. This approach is also feasible for the system where lab culture and isolation of symbionts are not possible (Kumar and Blaxter 2011).

The nematode S. khuongi is endemic to the environmental condition of the Flatwood region of Florida citrus orchards. This region has a devastating problem with the citrus weevil (Stuart et al. 2008)). The availability of the genome of the nematode and associated bacteria can serve as a valuable resource to analyze various genetic factors that enables this nematode to thrive in these specific environmental conditions, and that also further improve it as a more effective biological control agent. As we are reporting the draft genomes of the nematode and bacteria, we anticipate some genome variation between isolates sequenced in the future. Although methods like flow cytometry can accurately predict the size of the genome and check for heterozygosity, the genome reported here is similar to those within its genus. Because our genome has nearly 9,000 gaps, to improve the quality of the genome it is recommended to combine short reads like ours with additional sequencing of long-insert DNA libraries from other technologies. However, even in their current state, these genomes should be valuable tools for comparative and functional genomics. Rather than sequence members of a symbiotic association we sequenced, assembled, and analyzed the genome of both symbionts and host of the EPN in one sequencing. This method of experiment and analyzing the data saves the time and cost of sequencing, but also retrieves valuable information that is hard to recover using more conventional methods.

### Data availability

All genome sequencing data generated in this study for both nematode and bacteria are deposited at the NCBI database under the BioProject number PRJNA670677 for nematode and PRJNA670736 for bacteria.

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