Nanopore-Based Whole-Genome Sequencing Reveals a Bioluminescent and Non-Bioluminescent Bacterium Isolated from the Squid *Loligo forbesi* in the Red Sea

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Abstract

Common in aquatic life, symbiotic interactions with luminescent bacteria sometimes support bioluminescence. Though these interactions in cephalopods have been well studied in species such as *Euprymna scolopes*, not much is known about other cephalopods, most especially those of the Red Sea. This work separated and identified bioluminescent and non-bioluminescent bacterial strains from the ink sacs and eyes of *Loligo forbesii*, a squid species taken from the Zaafarana area of the Suez Canal in Egypt's Red Sea. Whole-genome sequencing based on nanopores turned up two distinct bacterial strains: *Shewanella baltica* (non-bioluminescent) and *Psychrobacter alimentarius* (bioluminescent). Under Alkanal Monooxygenase (FMN-linked), genome annotations in the *P. alimentarius* genome expose Alkanal Monooxygenase Alpha Chain bacterial bioluminescence. High similarity between the isolated *P. alimentarius* strain and publicly archived reference genomes was confirmed by comparative genomic study including ANI calculations tool and phylogenetic tree building. Moreover quite highly sequence conserved in GenBank between our *P. alimentarius* isolate and earlier published strains was whole-genome alignment with the Mauve tool. The findings suggest that *P. alimentarius* may be involved in the bioluminescence of *Loligo forbesii* and support current understanding of cephalopod bacteria associated with *Loligo forbesii*. The present work provides more information on the whole genome sequence including

genomes annotation of both bioluminescent and non bioluminescent strain and explores the bacterial symbiotic in cephalopod marine organisms inhabiting the Red Sea.

Keywords: Bioluminescence, *Loligo forbesii*, *Psychrobacter alimentarius*, *Shewanella baltica*, nanopore sequencing, bacterial symbiosis, Red Sea

1. INTRODUCTION

Including cephalopods, many terrestrial and aquatic species emit light. Moreover, bioluminescence (Haddock et al., 2010; Otjacques et al., 2021) functions as both a mechanism of attraction and defence. In the generation of light, a photophore is an organ. Although the autogenic organisms create light by themselves, the bacteriogenic organisms create light by the bioluminescent bacteria in a symbiotic relationship with the organism (Haddock et al., 2010). Recent studies on bioluminescent cephalopods—such as Euprymna scolopes—generated light by means of bioluminescent bacteria *Vibrio fischer*i. *Vibrio fischer*i extracellularly colonises euprymna scolopes along their apical surface of epithelium. Human microbiome cells interact with gut epithelia (Nyholm and McFall-Ngai, 2021) similarly to this mechanism. By means of the epithelia and the processes by which they survive over the lifetime of the host, the squid-Vibrio system can also help one understand the mechanisms behind the recruitment of gram-negative bacteria (Otjacques et al., 2021). Combining ventral photophorescts containing bioluminescent bacteria, the Uroteuthis genus of loliginid squids (Cephalopoda: Loliginidae): Several paired structures have been shown to exist in the ink sacs of female *Afrololigo mercatori*s and young *Loligo forbesi* (Alexeyev, 1992). Species of loliginid squid were categorised under subfamily Uroteuthinae (Alexeyev, 1992). These species have photophores, although the accessory nidamental glands of L. forbesi (Lum-Kong and Hastings, 1992; Vecchione et al., 1998) revealed a luminescent bacterial strain. A species of Psychrobacter (accession number LK9318) has revealed a fresh example of bioluminescence, that is, light emission.

The first known documentation of Bioluminescent within this class of bacteria comes from Found in symbiotic association with the rani fish (*Nemipterus japonicus*), the Bioluminescent Psychrobacter is Luminescence remarkably increased as the Psychrobacter genera in the culture entered the mid-logarithmic development phase. Under this phenomena called quorum sensing (Prakash et al., 2014), bacteria coordinate activities using a communication system based on population density. These days, quorum sensing is applied extensively by researchers to investigate related mechanisms in other species as well as the underlying cellular communication channels and biochemistry engaged (Chiu et al., 2007). It has been suggested to assist photoreactivate DNA repair systems, neutralise damaging reactive oxygen species, and offer an extra layer of protection against the negative effects of short-wavelength solar ultraviolet (UV) radiation. Additionally involving this light production are mutualistic interactions involving different cell types or to attract them. The supposed mutualistic symbiotic relationship between *Shewanella pealeana* and *Loligo pealeii* is one-sided. Linked to the last stage of egg development, the accessory nidamental gland (Bloodgood, 1977; Leonardo et al., 1999) is typified in molluscs by a gland rich in bacteria. Living in squid eggs, *Shewanella pealeana* guards against abiotic stresses, pathogens, predators, and parasites (Barbieri et al., 1997). Comprising the α (LuxA) and β (LuxB) subunits, LuxAB is the specific enzyme complex responsible for this light generation. Attached to a molecule called reduced flavin mononucleotide ($FMNH_2$), LuxAB generates light (Zarubin et al., 2012) concurrently with converting a long-chain aldehyde into a fatty acid using molecular oxygen. Studies on bioluminescence revealed a suite of bacterial species with rearranged lux operons. These species do not have luxB component. Introduced to a synthetic luxA gene derived from a simpler luxACDE operon in *Enhygromyxa salina*, *Escherichia coli* can develop luminescence. Taken all things considered, the esLuxA gene produces less light than the luciferase genes found in *Aliivibrio fischeri* (AfLuxAB).

Found in coastal Japan, the rather halophilic myxobacterium *Enhygromyxa salina* is not reported to be luminous. EsLuxA's luminosity is considerably less than that of typical heterodimeric luciferases (Yudenko et al., 2024). Most famously in *Vibrio fischer*i, bacterial bioluminescence—the emission of light by bacteria—has been extensively investigated in many marine and estuaries. Visibly luminous (producing high levels of light), non-visibly luminous (producing low levels of light, detectable only with a photometer), or nonluminescent (producing no light) are the several ways that bioluminescent bacteria might be classified. Fascinatingly, studies have found luciferase gene sequences in even nonluminescent and nonvisibly luminous strains. For instance, the reference strain *V. cholerae* ATCC 145 might generate just low levels of light even though it contains the luxA gene required for bioluminescence. This phenomena implies that the existence of luciferase genes does not always correlate with visible light emission, maybe due to a lack of other genes in the lux pathway, uncoordinated gene control, or changes in other genes involved in the bioluminescence process (Ramaiah et al., 2000). By means of nanopore-based whole-genome sequencing, the present work aims to identify a bioluminescent strain and a non-bioluminescent strain living symbiotally in several regions of the squid's body

2.MATERIALS AND METHODS

2.1 COLLECTION OF SAMPLE

As shown in Fig. 1, the samples of the squid individuals, L. forbesii, came from the Zaafarana area (Suez Canal, Red Sea, Egypt), which could provide bioluminescent bacteria. The squids were then kept in an icebox and transported to the Biotechnology Institute of the National Research Centre (NRC) for microbial genetic study. To get rid of sand or mud, the squids were gently cleaned using sterilised distilled water. The squids were then put in seawater, half submerged for several hours at 4°C to 8° refrigerated temperature.

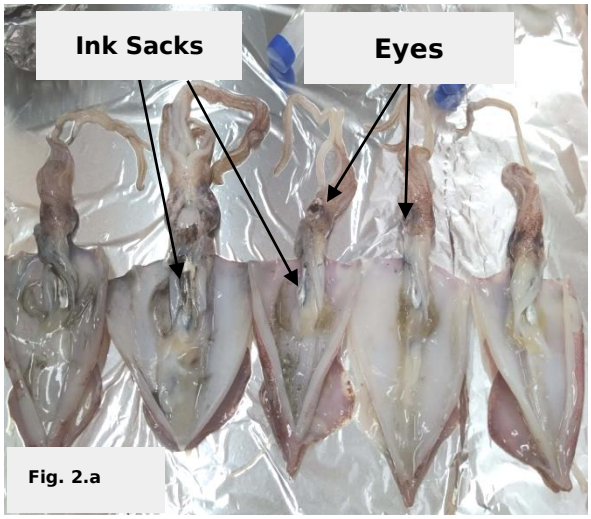
Fig. 1. The squid individuals, L. forbesii, were collected from the Zaafarana region.

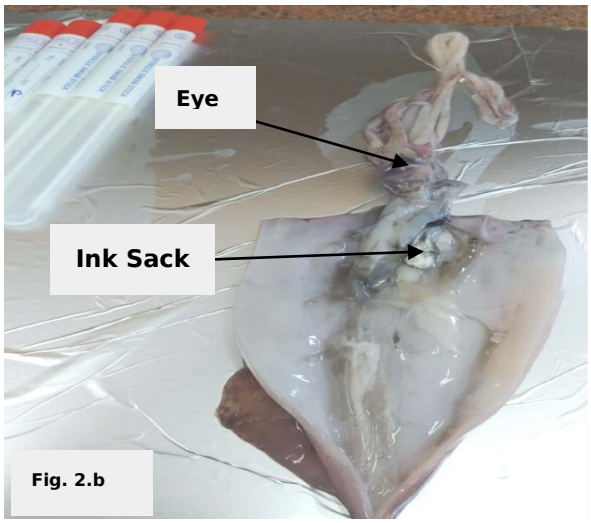
2.2 Bioluminescent media

This work used luminescent broth (LB) and a modified form of luminescent agar (LA) as described by Nealson, 1978. Per litre the luminescent agar and broth consists of 10g of peptone, 60% glycerol, 1g of $MgSO_4$, 4g of K_2HPO_4, 30g of NaCl, and 15g of bacteriological agar. The media was sterilised in an autoclave set on 121°C for fifteen minutes.

2.3 Isolation and culturing

Following dissection of their digestive system and ocular structures, ink samples were taken from the ink sacs and eyes of the squids (Fig. 2.a & 2.b). Serial dilutions were carried on until a 10^-5 dilution was obtained. Every dilution went on LA plates. Avoiding UV lamp illumination, the plates were incubated at 25°C for 24 hours, with luminescent colonies observed and recorded every 8 hours in a dark chamber of the Gel documentation device. Random selection produced brightest light-emitting colonies; these were streaked onto LA plates and incubated for another 24 hours at around 25°C.





Figs. 2.a and 2.b illustrate the position of a squid's eye and show its dissection. Black arrows show the particular sites—the eye and the ink sac (silver body)—from which samples were taken.

2.4 Subculture of specific isolates

Unique luminescent bacterial colonies were identified through luminescence observation and purified by sub-culturing on LA plates using standard streaking techniques. Pure cultures were subsequently stored in LB for further growth and analysis.

3.GENOMIC DNA ISOLATION AND 16s rDNA PCR REACTION

A modified approach (Williams et al., 2012) was used to extract high-molecular-weight (HMW) DNA. Using a NanoDrop microvolume spectrophotometer, DNA concentration was measured; quality was evaluated by loading samples onto a 0.8% agarose gel in 1× TAE buffer to validate integrity and absence of smudging. Three PCR reactions were optimised to amplify the 16S rDNA template using a melting temperature of 52°C to investigate whether the extracted HMW of genomic DNA degrades the 16s rDNA templates. Using the Rapid Barcoding Kit method, HMW DNA was delivered to a genomic services facility at the Children's Cancer Hospital for sequencing on the Nanopore platform (MinION Mk1C).

4. GENOME ASSEMBLY

MinION readings went through a multi-step quality control process. First FastQC (version 01-03-23) then adapter removal with Porechop (version 0.2.4) examined raw data. Further filtered using Filtlong (version 0.2.1), which used k-mer matching to a reference sequence for improved quality assessment, were reads Using Flye (version 2.9.5), a de novo assembler idealised for long-read data, filtered reads were reassessed under FastQC before assembly (Lin et al., 2016). Flye can tolerate high error rates and process varying sized genomes. Racon (Vaser et al., 2017) aligned reads to contigs and corrected mistakes, so improving the assembly. Bandage (Wick et al., 2015) visualised assembly graphs; assembly quality was assessed with QUAST (Gurevich et al., 2013).

4.1 Genome Contamination

Accessible via the Biocloud server, the ContEst16S online tool was used to evaluate the possible contamination risk so guaranteeing the purity and integrity of the genome. This tool identifies contamination by analyzing 16S rRNA gene sequences within the assembly (Lee et al., 2017).

4.2 Genome annotation using DFAST

The finalized assembled genome was annotated and subsequently submitted to the DNA Data Bank of Japan (DDBJ) at the National Institute of Genetics. This was accomplished using the DDBJ Fast Annotation and Submission Tool (DFAST), accessible at https://dfast.ddbj.nig.ac.jp.

4.3 Analyzing and visualizing genomic data with BV-BRC

The Bacterial and Viral Bioinformatics Resource Centre (BV-BRC) server (https://www.bv-brc.org/) annotated the whole genome. The circular viewer program proKsee (Grant et al., 2023) let only view annotated genomic sequences.

4.4 ANI calculator

OrthoANIu (https://www.ezbiocloud.net/tools/ani) is an improved method grounded on USEARCH that helped to estimate the average nucleotide identity (ANI) between genome sequences. Yoon et al. (2017) claim that this method presents a simpler and more successful substitute for the first developed OrthoANI approach.

4.5 Constructing Bacterial Phylogenetic Trees: Genomic and 16S rRNA Sequence Analysis

Using Mash/MinHash for the estimate of genetic similarity, the Similar Genome Finder in BV-BRC was used to identify bacterial genomes closely associated to the query genome (Ondov et al., 2016). Using the codon tree approach, which finds single-copy genes from BV-BRC PGFams and uses RAxML for analysis, a phylogenetic tree was built. MAFFT (version 7.526) generated alignments of 16S rDNA sequences that were visualised using Archaeopteryx (https://www.phylosoft.org/archaeopteryx).

4.6 Genome alignment (Mauve)

Whole-genome alignment was performed using Progressive Mauve (Darling et al., 2010) to identify conserved regions and genomic differences.

5.0 Bioluminescent Research Workflow: From Culturing to Genome Analysis

The process started with swab samples on LA media being developed from *Loligo forbesii* ink sacs. After enrichment of single colonies, fly-based de novo assembly and nanopore sequencing followed. Later actions included genetic similarity estimate with Mash/MinHash, genome alignment using Mauve, contamination checks with ContEst16S, and genome annotations using DFAST. One can see this thorough process in (Fig. 3.).

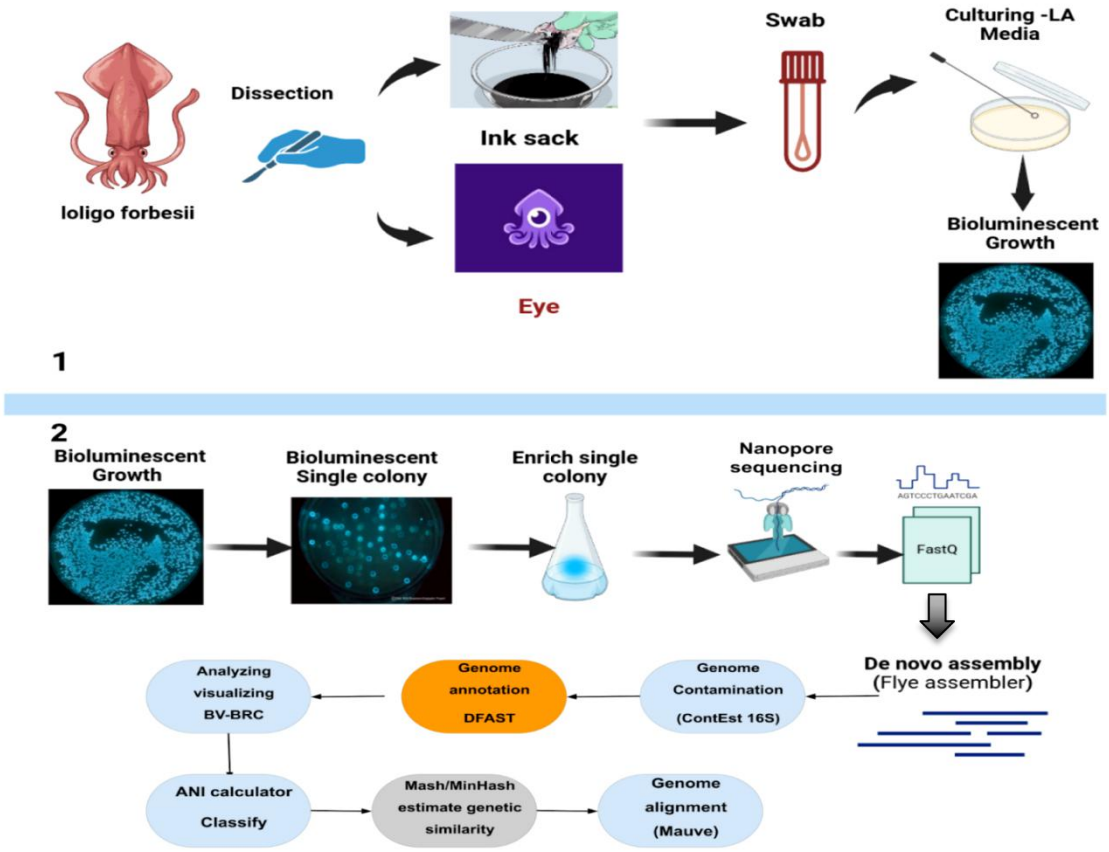
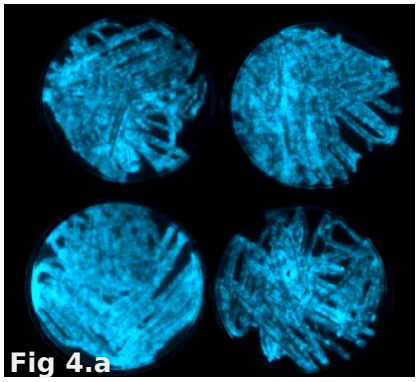


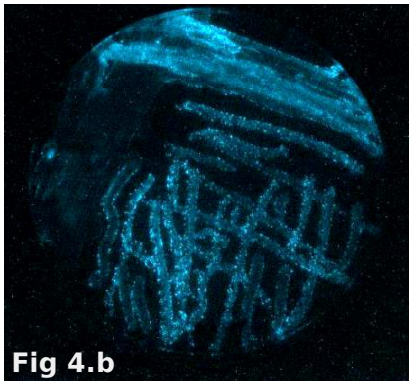
Fig. 3. Step 1: We begin with the dissection of *Loligo forbesii* to extract the ink sac. This ink is swabbed and cultured on LA media to encourage the growth of bioluminescent bacteria. Step 2: Bioluminescent growth is observed, and a single colony is selected for further enrichment. This enriched colony undergoes nanopore sequencing, generating FastQ data for genome analysis. The genome is then built from scratch using the Flye assembler, and after that, it is labelled and analysed further with tools like ANI calculator, Mash/MinHash, and Mauve for sorting and comparing.

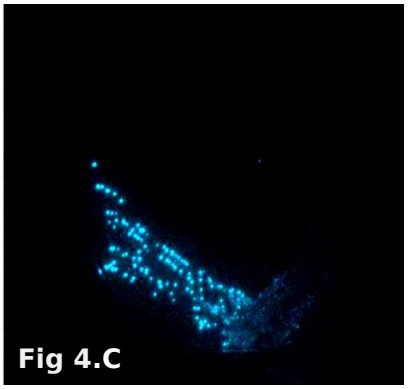
6. Results

6.1 Luminescent bacteria growth in medium

Separated bioluminescent bacteria linked to ink sacs and eyeballs were grown on plates as shown in (Fig. 4 a, b, c). **Figure** 5 (a, b) displays one colony. The colony shows up on the solid medium as a white dot under normal light. Dark settings cause the colony to show luminescence.







Figures 4.a and 4.b show luminescent bacteria taken from the ink sac grown on specialised luminescent agar in a dark surroundings. Particularly showing the streaking luminescent bacteria taken from the eye, grown on LB media, is **Figure** 4.c.

Figure 5.a and **Figure** 5.b illustrate the visualization of a single colony: a white dot on solid medium under normal light and a luminescent colony observed in the dark (Figures 5.a, b).

6.2 High molecular weight of the DNA and detection of 16s rDNA

We isolated high molecular weight (HMW) DNA from the luminescent broth of pure cultures. DNA concentration was 300 ng/µl and measured by NanoDrop. The presence of 16S rDNA in the bioluminescent bacteria was confirmed through three PCR reactions. A sharp band corresponding to the 16S rDNA gene was visualised on an agarose gel with an expected molecular weight of 1600 bp, as shown in Fig. 6.

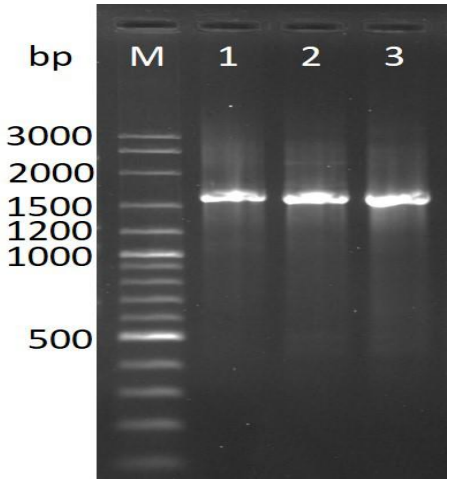


Fig. 6. Detection of PCR Products from 16S rDNA at 1600 bp on DNA Ladder Molecular Weight.

6.3 De novo assembly and quality control results

Following confirmation that the 16S rDNA PCR product was good, we generated high molecular weight (HMW) genomic DNA using the Nanopore Rapid Barcoding Kit and then put it onto a MinION Mk1C flowcell. Fastq files were then handled according to the assembly processes and quality control guidelines found in the Materials and Methods section. We handled the Fastq files according to the assembly and quality control procedures described in the Materials and Methods section. Generating assembly graphs and summary reports, we validated the final genomic assembly using the Bandage tool. As seen in Fig. 7 two main nodes were found: the smallest contig (3,256,386 bp) and the largest contig (4,992,825 bp). Reduced numbers of contigs, according to Quast, point to a better-quality assembly with less mistakes.

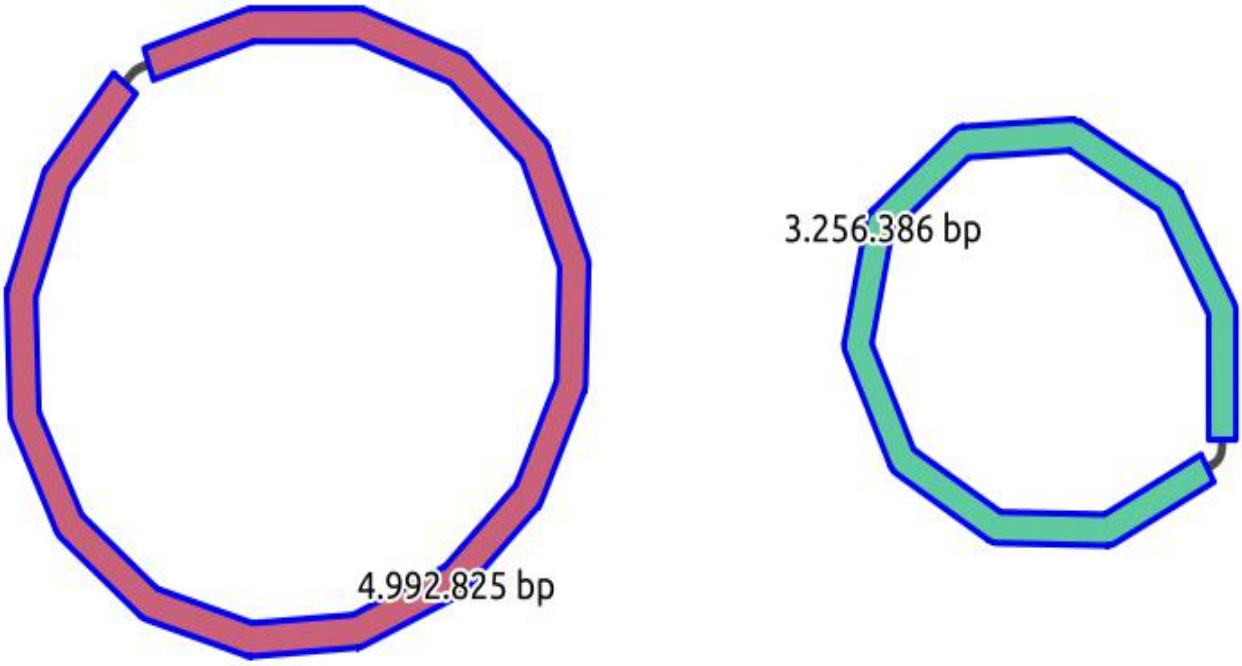


Fig. 7 shows the graphical view of assembled genomes using de novo assembly graphs and the Bandage tool.

6.4 Check the genome for contamination

ContEst16S assisted in evaluating the assembled genomes for contamination. Including GC content, genome length, and N count, Table 1 lists the genomic features for the smallest (3,256,386 bp) and largest (4,992,825 bp). ContEst16S matched the 16S rRNA gene segments from the assembled genomes to a database of 69,745 genomes including 596 contaminated entries. The produced genomes were confirmed to be uncontainted. While the smallest contig was tightly related to *Psychrobacter alimentarius,*, ContEst16S's maximum likelihood phylogenetic trees (Fig. 8 a, b) revealed that the biggest contig of the genome was more similar to *Shewanella baltica* than to other 16S rDNA fragments.

Table 1. Features of the assembled genomes analyzed using ContEst16S.

Contig	Total Length (bp)	A	C	G	T	N	GC Content (%)	Decision
Smallest contig	3,256,386	926,885	701,606	699,426	928,469	0	43.02	Not Contaminated
Largest contig	4,992,825	1,338,502	1,160,023	1,157,916	1,336,384	0	46.43	Not Contaminated

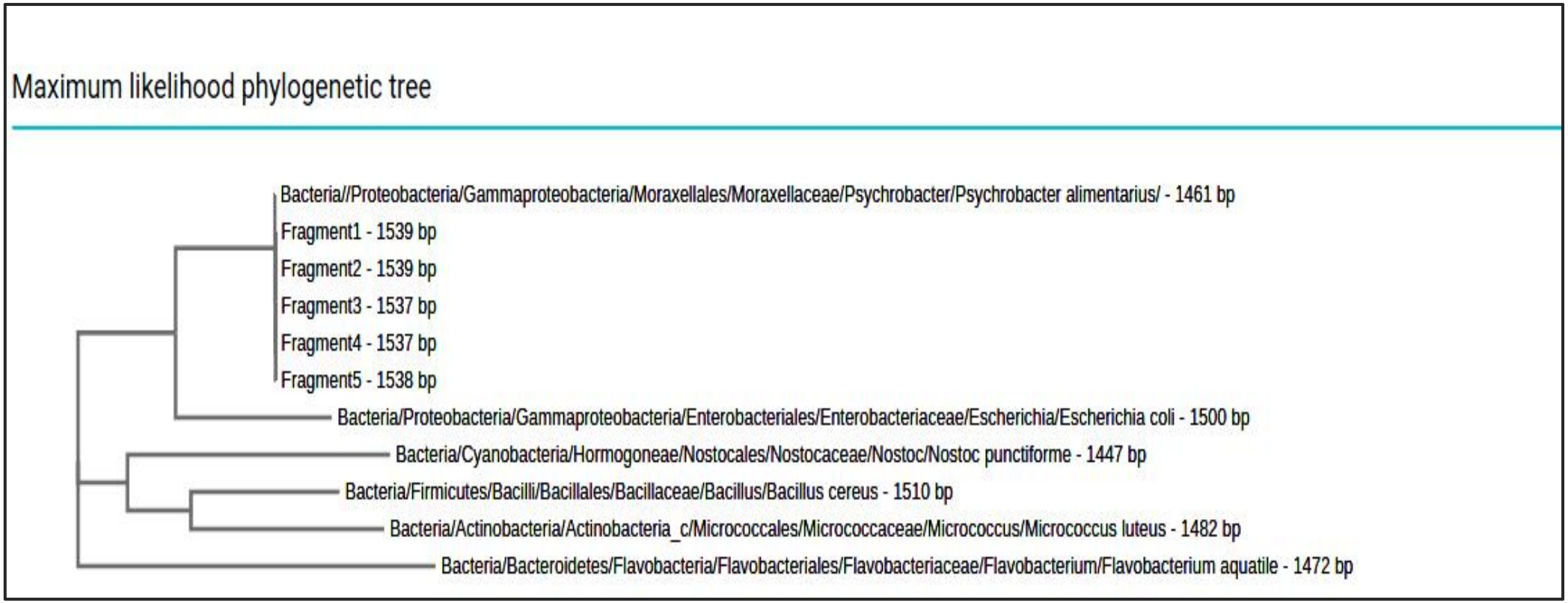


Fig. 8.a Analysis using maximum likelihood for the five fragments of 16S rDNA from the smallest contig (3,256,386 bp).

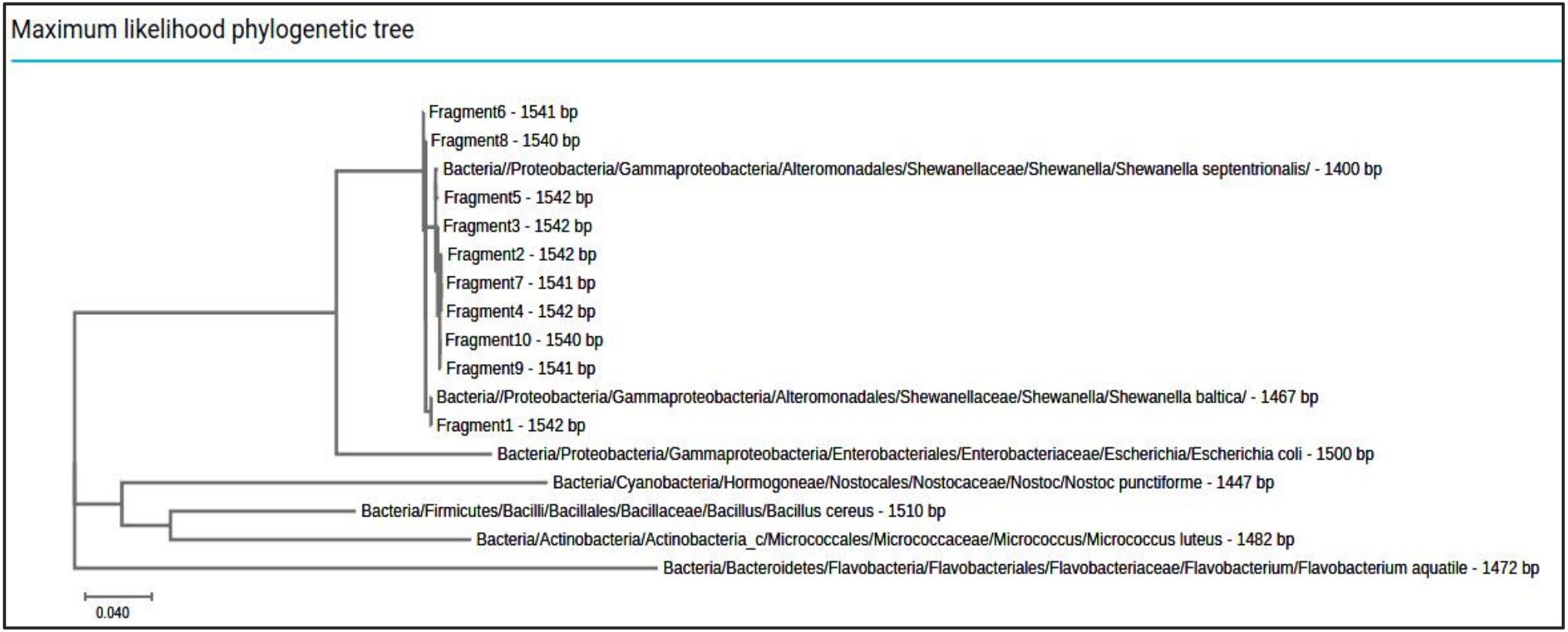


Fig. 8.b illustrates the maximum likelihood for the five fragments of 16s rDNA within the largest contig, which is (4,992,825 bp).

6.5 Fast annotation and submission tool (DFAST) to DDBJ

After annotations using the DFAST tool, the two assembled genomes were turned in to DDBJ's GenBank database. Measuring 3,256,386 bp, the smallest-sized genome was annotated as *Psychrobacter alimentarius* and assigned the accession number AP031495. By contrast, with 4,992,825 bp, the biggest genome was annotated as *Shewanella baltica* and assigned the accession number AP031461.1.

6.6 Annotate the complete genome

The two assembled genomes were annotated via uploading to the server of the Bacterial and Viral Bioinformatics Resource Centre (BV-BRC) to generate the output JSON file, which was visualised using Proksee as shown in the circular map view for *Psychrobacter alimentarius* (Fig. 9 a) and for *Shewanella baltica* (Fig. 9 b).

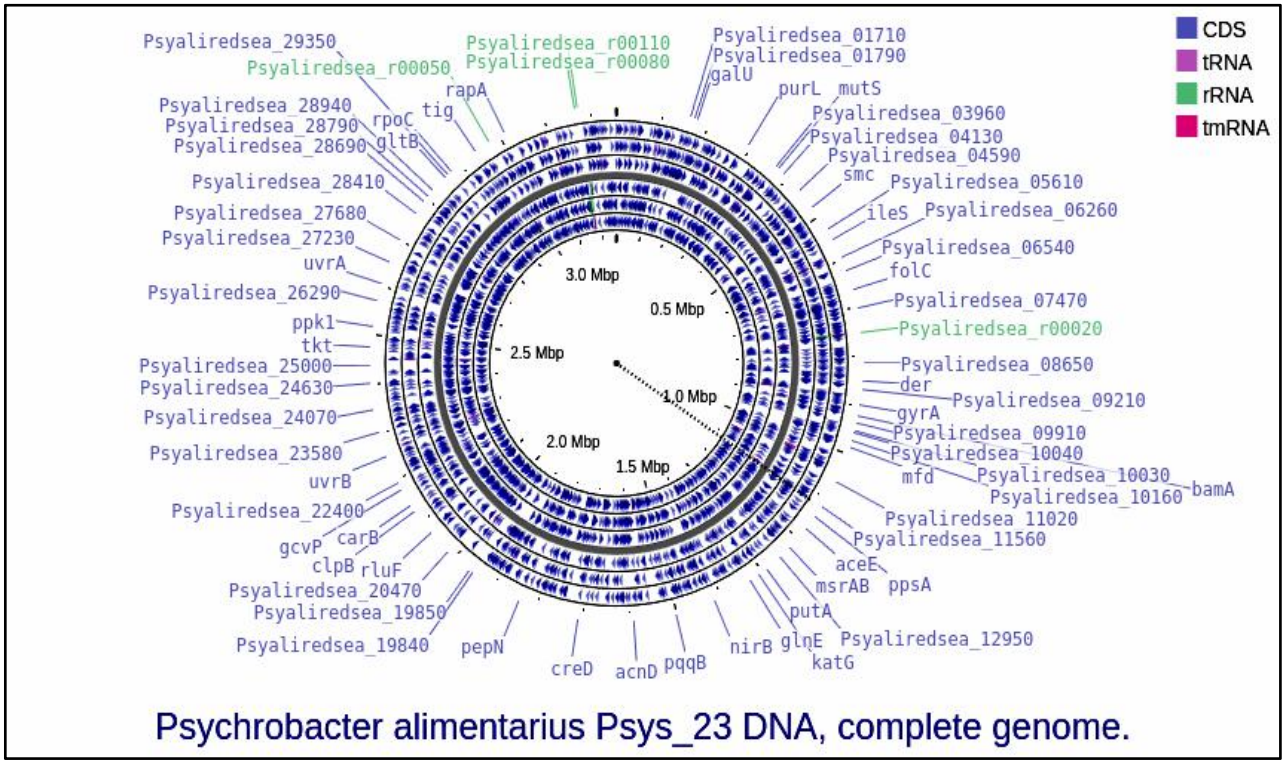


Fig. 9.a Circular map view of the complete genome of *Psychrobacter alimentarius* with accession number AP031461.1

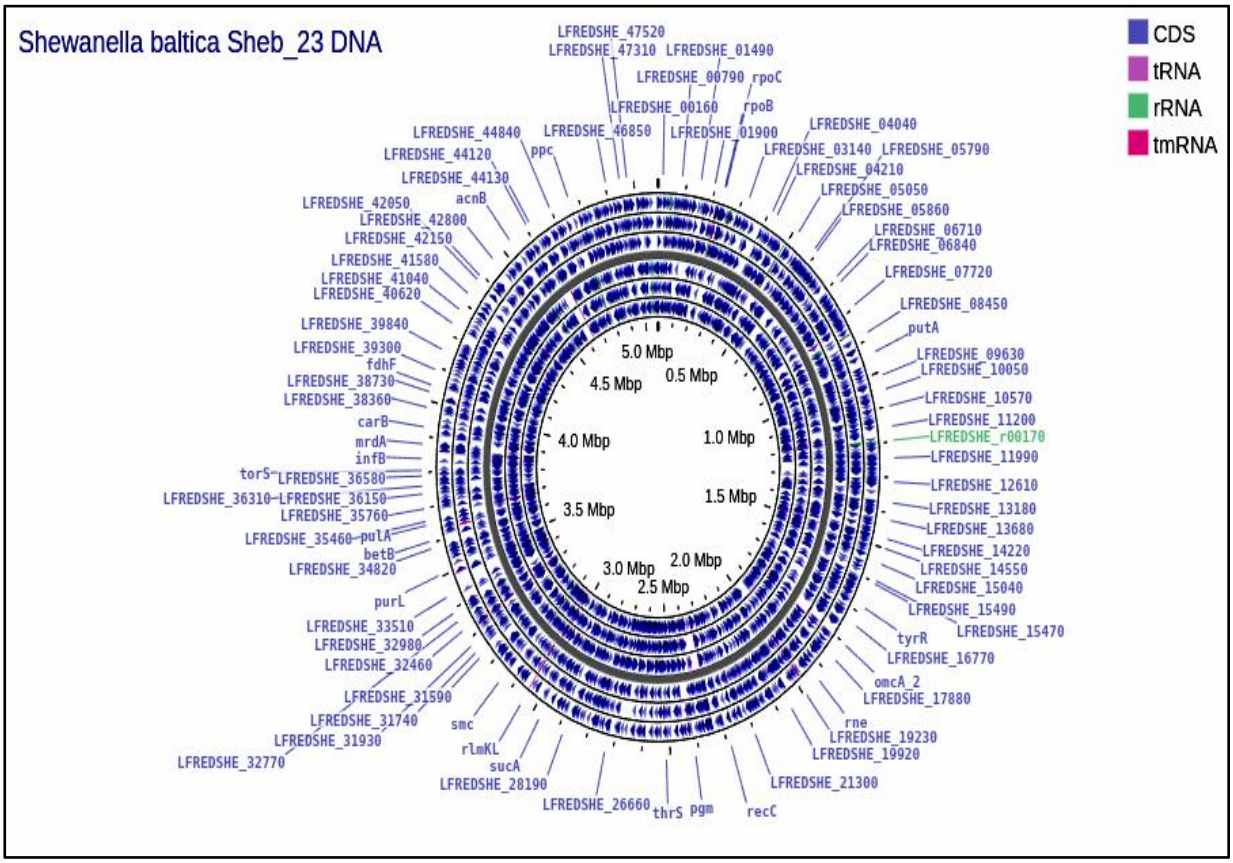


Fig. 9.b Circular map representation of the complete genome of *Shewanella baltica* and had obtained the accession number (AP031495.1).

6.7 Finding the bioluminescent gene in the complete genome of *Psychrobacter alimentarius*

Bioluminescent genes were identified using the PATRIC annotation tool included within the BV-BRC server. It identified the family of alkanal monooxygenase (FMN-linked) proteins by means of the GO term GO:0047646 Table 2 offers a synopsis of the annotations, including Alkanal Monooxygenase Alpha Chain's coding sequence (CDS).

Table 2. PATRIC annotation output for *Psychrobacter alimentarius*.

Feature Type	Start	End	Length (bp)	Length (AA)	Product
CDS	1,193,850	1,194,887	1,038	345	Alkanal Monooxygenase Alpha Chain

6.8 Finding genomes similar to the assembled *Psychrobacter alimentarius* bacterial strain

To evaluate similarity between public genomes, genome distances were computed applying the Mash/MinHash technique. With an OrthoANIu value of 97.55%, the assembled *Psychrobacter alimentarius* genome (AP031495) and the reference strain PAMC 27649 (CP014945) showed a high degree of similarity (Table 3). The assembled genome (*Psychrobacter alimentarius* 2024) is tightly related to *Psychrobacter alimentarius* PAMC 27889 according to a phylogenetic tree produced with the BV-BRC Bacterial Genome Tree Service. Together with accessions OR621168.1 and LK931862.1, fragments 1–5 (AP031495.1) clustered tightly to confirm that they are all bioluminescent strains (Fig. 10b).

Table 3. OrthoANIu comparison of *Psychrobacter alimentarius* genomes.

Metric	Value
OrthoANIu value (%)	97.05
Genome A length (bp)	3,255,840
Genome B length (bp)	3,348,660
Average aligned length (bp)	2,226,357
Genome A coverage (%)	68.38
Genome B coverage (%)	66.48

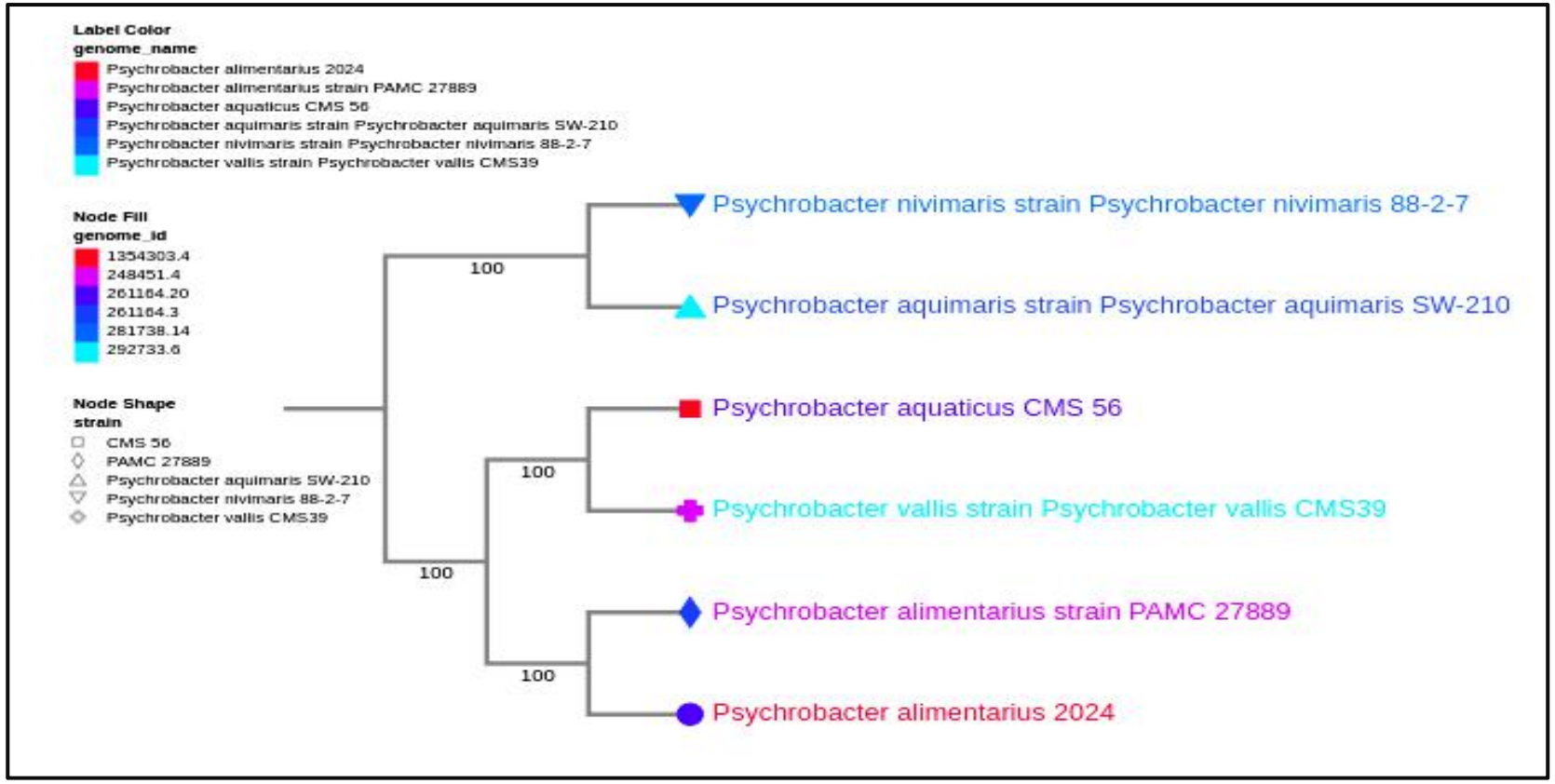


Fig. 10.a Psychrobacter strain phylogenetic links. With *P. alimentarius* strains (2024 and PAMC 27889) forming a strongly supported clade (bootstrapped >90%), The Codon Tree method picks single-copy BV-BRC reveals distinct clustering of five species. While P. aquimaris SW-210 shows as an evolutionary outlier with longer branch lengths, implying possible niche specialisation, moderate genetic distances separate P. aquaticus CMS 56 from this group. Node forms serve as strain identifiers; filled circles show strains incorporated in downstream analyses. The strong bootstrap values (>85% for all major nodes) highlight both conservation within species and divergence between them, most likely reflecting adaptations to different cold environments, so confirming the dependability of these phylogenetic relationships.

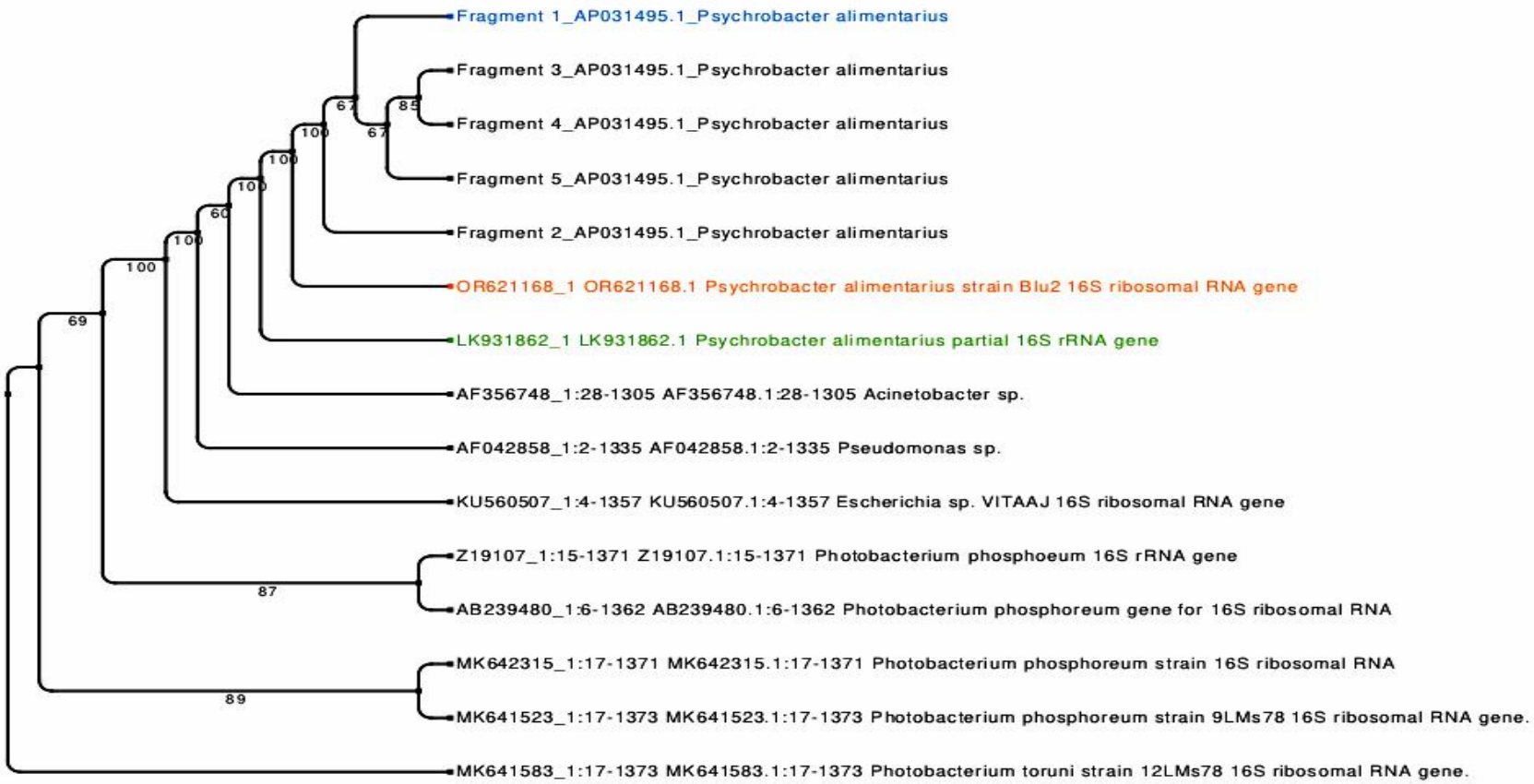


Fig. 10.b phylogenetic tree built by MAFFT alignment and neighbor-joining of 16S rRNA sequences placed five *P. alimentarius* fragments (AP031495) in a strongly supported clade (bootstrap >95%), so verifying their taxonomic classification. Indicating great conservation, close relatives (strains Blu2 [OR621168.1] and LK931862.1) grouped within this cluster.While Pseudomonas sp. and Escherichia sp. provided outgroups, Photobacterium spp. developed a clear sister clade with horizontal transfer of bioluminescent genes to Escherichia. Branch lengths proposed higher variation among Photobacterium strains than in *P. alimentarius*.

6.9 Genome alignment (Mauve)

The Mauve tool brought the reference genome (CP0149) into line with the recently produced genome (AP031495.1) of *Psychrobacter alimentarius*. Local collinear blocks (LCBs) found here revealed conserved genomic segments with structural integrity. Figures 11 shows two LCB displaying homologous backbone sequences.

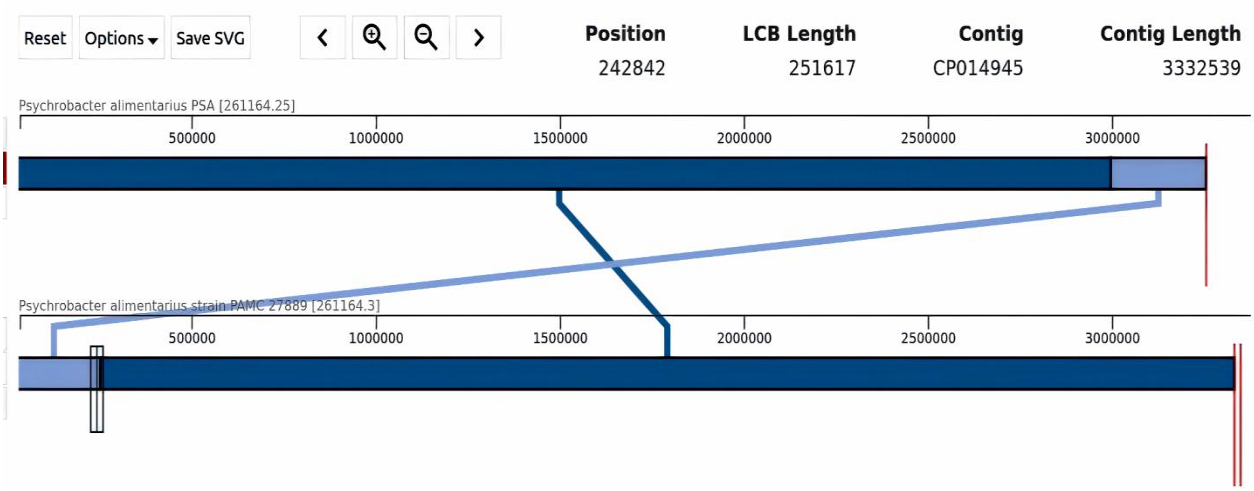


Fig. 11. Locally Collinear Block (LCB), which is considered as backbone sequences that are homologous.

7. Discussion

Symbiotic interactions between cephalopods and bioluminescent bacteria best illustrate the essential ecological adaptation of bioluminescence in marine life. Although the Uroteuthis genus is well-documented for such symbioses, their frequency throughout the Loliginidae family is yet unknown (Anderson et al., 2014; GenBank OR621168.). Our work separates and identifies bioluminescent bacteria from *Loligo forbesi* in the Red Sea, so offering genomic and ecological understanding of these symbiotic interactions and their evolutionary consequences.

Important discoveries and genomic perspectives

We discovered two bacterial symbionts in L. forbesi using nanopore sequencing: *Shewanella baltica* and *Psychrobacter alimentarius*. The effectiveness of long-read sequencing for resolving complex microbial genomes was highlighted by the discovery of few dark regions in high-quality genome assemblies (Ebbert et al.,

2019). Our isolates and reference strains (*P. alimentarius* CP014945.1, S. baltica CP002384.1) were found to have close relationships, according to phylogenetic analysis. Locally collinear blocks (LCBs) highlighted conserved genomic architecture (Fig. 11). In line with earlier reports of low-light emission in this genus, *P. alimentarius* was notable for having a LuxA domain associated with dim bioluminescence (Prakash et al., 2014). By identifying bioluminescent *P. alimentarius* in L. forbesi for the first time, its known associations within Loliginidae are expanded.

Ecological and evolutionary implications

Shewanella and Psychrobacter have clearly established mutualistic roles in marine symbioses. While Psychrobacter adds to host fitness by oxidative stress reduction, Shewanella spp. guard squid eggs from pathogens and abiotic stress (Barbieri et al., 1997). Our identification of bioluminescence-related genes in *P. alimentarius*—including the alkanal monooxygenase family (GO:0047)—indices adaptive advantages in dimly lit environments, such UV protection or prey attraction. These results support current studies on bioluminescent *P. alimentarius* in Loligo vulgaris (GenBank OR6211), so highlighting the ecological adaptability of the genus.

Bioluminescence as a Trait Under Conservation

Fascinatingly, non-luminous bacteria such as Vibrio cholerae retained truncated lux operons, generating barely detectable light to humans. This is consistent with observations in *Enhygromyxa salina* whereby low-intensity luminescence (Yudenko et al., 2024) results from a simplified luxACDE operon. Even in species with functional expression lost, such "cryptic" bioluminescence suggests evolutionary conservation of light-production machinery (Ramaiah et al., 2000). This phenomena can be explained by regulatory disturbances, incomplete lux paths, or ecological niche shifts; hence, research on gene expression dynamics is justified.

Future Directions

Our work emphasises the need of investigating bioluminescence control among bacterial taxa and surroundings. Comparative research of lux operon organisation, transcriptional control, and ecological pressures will help to define its evolutionary path. Furthermore underdeveloped are the uses of bioluminescent bacteria in environmental monitoring, including biofilm indicators or pollution biosensors.

In conclusion

Finally, this work reveals genomic preservation in Psychrobacter and cryptic lux genes in non-luminous taxa, so advancing knowledge of bioluminescent symbioses in cephalopods. We highlight the dynamic adaptive function of bioluminescence in marine ecosystems by linking genomic, ecological, and evolutionary points of view.

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