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Genetic Detection of a Novel Geosmin-Producing *Oscillatoria lutea* Isolated from the Euphrates River, Iraq

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Abstract

In freshwater environments, cyanobacteria are responsible for the majority of taste-and-odour compounds that compromise water quality. Geosmin, a widespread bicyclic tertiary alcohol, is one of the most problematic of these compounds, imparting a persistent earthy–musty character to drinking water. Because the timely surveillance of geosmin producers is central to any early-warning system, molecular tools capable of rapidly detecting the responsible organisms offer a practical alternative to conventional taxonomic and instrumental approaches. In the present study, a cyanobacterial strain isolated from the Al-Shamiya River, a branch of the Euphrates in central Iraq, was characterised morphologically and then confirmed genetically using the 16S rRNA gene. The isolate shared 100% sequence identity with *Oscillatoria lutea* deposited in the National Center for Biotechnology Information (NCBI) GenBank database. Amplification and sequencing of the geosmin synthase gene revealed 100% identity with the putative geosmin synthase (geoL) gene of *Oscillatoria* sp. PCC 6506 and progressively lower identity with the homologous gene of other cyanobacteria, reflecting the conserved architecture of the geosmin-biosynthetic machinery. At the amino-acid level, BLAST analysis further demonstrated pronounced homology among taxonomically distant geosmin producers, including actinomycetes, cyanobacteria, fungi and myxobacteria. These findings confirm that genetic analysis is an accurate, inexpensive and rapid means of identifying cyanobacterial species and of predicting the occurrence of taste-and-odour compounds in water resources.

Keywords: *Cyanobacteria; Geosmin; Genetic Analysis; Iraq; Oscillatoria lutea; 16S rRNA.*

I. INTRODUCTION

Harmful algal blooms can develop at almost any time, and the excessive accumulation of algal biomass frequently impairs the use of water resources. Freshwater systems are, in particular, dominated by cyanobacteria, which constitute the principal group of harmful bloom-forming algae. During cyanobacterial blooms, recreational activities may be restricted by surface scums, while public water supplies can be compromised by the release of taste-and-odour compounds [1]. Cyanobacteria exert a range of detrimental effects on water quality: in addition to producing objectionable taste-and-odour compounds, several species synthesise potent toxins capable of harming humans and livestock [2]. Taste-and-odour compounds are of particular concern because they impart an unpleasant character to the water of rivers, lakes and reservoirs used for recreation or as drinking-water sources; the resulting musty or earthy flavours not only increase treatment costs but can also taint fish and other aquatic organisms, leading to losses in aquaculture and recreational revenue [3]. Among these compounds, 2-methylisoborneol (2-MIB) and geosmin are the two most commonly associated with cyanobacterial taste-and-odour episodes; although generally non-toxic, they markedly degrade the aesthetic quality of water sources and supplies [4].

Geosmin (trans-1,10-dimethyl-trans-9-decalol) is a secondary metabolite produced by cyanobacteria and actinobacteria in aquatic environments and is responsible for a characteristic musty–earthy odour. As a tertiary alcohol it occurs as (+)- and (−)-enantiomers, of which the naturally dominant (−)-form is responsible for odour episodes and is approximately ten times more potent than the (+)-enantiomer [5].

The Al-Shamiya River is one of the branches of the Euphrates River in Iraq and constitutes an important source of drinking water and irrigation. It takes its name from the Al-Shamiya district of the Al-Diwaniyah region, through which it flows, and enters the governorate from the north-west after branching from the Al-Hindiya River south of Kufa. Numerous drinking-water stations are distributed along its course, and its water irrigates approximately 38,400 hectares of agricultural land. The river is prone to cyanobacterial blooms, particularly when water levels fall during the summer, giving rise to taste-and-odour problems that render the water unpalatable. One of the earliest investigations of taste-and-odour compounds in this river isolated and identified a geosmin-producing cyanobacterium, *Phormidium retzii* [6].

The detection of taste-and-odour compounds by genetic analysis plays an important role in identifying the microorganisms that generate them, as demonstrated by several studies. Genetic analysis has been used as a complementary and essential approach alongside instrumental (chromatographic) assessment; in this way the gene associated with geosmin production was detected in *Phormidium retzii* isolated from the Al-Shamiya River [7]. A quantitative PCR (qPCR) assay was likewise shown to provide a rapid, high-throughput alternative to gas chromatography–mass spectrometry (GC/MS) for identifying geosmin-producing *Anabaena circinalis*, enabling water utilities to characterise a geosmin episode more quickly and to select appropriate management and treatment options [8]. qPCR techniques have been reported to shorten the time to result from days to hours when quantifying geosmin-producing *Anabaena* sp. in freshwater systems, while requiring considerably less taxonomic expertise than GC/MS-based approaches [9]. The geosmin synthase gene and its expression product have also been characterised from cyanobacteria, improving the understanding of the basic biological mechanism of geosmin biosynthesis [10].

Nucleotide sequences linked to the geosmin synthase gene have been examined in numerous studies. The growth conditions influencing geosmin synthase gene expression in *Anabaena circinalis* AWQC318 have been investigated in detail [11], and RT-PCR and taxon-specific PCR assays have been developed to provide early detection of potential geosmin releases and to identify the likely biological sources when examining the gene responsible for geosmin production in *Anabaena lemmermannii* [12].

The aim of the present study was to apply genetic analysis to the identification of the species and of the gene responsible for geosmin production in a cyanobacterium isolated from the Al-Shamiya River. As indicated by earlier work, genetic analysis is simpler and faster than traditional algal identification, taxonomic analysis and instrumental methods.

II. MATERIALS AND METHODS

➢ *Study Area and Sampling*

The cyanobacterial sample examined in this study was collected from the water of the Al-Shamiya River into a 50 mL polyethylene container containing BG11 medium prepared according to Rippka et al. [13].

➢ *Isolation and Purification*

A unialgal culture was obtained by the agar-streaking method described by Stein et al. [14] and was repeated once to ensure unialgal purity. An axenic culture was then generated by density-gradient centrifugation. A 15 mL thick-walled tube was inoculated with 10 mL of the unialgal culture and centrifuged at 3,000 rpm for 5 min; the supernatant was discarded, and the pellet was washed thoroughly with sterile distilled water and re-centrifuged. This washing–centrifugation cycle was repeated at least twelve times until an axenic culture was obtained [15]. The axenic culture was subsequently transferred onto nutrient agar and incubated at 37 °C for 72 h to verify that it was free of contamination [16]. Stock cultures were maintained in 250 mL sterile BG11 medium in glass flasks under an 8:16 h light:dark cycle at 25 °C and a photon flux density of 40 μmol photons m^{-2} s^{-1}.

➢ *Morphological Identification*

The isolate was first examined by light microscopy and identified on the basis of the taxonomic classification key of Guiry & Guiry [17].

➢ *DNA Extraction, PCR Amplification and Sequencing*

Genomic DNA was extracted from the axenic culture using [DNA extraction method/kit]. The 16S rRNA gene was amplified with the cyanobacteria-specific primers CYAL106F and CYA781R [18], and the geosmin synthase (geoA) gene with the primers geo78F and geo982R [19]; the primer sequences and expected product sizes are listed in Table 1. Each PCR was performed in a final volume of [reaction volume] μL containing [DNA polymerase/master mix, dNTPs, $MgCl_2$ and primer concentrations]. Amplification was carried out with an initial denaturation at [temperature] for [time], followed by [number] cycles of denaturation at [temperature] for [time], annealing at [temperature] for [time] and extension at [temperature] for [time], with a final extension at [temperature] for [time]. Amplification products were separated by electrophoresis on a [concentration]% (w/v) agarose gel alongside a 100 bp DNA ladder and visualised under ultraviolet illumination. Purified amplicons were sequenced using [sequencing platform/service provider].

Table 1 Primer Pairs Used for PCR Amplification and Sequencing of the 16S rRNA and geoA (Geosmin Synthase) Genes.

Target gene	Primer	Sequence (5′→3′)	Product size (bp)	Reference
16S rRNA	CYAL106F	CGGACGGGTGAGTAACGCGTGA	519	Nübel et al. [18]
	CYA781R	GACTACTGGGGTATCTAATCCCATT		
geoA	geo78F	GCATTCCAAAGCCTGGGCTTA	905	Suurnäkki et al. [19]
	geo982R	ATCGCATGTGCCACTCGTGAC		

➢ *Sequence and Phylogenetic Analysis*

The nucleotide sequences obtained were compared with homologous sequences in the NCBI database using the BLASTn algorithm, and the corresponding amino-acid sequences were analysed with BLASTP (version 2.2.31+) against the UniProtKB database. Multiple sequence alignment and phylogenetic reconstruction were carried out for both the 16S rRNA and geosmin synthase genes using the unweighted pair-group method with arithmetic mean (UPGMA) implemented in MEGA version 6.0, with [number] bootstrap replicates. The 16S rRNA and geosmin synthase sequences determined in this study were deposited in the NCBI GenBank database.

III. RESULTS AND DISCUSSION

The isolate was identified primarily by light microscopy using the classification key of Guiry & Guiry [17]. Morphological examination indicated that the strain under investigation was *Oscillatoria lutea* (Fig. 1), a member of the cyanobacteria (blue-green algae). When the isolate was compared with the descriptions provided in the same taxonomic key [17], minor morphological differences were noted; these are likely to reflect phenotypic plasticity, which is frequently displayed by laboratory-cultured isolates over extended periods, as it is not unusual for cultured strains to alter their features under controlled conditions relative to those observed in nature [20].

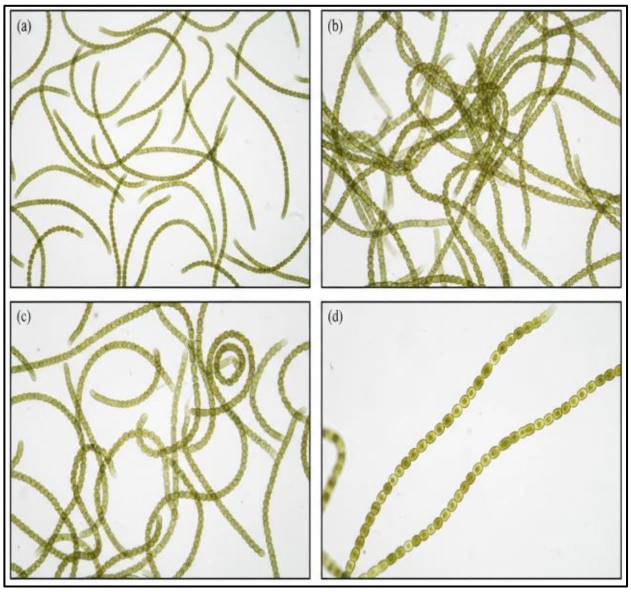

Fig 1 Filaments of *Oscillatoria lutea* Isolated from the Al-Shamiya River, Observed Under Light Microscopy (×40). Panels (a–d) Show Representative Fields of the Trichomes.

Molecular identification based on the 16S rRNA gene was then used to confirm the morphological classification. Using the specific primers listed in Table 1, amplification of the 16S rRNA gene yielded a single product of 519 bp (Fig. 2, lane 1), while amplification of the gene associated with geosmin production produced a single band of approximately 905 bp (Fig. 2, lane 2); a 100 bp DNA ladder was run in lane M.

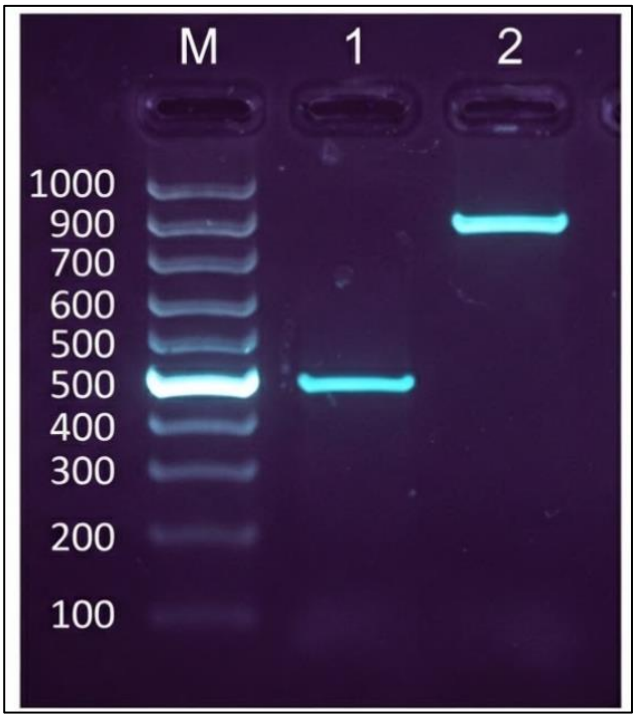

Fig 2 Agarose-Gel Electrophoresis of PCR Products for the Isolate *Oscillatoria lutea*. Lane 1, 16S rRNA Gene (519 bp); Lane 2, Geosmin Synthase Gene (905 bp); Lane M, 100 bp DNA Ladder.

The nucleotide sequences obtained were identified by comparison with homologous sequences using the NCBI BLAST search program, and multiple sequence alignment and phylogenetic analysis were performed for both the 16S rRNA and geosmin synthase genes using the UPGMA method in MEGA (version 6.0). Phylogenetic analysis showed that the 16S rRNA sequence of the isolate matched that of *Oscillatoria lutea* completely (Fig. 3), with 100% genetic similarity between the strain under investigation and the *O. lutea* sequence available in the NCBI database. This close agreement supported the morphology-based identification, and it confirms that the discrepancies occasionally observed between GenBank sequences and newly determined sequences do not necessarily indicate misidentification; on the contrary, the genetic analysis validated the morphological result, showing that the cyanobacterium belongs to *O. lutea*. In the study of bacterial evolution, the 16S rRNA gene is regarded as a molecular clock and is the most widely used genetic marker [21]. BLAST assessment revealed only limited variation between the 16S rRNA sequence of *O. lutea* and those of other cyanobacterial strains in GenBank. The 16S rRNA gene is useful for distinguishing individual species and broader taxonomic groups and is routinely employed for the detection of cyanobacteria [22]; direct analysis of this region has been essential in cyanobacterial phylogenetics and has contributed greatly to the molecular information now available in databases such as GenBank [23].

Fig 3 Phylogenetic Tree of the 16S rRNA Gene of the Cyanobacterium Under Investigation and other Cyanobacterial Species. The Tree was Constructed Using the UPGMA Algorithm; only Bootstrap Values Equal to 100% are Shown.

The results further demonstrated that the *O. lutea* strain possesses the geosmin synthase gene, indicating that it is capable of producing the odorous compound. Cyanobacteria synthesise a variety of harmful substances, and both toxins and off-flavour compounds are considered to pose environmental and economic risks in freshwater systems [24]; some of the principal producers of off-flavour compounds, such as *Oscillatoria*, are widely distributed and are known to cause water-quality problems. In the present study, the potential of the isolate to produce undesirable secondary metabolites was assessed using molecular techniques, which provide cost-effective tools for detecting cyanobacteria that generate taste-and-odour or toxic compounds and can be used to provide early warning of nuisance blooms and to support research on the factors governing bloom development and the production of these compounds [25]. Geosmin belongs to the terpenoid family and is formed through terpene biosynthesis [26]; the genes involved in its biosynthesis have only recently been identified in cyanobacteria, and geosmin is produced through the cyclisation of farnesyl diphosphate by a synthase encoded by the geoA gene [27]. When the geosmin synthase sequence of the isolate was compared with nucleotide sequences in the NCBI database using the online BLAST program, the phylogenetic tree (Fig. 4) revealed high similarity between the geosmin synthase gene of *O. lutea* and the homologous gene of other cyanobacterial strains deposited in GenBank, in agreement with previous reports of sequence homology among cyanobacterial geosmin synthase genes [28, 29]. Phylogenetic analysis indicated that the geosmin synthase gene of *O. lutea* was most similar to the putative geosmin synthase (geoL) gene of *Oscillatoria* sp. PCC 6506, with an identity of 100%, and less similar to the corresponding gene of other cyanobacterial species, reflecting the conservation of the gene structure responsible for geosmin production among cyanobacteria. It has been reported that the amino-acid sequence of geosmin synthase resembles that of certain cyanobacterial sesquiterpene synthases, and that geoA may in fact represent a sesquiterpene synthase gene [30]; a single gene encoding the geosmin synthase enzyme, including the N-terminal domain common to all geosmin synthases, has been demonstrated in cyanobacteria [27]. Cloning of geoA PCR products has revealed a strong resemblance among cyanobacterial geoA sequences [31], and homologous alignment has shown that geoA is highly conserved among the cyanobacterial species examined [32].

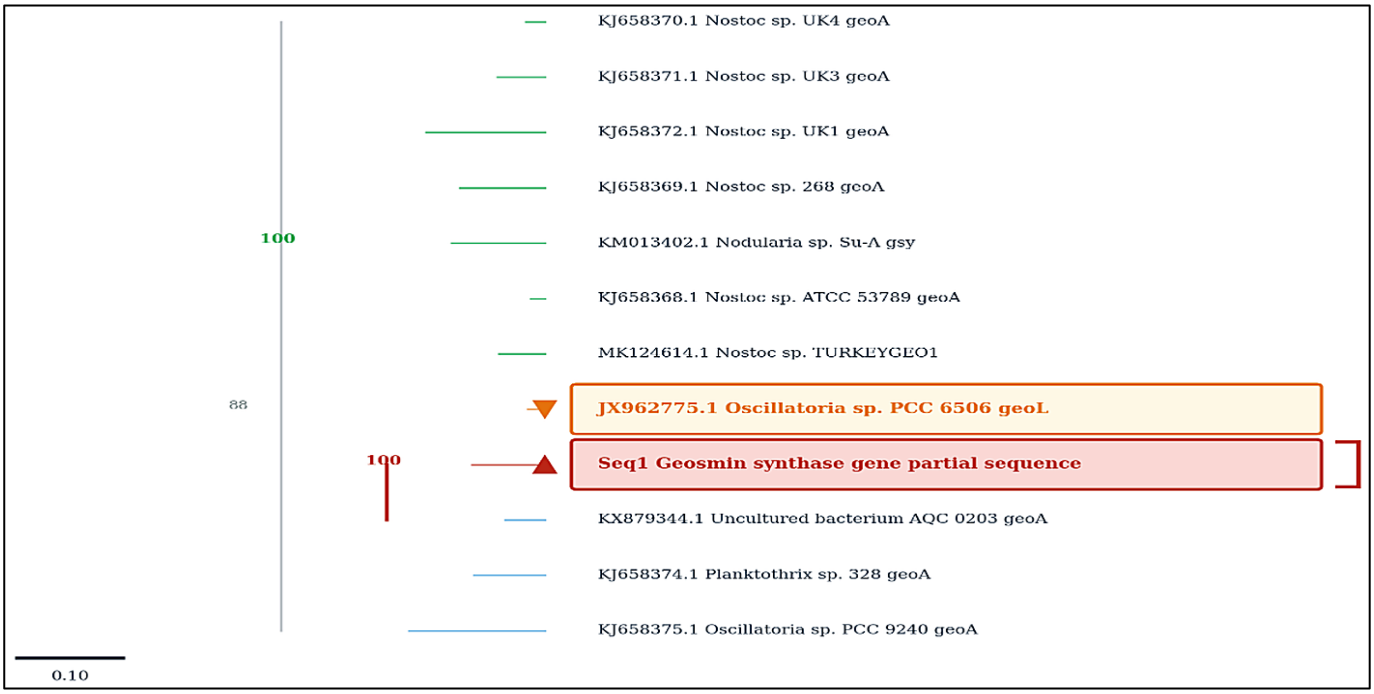

Fig 4 Phylogenetic Tree of the Geosmin Synthase Gene of the Cyanobacterium Under Investigation and other Cyanobacterial Species. The Tree was Constructed Using the UPGMA Algorithm; Only Bootstrap Values Equal to 100% are Shown.

The 16S rRNA and geosmin synthase sequences generated in this study were deposited in the NCBI GenBank database under the accession numbers MK771141.1 (16S rRNA gene) and MK759879.1 (geosmin synthase gene). BLAST analysis of the amino-acid sequence of the *O. lutea* geosmin synthase, performed with BLASTP (version 2.2.31+) against the UniProtKB database, revealed a high degree of similarity among the cyanobacterial species that produce geosmin and other bacteria carrying this gene. The alignment of these amino-acid sequences, together with the corresponding protein-based phylogenetic tree, indicated a substantial similarity between the taxa harbouring the gene responsible for geosmin production (Figs. 5 and 6). In particular, the amino-acid sequences of the *O. lutea* geosmin synthase and those of other actinomycetes, myxobacteria and cyanobacteria were closely aligned, pointing to similarities in the biochemical processes of geosmin biosynthesis and in the genetic regulation of the synthesis genes among these microorganisms. Consistent with this, the geosmin synthase genes of actinomycetes and cyanobacteria have been found to share a high degree of homology [33, 34], and codon-usage analysis together with geoA-based phylogenetic reconstruction has revealed a monophyletic branch with a common ancestor and origin for actinomycetes, myxobacteria and cyanobacteria [32].

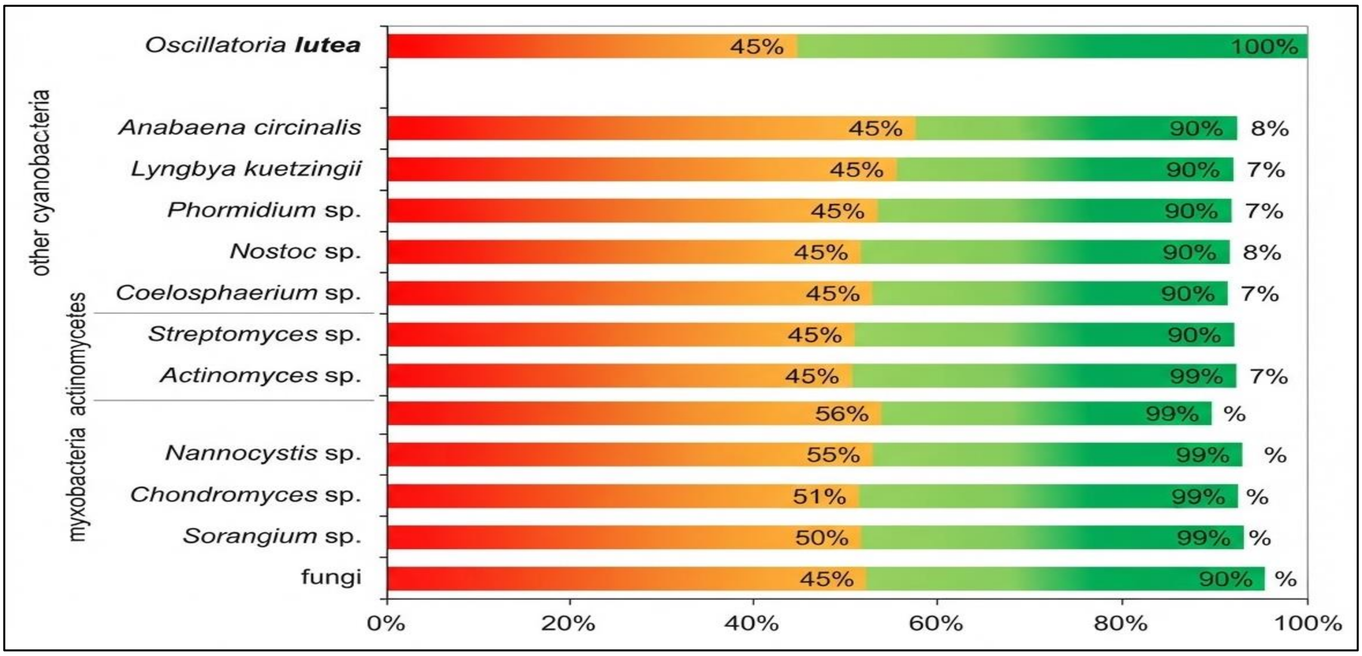

Fig 5 Graphical Representation of the Amino-Acid Sequence Identity between the Geosmin Synthase of *Oscillatoria lutea* and Representatives of other Geosmin-Producing Microbial Groups, Obtained with the BLASTP Algorithm.

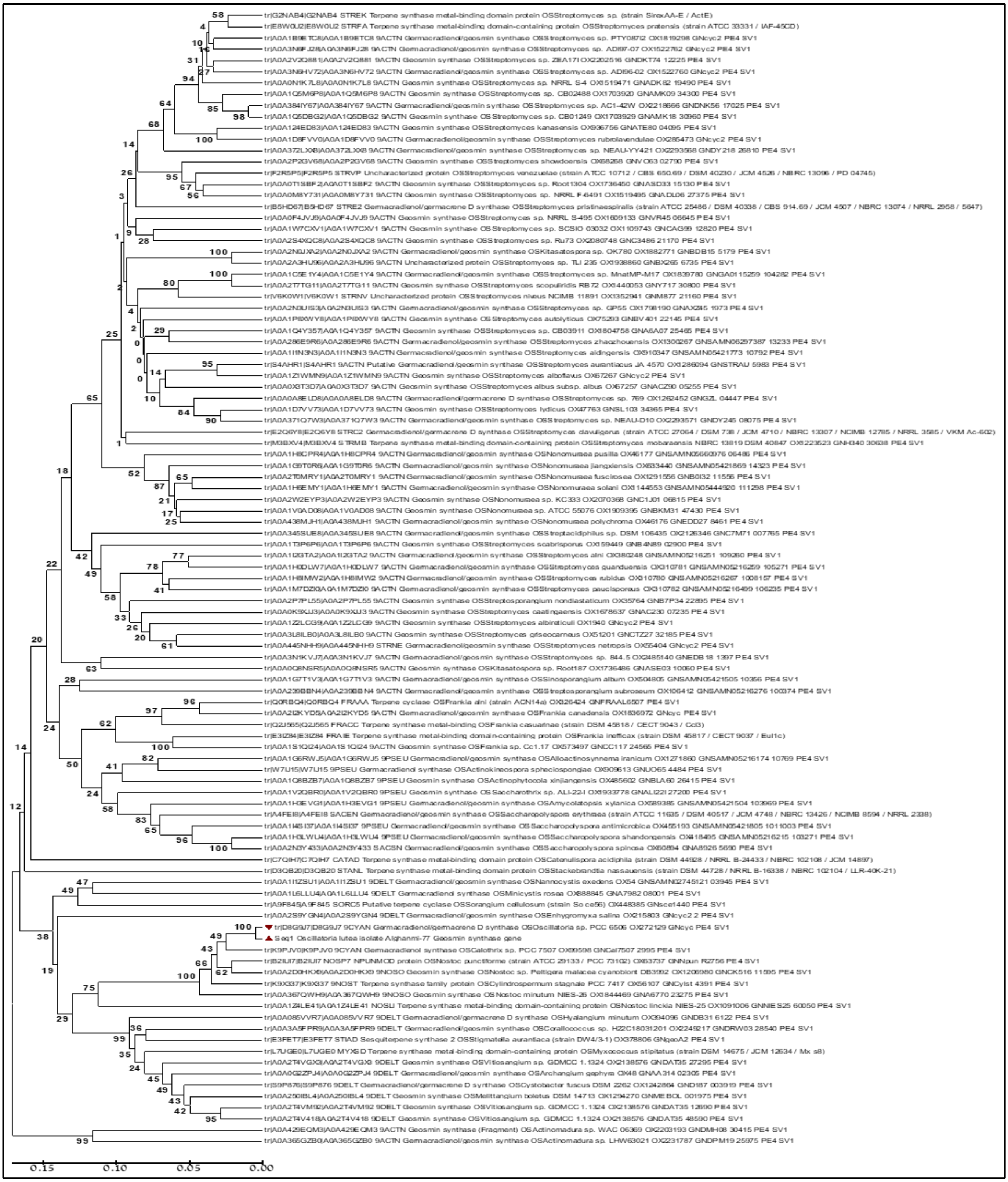

Fig 6 Protein-Based Phylogenetic Tree Showing the Evolutionary Relationships Between the Examined Taxa and *Oscillatoria lutea* with Respect to the Geosmin Synthase Gene. The Tree was Constructed using the UPGMA Method; Only Bootstrap Values Equal to 100% are Shown.

Overall, molecular approaches provide a valuable means of tracking potential cyanobacterial geosmin producers and of predicting the distribution of taste-and-odour compounds in water quickly and inexpensively, without recourse to costly conventional methods. When combined with physical, physicochemical and chemical measurements, quantification of the abundance of geosmin synthase genes in a water body may provide important information for water-storage prediction models and can be used to anticipate the frequency of taste-and-odour incidents before they become operational problems [27]. PCR-based techniques allow geosmin producers to be monitored rapidly and represent an excellent alternative to previously established standard methods [35]. A molecular method has also been developed to track geosmin-producing strains in aquatic ecosystems in order to mitigate the earthy odour caused by *Coelosphaerium* sp. in brackish Lake Shinji, Japan [36]. Taken together, these findings indicate that genetic analysis is a useful, cost-effective and rapid tool for controlling blooms and for detecting the cyanobacteria responsible for producing taste-and-odour or toxic compounds.

IV. CONCLUSION

The present study, conducted on the Al-Shamiya River in central Iraq, clearly demonstrated that genetic analysis is an essential complement to morphological examination for the reliable identification of cyanobacteria and their taste-and-odour potential. Light-microscopic examination identified the isolate as Oscillatoria lutea, and this assignment was confirmed by the 16S rRNA gene, which showed 100% sequence identity with O. lutea in the NCBI GenBank database. Amplification and sequencing of the geosmin synthase gene further established that the strain carries the biosynthetic machinery for geosmin production, showing 100% identity with the putative geosmin synthase (geoL) gene of Oscillatoria sp. PCC 6506. At the amino-acid level, BLAST analysis revealed pronounced homology among taxonomically diverse geosmin producers, including actinomycetes, cyanobacteria, fungi and myxobacteria, pointing to a conserved biosynthetic pathway across these groups. Collectively, these findings confirm that genetic analysis offers a simple, inexpensive and rapid means of detecting geosmin-producing cyanobacteria and of predicting the occurrence of taste-and-odour episodes in water resources, and they support the wider adoption of molecular tools within early-warning and water-quality monitoring programmes.

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