




SHORT COMMUNICATION

First Molecular Detection of *Mycobacterium ulcerans pseudoshottsii* From Gulf Strain Striped Bass (*Morone saxatilis*)

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ABSTRACT

Mycobacterium ulcerans pseudoshottsii is a mycolactone-producing bacterium previously isolated from Striped Bass (*Morone saxatilis* (Walbaum)) from Chesapeake Bay and adjacent waters of the Atlantic Coast of North America. We report the first molecular detection of this pathogen in the native Gulf strain of *Morone saxatilis* collected from the Pearl River, Mississippi (USA). Molecular identification was conducted using a novel PCR assay targeting the parA-625 intergenic spacer of the virulence-associated pMUM plasmid. The isolate was unambiguously assigned to *M. u. pseudoshottsii* based on diagnostic single nucleotide polymorphisms (SNPs) and phylogenetic analysis. This report expands the known range of *M. u. pseudoshottsii* to include Gulf Coast watersheds and highlights the need for enhanced surveillance in wild and aquacultured fish populations of the southern United States.

1 | Introduction

Mycobacterium ulcerans (MU) is a Mycolactone Producing Mycobacteria (MPM) that causes skin lesions and, less frequently, systemic infection. The species currently contains one subspecies (*M. u. shinsuense*), one “ecovar” (MU ecovar Liflandii), and multiple uncharacterized lineages differentiated by host range and mycolactone structural variants, “A through F” (Doig et al. 2012; Hammoudi et al. 2020; Röltgen et al. 2012). Human disease is predominantly associated with

the MPMs that produce mycolactones A and B, but several MPMs are known to infect non-human hosts (Pidot et al. 2010; Stinear et al. 2007; Williamson et al. 2014). Among these is a mycolactone F-producing species that has been associated with mycobacteriosis outbreaks in aquacultured and wild fish species (Komine et al. 2022; Mugetti et al. 2020; Nakanaga et al. 2012; Stathopoulou et al. 2020; Stine et al. 2009). It was first isolated from diseased Striped Bass (*Morone saxatilis* (Walbaum)) in the Chesapeake Bay, USA (Rhodes et al. 2005). Since then, multiple studies have recognised that genetic similarity between *M.*

ulcerans and *M. pseudoshottsii* is below accepted thresholds for bacterial species differentiation (Doig et al. 2012; Pidot et al. 2010; Tortoli 2012). Genomic comparisons have revealed high average nucleotide identity (ANI > 98.5%), shared pathogenicity islands, and congruent ecological associations with *M. ulcerans* sensu stricto (Doig et al. 2012; Käser et al. 2009; Yip et al. 2007). In light of these similarities, we propose referring to this MPM as *Mycobacterium ulcerans pseudoshottsii*, recognising it as a subspecies-level taxon within the *M. ulcerans*–*M. marinum* complex. Thus, we follow the taxonomic recommendations of Doig et al. (2012), but recognise monophyletic clades as subspecies. To our knowledge, this is the first study to adopt this trinomial nomenclature, which reflects both phylogenetic continuity and host-specific ecological divergence.

The ecology and geographic distribution of MPMs remain incompletely understood (Chevillon et al. 2024; Guégan et al. 2025; Hennigan et al. 2013). Most surveillance efforts have focused on regions with known human disease prevalence, often overlooking the pathogen carriage by a wide range of ectothermic and aquatic species that may serve as indicators of environmental presence (Receveur et al. 2022). A recent study has highlighted the potential for multiple MPMs to occur in sympatry in southern USA rivers, suggesting a broader and more complex biogeography than initially appreciated (Dogbe et al. 2025). In this context, the development of molecular tools capable of resolving strain-level variation among MPMs is critical for ecological, epidemiological, and diagnostic studies.

Here, we report the first detection of *M. u. pseudoshottsii* from Gulf Strain Striped Bass (*Morone saxatilis*) collected from the Pearl River in Mississippi, extending the known range of this fish-associated MPM in the Gulf of Mexico. Molecular identification was performed using a novel PCR/Sanger sequencing assay targeting a single-copy intergenic spacer of the pMUM

virulence plasmid, allowing for phylogenetic assignment. This finding provides new insight into the geographic and host range of *M. u. pseudoshottsii*, with implications for fish health and aquatic biosecurity in Gulf Coast ecosystems.

2 | Methods

2.1 | Sample Acquisition and Preparation

Two deceased adult *Morone saxatilis* with necrotic dermal lesions were submitted by the Mississippi Department of Wildlife, Fisheries, and Parks (MDWFP) from the Pearl River, Mississippi (Figure 1). Mucosal scrapings from affected areas were collected using sterile technique and preserved in 100% EtOH at -20°C . Cultured isolates of MPMs were obtained from curated collections (Table 1). DNA was extracted from mucosal samples and bacterial cultures using a standard phenol-chloroform precipitation (Supporting Information). Attempts to type isolates by performing variable number tandem repeat (VNTR) PCR as previously described (Williamson et al. 2008) were inconclusive (Figure S1); this prompted the design of a new nested PCR assay.

2.2 | PCR Assay Design

We compared the new partial plasmid sequence to publicly available orthologous sequences from NCBI Nucleotide to identify regions of high single-nucleotide variability. After aligning the contigs to 10 orthologous plasmid genomes using MAFFT v7 (Katoh et al. 2002; Katoh and Standley 2013), we identified a variable non-coding region of 802–804 bp between the 5' ends of the gene encoding the Plasmid Partition A protein (parA, CP110684:60171–60713) and a gene encoding an uncharacterized protein with locus tag OIO89_00625 (CP110684:59122–59367).

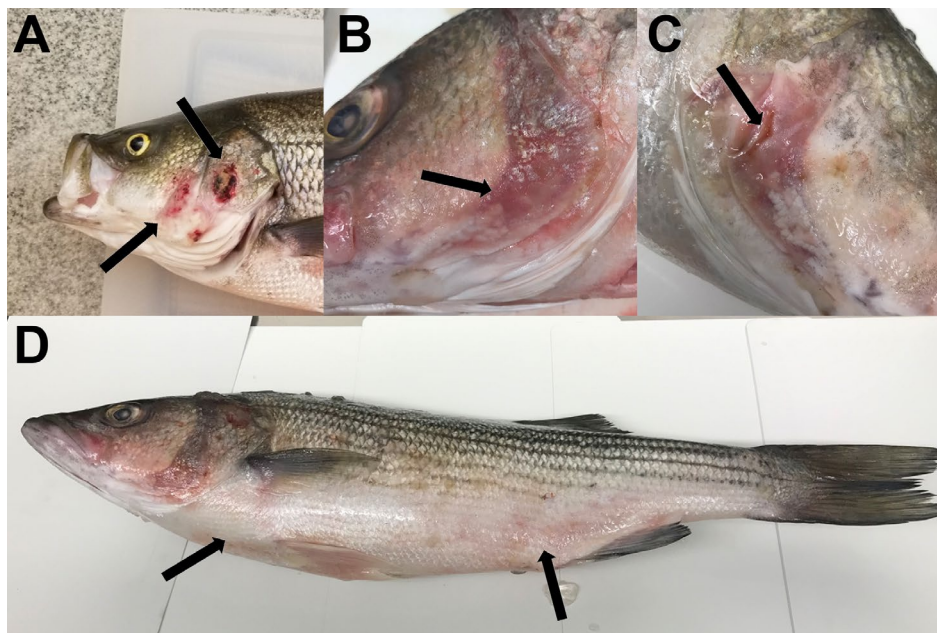


FIGURE 1 | Two deceased Gulf Strain Striped Bass (*Morone saxatilis*) specimens with necrotic dermal lesions submitted by Mississippi Department of Wildlife, Fisheries, and Parks (MDWFP) from Pearl River, Mississippi, USA. (A–C) Erythematous lesions of the operculum. (D) Disseminated ventral erythema and scale loss.

TABLE 1 | *Mycobacterium ulcerans* (MU) lineages and outgroups used to optimise the parA-625 PCR assay for MU detection and phylogenetic analysis.

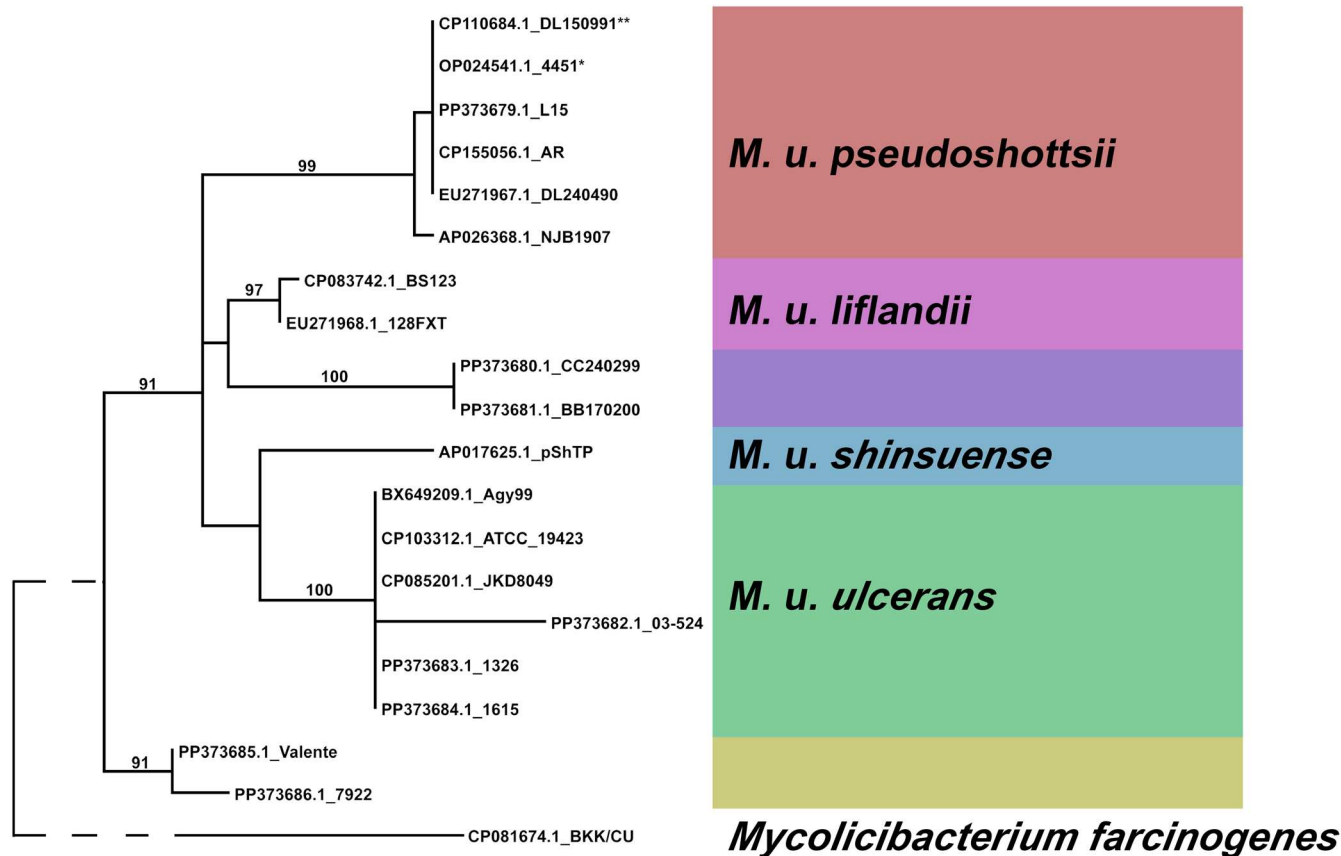
GenBank accession	Start	Stop	Sample ID	Taxon	Source	Country	References
AP017625.1	2999	4999	ATCC33728	<i>M. u. ulcerans</i>	Human	Japan	(Nakanaga et al. 2007)
AP026368.1	47,127	49,128	NJB1907-Z4	<i>M. u. pseudoshottsii</i>	Fish	Japan	(Komine et al. 2022)
BX649209.1	3000	5000	Agy99	<i>M. u. ulcerans</i>	Human	Ghana	(Stinear et al. 2004, 2007)
CP081674.1	78,019	78,950	BKK/CU	<i>Mycolicibacterium farcinogenes</i>	Human	Thailand	(Sodsai et al. 2021)
CP083742.1	48,426	50,426	BS123	<i>M. u. liflandii</i>	Fish	China	(Luo et al. 2022)
CP085201.1	156,799	158,799	JKD8049	<i>M. u. ulcerans</i>	Human	Australia	(Omansen et al. 2015)
CP103312.1	41,285	43,285	ATCC 19423	<i>M. u. ulcerans</i>	Human	Japan	(Igarashi et al. 2023)
CP110684.1	59,090	61,070	DL150991	<i>M. u. pseudoshottsii</i>	Fish	Israel	(Ucko and Colorni 2005)
CP155056.1	62,032	64,033	AR	<i>M. u. pseudoshottsii</i>	Fish	Greece	(Stathopoulou et al. 2020)
EU271967.1	56,249	58,250	DL240490	<i>M. u. pseudoshottsii</i>	Fish	Israel	(Ucko and Colorni 2005)
EU271968.1	2992	4992	128FXT	<i>M. u. liflandii</i>	Frog	USA	(Pidot et al. 2008)
OP024541.1	1	521	4451	<i>M. u. pseudoshottsii</i>	Fish	USA	This study
PP373677.1	1	1065	Agy99	<i>M. u. ulcerans</i>	Human	Ghana	(Stinear et al. 2007)
PP373678.1	1	1066	DL150991	<i>M. u. pseudoshottsii</i>	Fish	Israel	(Ucko and Colorni 2005)
PP373679.1	1	1365	L15	<i>M. u. pseudoshottsii</i>	Fish	USA	(Rhodes et al. 2005)
PP373680.1	1	683	CC240299	Other	Fish	Israel	(Ucko and Colorni 2005)
PP373681.1	1	683	BB170200	Other	Fish	Israel	(Ucko and Colorni 2005)
PP373682.1	1	683	03-524	<i>M. u. ulcerans</i>	Human	Papua New Guinea	(Stragier et al. 2005)
PP373683.1	1	683	1326	<i>M. u. ulcerans</i>	Human	Australia	(Mve-Obiang et al. 2003)
PP373684.1	1	683	1615	<i>M. u. ulcerans</i>	Human	Malaysia	(George et al. 1999)
PP373685.1	1	683	Valente	Other	Human	South America	Unpublished
PP373686.1	1	683	7922	Other	Human	French Guiana	(Stragier et al. 2005)
PP373687.1	1	521	128FXT	<i>M. u. liflandii</i>	Frog	USA	(Pidot et al. 2008)

This intergenic region, hereafter “parA-625,” contains at least 45 single nucleotide polymorphisms (SNPs) and a single-nucleotide indel. We developed an initial PCR protocol that amplifies a 682–684bp region from 34bp upstream of the parA start codon to 87bp upstream of the OIO89_00625 start codon. A nested primer pair (Table 2) targets a secondary 521bp amplicon beginning 133bp upstream of parA and terminating 149bp upstream of OIO89_00625. The 521bp amplicon excludes the indel, but it includes 39 of the 45 SNPs identified within the intergenic region. The initial amplicon is compatible with Sanger sequencing of isolates from pure cultures, as well as third-generation

sequencing platforms. In contrast, the nested PCR protocol is designed for environmental samples of unknown composition, offering increased sensitivity and improved tolerance to PCR inhibitors commonly present in such matrices (Englund et al. 1999; Gauthier et al. 2008). Additionally, the nested amplicon excludes the indel present in the full-length target, enabling the generation of interpretable Sanger sequence data from potentially heterogeneous environmental samples containing multiple MPMs. To assess potential cross-amplification from paralogs and off-target loci, we ran PrimerBlast against the alignment of nine pMUM sequences (Table 1) and against the NCBI Nucleotide database.

TABLE 2 | Primer set used in nested PCR amplification of the plasmid parA-625 region for detection of all Mycolactone Producing Mycobacteria (MPM).

	Primer Name	Sequence (5' → 3')
External Primers	pMUM3911F	GTGTTGGAATCGGGAGAATG
	pMUM4639R	GAGCACGCATAAGTGTTTGG
Internal Primers	pMUM4015F	TCGGTGTTCATATCCCTACTCG
	pMUM4576R	TTATGGCAGCTACCGCTATG

**FIGURE 2** | Maximum likelihood phylogenetic tree of the *Mycobacterium ulcerans* parA gene and intergenic spacer sequence, reconstructed using IQ-TREE v2.3.2 under the HKY + F substitution model and 10,000 bootstrap replicates. Numbers on nodes are bootstrap support values for each known subspecies. All other node values are presented in the tree file in [Supporting Information](#). The sequence obtained from the Gulf Strain Striped Bass (*Morone saxatilis*) from the Pearl River, Mississippi, USA is indicated with an asterisk. The sequence obtained from long-read genome assembly is indicated by two asterisks. The purple and tan rectangles represent uncharacterized MPM lineages. The tree is rooted with the parA gene sequence of *Mycolicibacterium farcinogenes* isolated from a human facial abscess in Thailand (Sodsai et al. 2021).

2.3 | PCR Typing

The initial PCR was performed in a 25 μ L reaction: 1X Ex Taq Buffer, 200 μ M each dNTP, 0.2 μ M external primers (Table 2), 0.45 mg/mL BSA, 1 U Taq polymerase, and Ultrapure water to volume. PCR experiments were performed on a GeneAmp 9700 thermal cycler, including a no template control. After initial DNA denaturation at 95°C for 2 min, the PCR was performed for 35 cycles of 30 s at 94°C, 45 s at 56°C, and 1 min at 72°C. A final incubation was performed at 72°C for 10 min. The nested PCR was performed as described above using 0.5 μ L of template PCR and internal primers. PCR products were visualised using

gel electrophoresis with a 1.5% agarose gel stained with ethidium bromide.

2.4 | Phylogenetic Analysis

PCR amplicons were sequenced by a commercial service provider (Eurofins) in forward and reverse directions using the internal PCR primer set. Chromatographic sequence data were imported and aligned in Geneious Prime (2022.2) and consensus sequences checked manually in BioEdit v7.2.5 (Hall 1999; Hall and Alzohairy 2011). Sequences were aligned with public

TABLE 3 | Diagnostic variants within the pMUM plasmid (reference NC_005916.1:3000–5000) that distinguish *Mycobacterium ulcerans pseudoshottsii* from all other subspecies. The initial PCR includes (“+”) nine SNPs and one indel, and the nested PCR excludes (“–”) the indel to facilitate Sanger sequencing.

Position	Reference	Variant	Region	Initial PCR	Nested PCR
3284	C	A	ParA	–	–
4005	G	GC	Spacer	+	–
4155	C	A	Spacer	+	+
4226	G	A	Spacer	+	+
4330	G	A	Spacer	+	+
4378	C	A	Spacer	+	+
4391	T	A	Spacer	+	+
4392	G	A	Spacer	+	+
4439	C	T	Spacer	+	+
4519	C	T	Spacer	+	+
4546	G	A	Spacer	+	+
4761	A	G	Spacer	–	–
4953	G	A	Spacer	–	–

data (Table 1) using MAFFT v7 and trimmed to the 521-bp target sequence. Phylogenetic relationships were inferred using a Maximum-likelihood HKY+F substitution model with IQ-TREE v2.3.2 (Minh et al. 2020) through the CIPRES Science Gateway (Miller et al. 2010) and run with 10,000 ultrafast bootstraps (Hoang et al. 2018). The tree was annotated in Dendroscope v3.5.7 (Huson and Scornavacca 2012).

3 | Results

PrimerBlast searches identified no paralogous or orthologous loci with potential for cross-amplification in silico. Primary and nested PCR of cultured MU isolates yielded single bands, which were sequenced and accessioned to NCBI nucleotide (Table 1). All amplicon chromatogram were homogeneous (i.e., no nucleotide ambiguity) and the resulting alignment included 39 SNPs.

Three amplicon sequences from cultured bacteria (i.e., Agy99, 128FXT, and DL150991) were identical to published whole plasmid sequences and were excluded from phylogenetic analysis for clarity. Phylogenetic analysis (Figure 2) placed the isolate from *M. saxatilis* within the *M. u. pseudoshottsii* clade with bootstrap support of 99%. SNP analysis confirmed the presence of nine SNPs and a single-nucleotide indel as diagnostic for *M. u. pseudoshottsii* (Table 3). This is the first report of *M. u. pseudoshottsii* in Gulf Coast watersheds and in the Gulf strain of *Morone saxatilis*.

4 | Discussion

This work builds upon earlier findings from the Chesapeake Bay, extending the range of *M. u. pseudoshottsii* into the Gulf of Mexico. The discovery raises questions about transmission routes and pathways and its potential environmental persistence

in southern USA freshwater systems and suggests potential fish health implications for Gulf fisheries and aquaculture, warranting routine screening.

This study demonstrates the utility of a plasmid-targeted PCR/Sanger assay for simultaneous detection and differentiation of MPMs from environmental and clinical sources. An intergenic sequence was selected for SNP typing because this region is less conserved than transcribed sequences, allowing accumulation of polymorphisms over time (Cicala et al. 2018). Intergenic spacers have been used to infer phylogenetic relationships of pathogenic strains whose functional genes are nearly identical due to evolutionary constraints, and have been successfully used in phylogenetic studies of monomorphic bacteria (Drancourt et al. 2004; Gürtler and Stanisich 1996). The pMUM-625 spacer exhibits variability at >8% of nucleotides, which exceeds sequence variation of chromosomal loci by an order of magnitude. The ability of parA-625 to differentiate MPMs makes this marker more specific than chromosome-targeted PCR/Sanger assays.

5 | Conclusion

The parA-625 assay enabled identification of *M. u. pseudoshottsii* DNA from Gulf Strain Striped Bass. This finding extends the known geographic distribution and corroborates a host association of this subspecies. Surveillance and management strategies should include plasmid-targeted assays to monitor MPMs in ectothermic hosts of economic and ecological value.

Author Contributions

DNA extraction was performed by A.W.R.; PCR and analyses were performed by A.W.R. and K.M.F. The paper was written by A.W.R. with

contributions from all authors. M.W.S. served as the principal investigator for the project. Finalised approval for this manuscript version by all authors has been given, with full agreement to be held accountable for the contents of this manuscript in all aspects.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data supporting the findings of this study are available on the open-source platform Genbank of NCBI, and can be found at (<https://www.ncbi.nlm.nih.gov/>) (CP110681–CP110687, OP024541, and PP373677–PP373687). The BioProject number is PRJNA892090; the SRA number is SRX17966086; and the BioSample number is SAMN31367647.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **FIGURE S1:** Gel electrophoresis results of variable number tandem repeat (VNTR) PCR of four loci (locus 6, locus 19, MIRU1, and ST1) for Gulf Strain Striped Bass (*Morone saxatilis*) from the Pearl River, Mississippi, USA. Well on the right includes a 100 bp ladder. **TABLE S1:** List of seven plasmid contigs resulting from Oxford Nanopore de novo sequencing of *Mycobacterium ulcerans* ecovar Pseudoshottsii strain DL150991.