MAESTRÍA EN
CIENCIAS AMBIENTALES

PROGRESS AND RESULTS REPORT

MVZ DIANA RUBI RIOS HUERTA

Molecular Detection of Fusarium in lepidochelys olivacea eggs shells

OBJECTIVE

To detect and molecularly characterize the presence of *Fusarium* spp., with emphasis on the *Fusarium solani* species complex, in eggshells from unhatched hatchlings of *Lepidochelys olivacea* using DNA extraction, mycological culture, and PCR techniques, in order to contribute to the understanding of the role of fungal infections in hatching success.

Specific objectives

• To extract DNA from eggshells and fungal cultures using both commercial kits and modified in-house methods optimized for filamentous fungi.

• To standardize the PCR technique using primers targeting the **TEF-1α gene** for the detection of *Fusarium solani*.

• To evaluate the quality and concentration of DNA obtained from different extraction methods: a commercial Zymo kit, a modified in-house protocol using clinical samples, and a modified in-house protocol using mycological cultures.

• To confirm the presence of fungal structures through mycological culture and direct diagnosis using KOH preparation.

• To generate preliminary evidence to identify potential *Fusarium* species associated with non-viable eggshells.

COLLECTION SITE AND PERMITS

This research is part of the following research line:

Ecology, Health, and Environmental Contamination in the Olive Ridley Sea Turtle (LEPIDOCHELYS OLIVACEA) in the Mexican Pacific, conducted under a scientific collection permit issued by **SEMARNAT**.

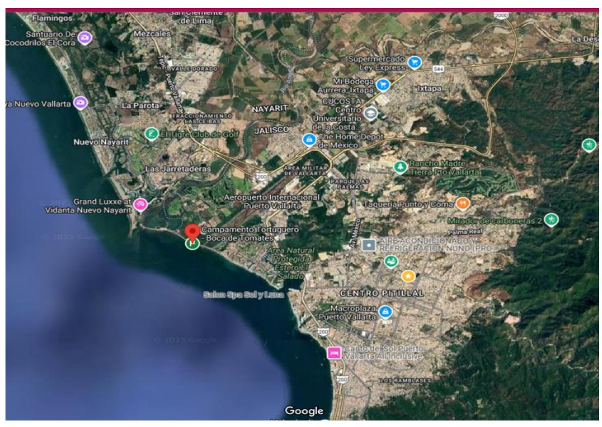
PERMIT INFORMATION

• **Permit number / official reference:** SBRA/DGVS/01065/25
• **Institutions:** Universidad Autónoma de San Luis Potosí & Nakawe A.C.
• **Permit validity:** July – September 2025
• **Legal responsible:** Dr. Milagros Gonzalez Hernandez
• **Collection site:** Boca de Tomates Sea Turtle Conservation Camp, Puerto Vallarta, Jalisco, Mexico
• **Coordinates:** 20°40′06.9″ N, 105°16′17.1″ W

This research is also part of the project **“Protecting Sea Turtle Hatchlings: Evaluating Nest Health Success and Threats in Puerto Vallarta, Mexico”**, funded by the **Rufford Foundation (UK)**. The main objective of this project is to identify factors that may affect hatchling viability and/or hatching success.

These factors include:

•**Physical factors:** Technical nest management and temperature conditions.
•**Chemical factors:** Environmental contaminants that bioaccumulate in the organism.
•**Biological factors:** Infectious agents such as FUSARIUM spp., with potential zoonotic risk, as well as congenital malformations.



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Protecting Sea Turtle Hatchlings: Evaluating Nest Health Success and Threats in Puerto Vallarta, Mexico

17 JUL 2025 Boca de Tomates Sea Turtle Camp, Puerto Vallarta, Jalisco, México, Central and Latin America Communities | Marine | Turtles

Diana Rubi Rios Huerta

This project will evaluate nest health and hatching success in Lepidochelys olivacea in Puerto Vallarta, Mexico, by integrating environmental and biological indicators. We will monitor 20 nests, record emergence success and assess congenital malformations, fungal infections, and heavy metal contamination (lead, mercury, cadmium, arsenic) in stillborn malformed hatchlings.

The project will also evaluate nest excavation, moisture accumulation and fungal growth. Results will inform improved nest management, detection of fungal threats, and pollutant monitoring. Educational outreach with local communities and volunteers will promote conservation awareness and science-based decision-making for sea turtle protection.

PROJECT UPDATES

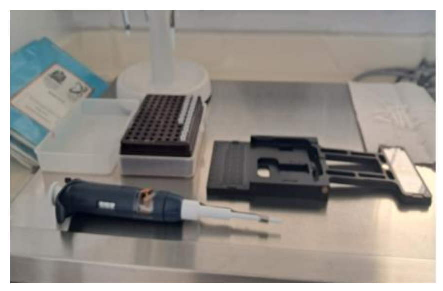
MOLECULAR ANALYSIS PROGRESS

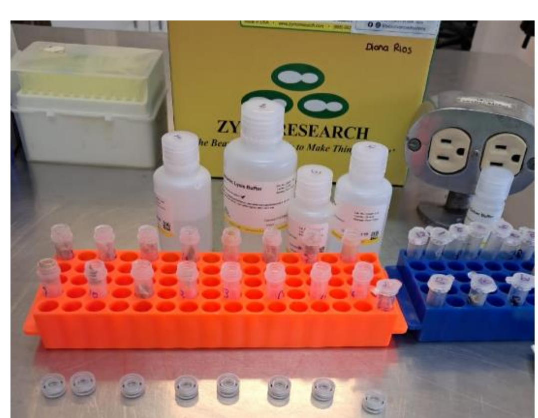
Thirty eggshell samples of the olive ridley sea turtle (*Lepidochelys olivacea*) were obtained from unhatched (non-viable) hatchlings originating from 10 different nests during the 2025 summer nesting season at the Boca de Tomates Sea Turtle Camp, Puerto Vallarta, Jalisco, Mexico.

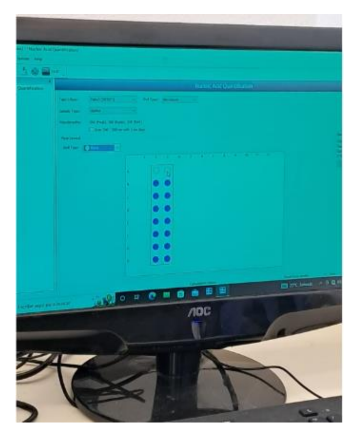
The eggshells exhibited pink discoloration with a multifocal pattern, and some contained embryos at different developmental stages. These macroscopic findings were consistent with lesions suggestive of infection by Fusarium species belonging to the Fusarium solani species complex.

For the initial DNA extraction, the Zymo DNA Fecal/Soil Kit was used, processing fragments of approximately 1.5 × 1.5 cm from each eggshell. Subsequently, DNA quantification (ng/µL) was performed using a Gene5 plate reader, yielding variable DNA concentrations in the 26 samples processed with this method.

DNA CONCENTRATION RESULTS USING THE GENE5 PLATE READER WITH THE ZYMO SOIL/FECAL DNA EXTRACTION KIT







	2	3	
A			260
			280
			260/280
			ng/µL
B	0.01	0.017	260
	0.003	0.009	280
	3.065	1.963	260/280
	9.879	**16.778**	ng/µL
C	0.013	0.014	260
	0.008	0.007	280
	1.685	1.845	260/280
	12.947	**13.789**	ng/µL
D	0.012	0.012	260
	0.008	0.007	280
	1.616	1.681	260/280
	12.403	**12.231**	ng/µL
E	0.009	0.014	260
	0.006	0.008	280
	1.66	1.743	260/280
	9.298	**13.642**	ng/µL
F	0.017	0.011	260
	0.011	0.005	280
	1.553	2.061	260/280
	16.885	**10.756**	ng/µL
G	0.007	0.008	260
	0.008	0.003	280
	0.897	2.448	260/280
	7.328	**7.504**	ng/µL
H	0.004	0.009	260
	0.002	0.006	280
	1.636	1.483	260/280
	3.714	**8.99**	ng/µL

	2	3	
A			260
			280
			260/280
			ng/µL
B			260
			280
			260/280
			ng/µL
C	0.009	0.015	260
	0.006	0.006	280
	1.5	2.339	260/280
	8.842	**14.526**	ng/µL
D	0.005	0.013	260
	0.007	0.005	280
	0.708	2.711	260/280
	4.835	**12.864**	ng/µL
E	0.006	0.01	260
	0.003	0.006	280
	2.143	1.75	260/280
	6.34	**10.364**	ng/µL
F	0.007	0.009	260
	0.005	0.004	280
	1.455	2	260/280
	6.754	**8.733**	ng/µL
G	0.006	0.011	260
	0.003	0.005	280
	2.037	2.163	260/280
	5.758	**11.203**	ng/µL
H	0.006	0.015	260
	0.005	0.007	280
	1.341	2.121	260/280
	6.087	**14.635**	ng/µL

PRIMERS

The primers TEF-Fs4 and TEF-Fs4r, targeting the translation elongation factor alpha gene (TEF-1α), described by Arif et al. (2011), were used. These primers amplify an expected 658 bp fragment specific for Fusarium solani. The PCR conditions reported include 40 cycles consisting of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min.

These primers have been previously validated for the specific detection of Fusarium solani, as demonstrated in the amplification profile described in the original publication.

Primer code	Primer sequence (5'→3')	Gene	Amplified product size	Specificity	PCR programme
TEF-Fs4f	**ATCGGCCACGTCGACTCT**	TEF-1α	658 bp	*F. solani* (Fig.1)	40 cycles; 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min
TEF-Fs4r	GGCGTCTGTTGATTGTTAGC				

Development of specific primers for genus *Fusarium* and *F. solani* using rDNA sub-unit and transcription elongation factor (TEF-1α) gene

Mohammad Arif[1]*, Shilpi Chawla[2], N. W. Zaidi[1], J. K. Rayar[3] M. Variar[4] and U. S. Singh[1]

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[2]Department of Plant and Soil Science, Texas Tech University, Lubbock, Texas 79409, USA.
[3]Department of Molecular Biology and Genetic Engineering, G. B. Pant University of Agriculture & Technology, Pantnagar 263145, Uttarakhand, India.
[4]Central Rainfed Upland Rice Research Station, Post Box 48, Hazaribag 825301, India.

Accepted 19 December, 2011

Figure 1. Profile of *F. solani* specific marker obtained using primer TEF-Fs4. Lane M is 100 bp ladder and lanes 1 to 17 represent different strains as listed in Table 1.

Calculations were performed to prepare the **primer stock solutions**, determining the volume of **sterile H_2O required to obtain an initial concentration of 100 µM**, based on the **molecular weight and absorbance values provided by the manufacturer**.

The following formula was used to calculate the **primer concentration**:

$$X = \frac{\left(\frac{\text{Peso molecular}}{10}\right)}{\text{Densidad óptica} \times 33}$$

Subsequently, the final reconstitution volume of the stock solution was determined using the following formula: $\frac{1000}{X}$

Examples:

EF1F

$$713 \div 10 = 71.3$$

$$(18.8)(33) = 389.4$$

$$X = 1.84$$

$$\frac{1000}{1.84} = 543.47\,\mu L$$

EF1R

$$611 \div 10 = 61.1$$

$$X = 1.88$$

$$\frac{1000}{1.88} = 531.9\,\mu L$$

AGAROSA Y TAE 1X

TAE 1X buffer was prepared by diluting the TAE 10X stock solution with distilled water (1:10):

$$C_1V_1 = C_2V_2$$

$$(10X)(V_1) = (1X)(100\,mL)$$

$$V_1 = 10\,mL$$

We mixed:

- 10 mL de TAE 10X
- 90 mL de H_2O destilada

para obtener 100 mL de TAE 1X.

AGAROSE:

For gel preparation, the following proportion was used.

$$1.2\% = \frac{1.2\,g}{100\,mL}$$

Therefore, **1.2 g of agarose were dissolved in 100 mL of 1X TAE buffer**.

ELECTROPHORESIS CONDITIONS

The gel was run in a **small electrophoresis chamber for 55 minutes at 80 V**.

PCR Reaction Setup

Eight PCR reactions were prepared in **1.5 mL microtubes** using primers at a **final concentration of 100 nM**. The reaction mixture was prepared as follows:

• **Roche Master Mix:** 30 µL × 8 = 240 µL
• **Primer EF1F:** 1 µL × 8 = 8 µL
• **Primer EF1R:** 1 µL × 8 = 8 µL

Total mixture volume: 256 µL, corresponding to **32 µL per reaction**.

The mixture containing the primers was distributed into **seven 200 µL tubes**.

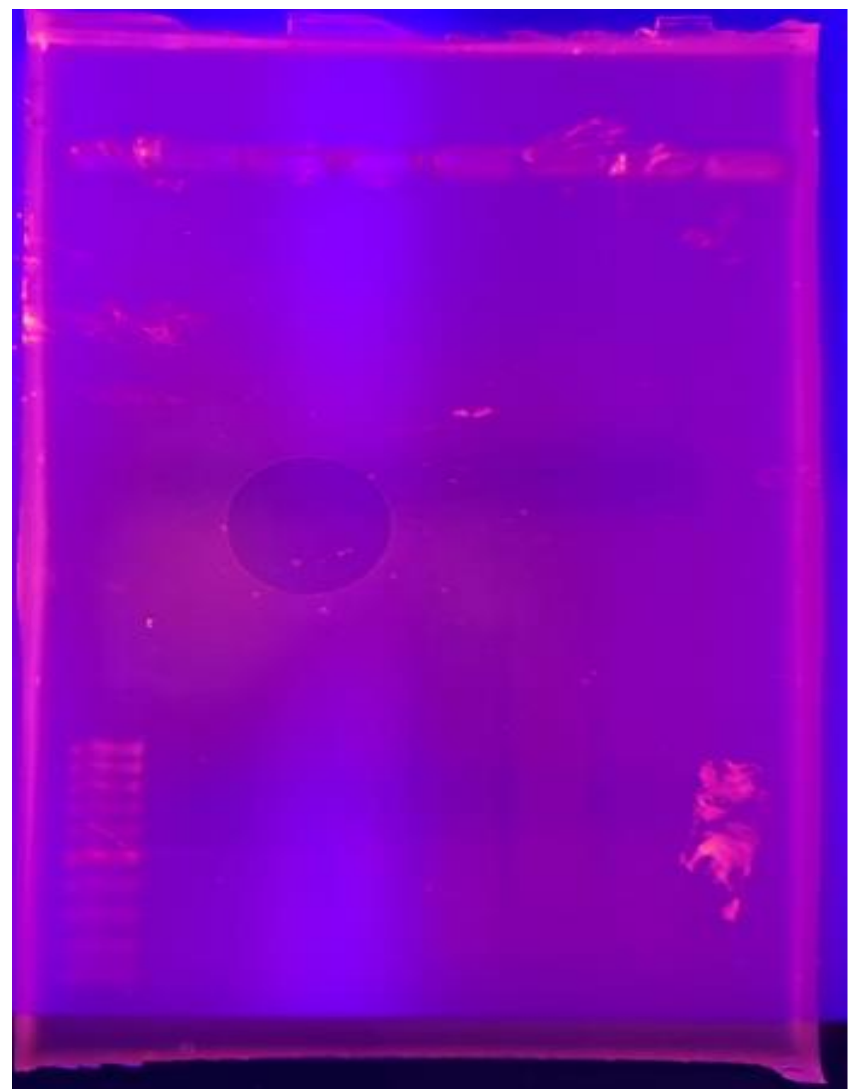
Each PCR reaction consisted of:

• **32 µL of primer-containing mixture**
• **3 µL of DNA (Zymo Kit extraction)**

For electrophoresis, **10 µL of the PCR product** were loaded together with **2 µL of loading buffer**, resulting in a **final volume of 12 µL per well**

Well	Concentration
1	Kb (7 µL)
2	C+
3	C+ 5.8S F/R
4	Sample 1
5	Sample 2
6	Sample 3
7	Sample 4
8	Sample 5

The procedure was repeated three additional times; however, no detectable amplification was observed in the clinical samples.

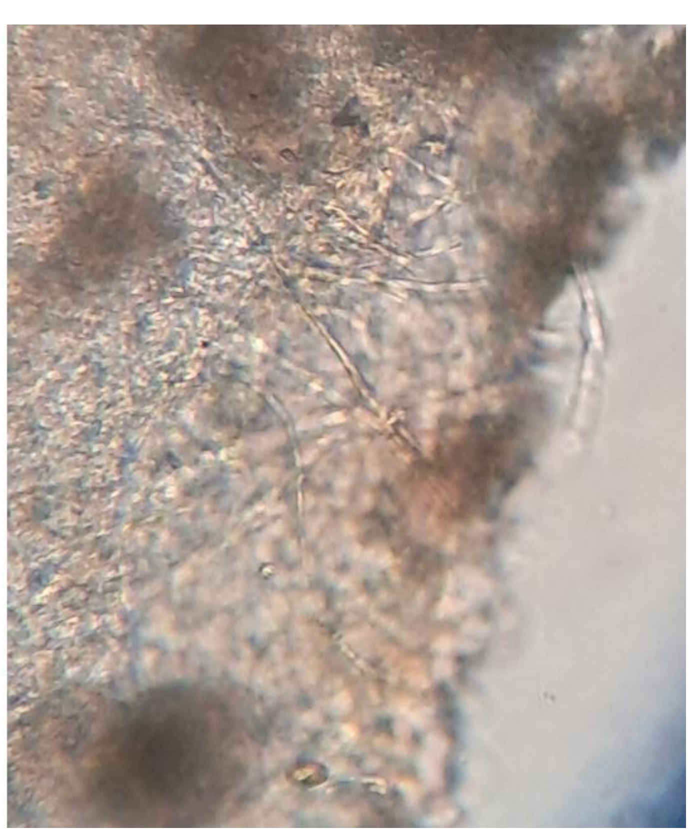


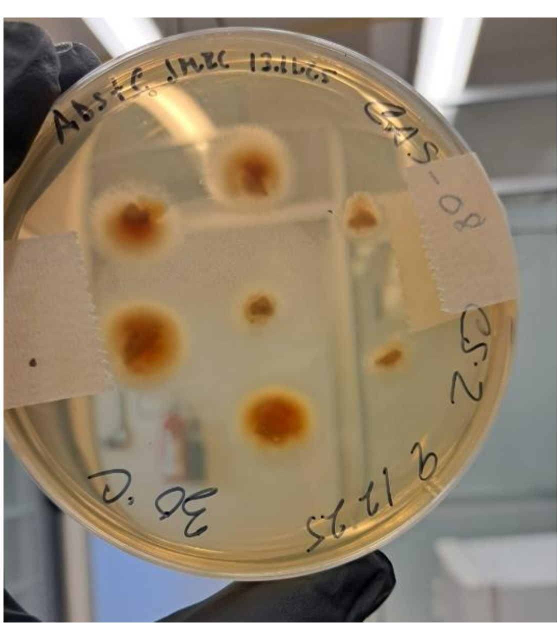
DECEMBER 2025

DNA extraction was repeated directly from the clinical sample (eggshell) using a modified in-house method optimized for filamentous fungi.

In parallel, mycological culture of sample 8 was initiated on dextrose and Sabouraud media, where fungal growth was observed after four days, with colonies showing a pinkish coloration.

Additionally, a direct diagnostic examination using KOH was performed from the clinical sample, revealing filamentous structures compatible with fungal hyphae (Dr. Carolina recorded clearing times of approximately three days).





PCR Reaction Mixture

The PCR reaction mixture was prepared using Roche Master Mix as follows:

- Roche Master Mix: 25 µL
- Forward primer: 1 µL
- Reverse primer: 1 µL

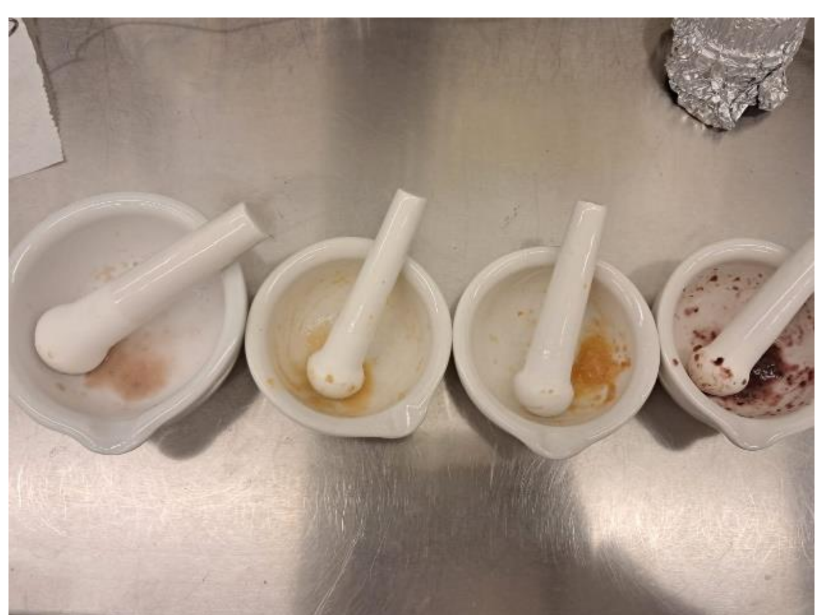
Partial volume: 27 µL, to which 2 µL of DNA (in-house extraction) were added, resulting in a final reaction volume of 29 µL per reaction.

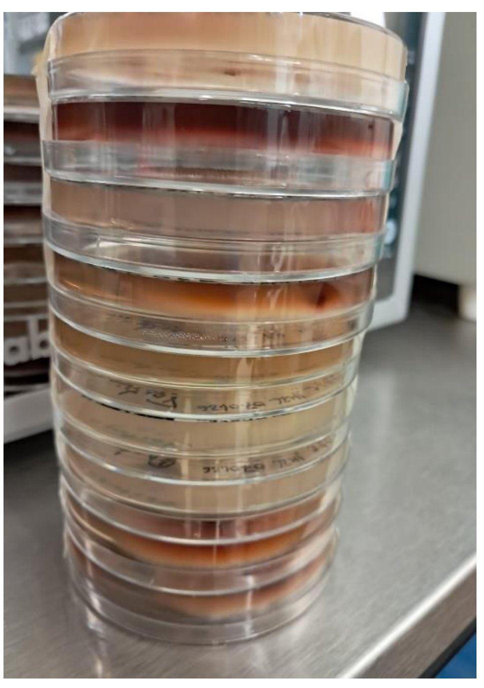
For electrophoresis, 10 µL of the PCR product were loaded mixed with 3 µL of loading buffer. In well 1, 7 µL of the molecular weight marker were loaded.

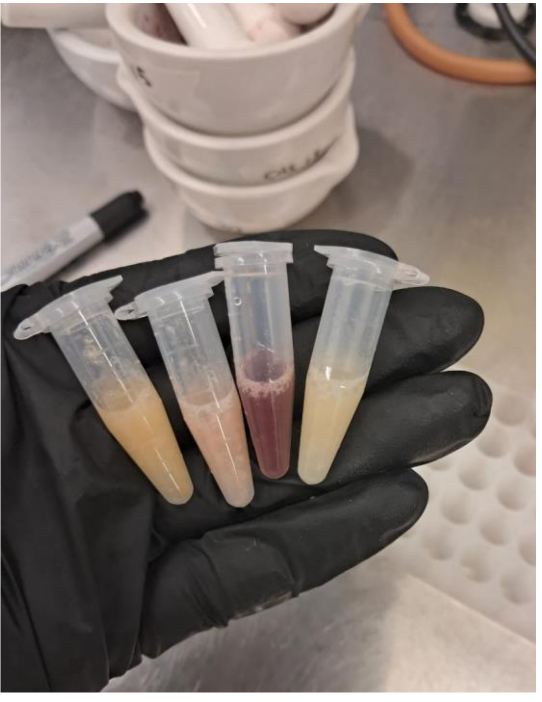
Two faint amplification bands were observed corresponding to lane 4 (sample 7) and lane 6 (sample 11). The assay was repeated using the same samples, obtaining similar results. Re-amplification of the PCR products was attempted, **but was unsuccessful.**

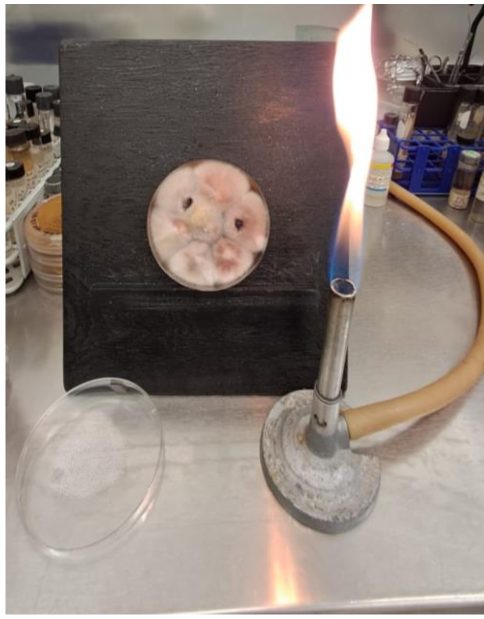
15 JANUARY 2026

Additional mycological cultures were initiated to obtain purer fungal DNA for subsequent molecular assays.









On the same day, a Qiagen Taq DNA polymerase available for a limited number of reactions was used as a substitute for the Roche Master Mix. The Qiagen kit includes the additive Q-solution, which is intended to improve the amplification of templates with secondary structures or high GC content.

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Initially, PCR assays were performed using primers at a 1:100 stock dilution. However, based on technical recommendations aimed at improving specificity and avoiding non-specific amplification or smeared bands, the primer concentration was adjusted to 1:10 in subsequent reactions.

The final reaction mixture (25 μL) was prepared as follows:

H2O	8ul
Buffer 10x	2.5 ul
Mgcl2 (25 mM)	2 ul
dNTPS (10mM c/u)	0.5ul
Primer F (10)	0.5ul
Primer R (10)	0.5ul
Q solution 5x	5ul
DNA	5ul
Taq DNA pol	1ul

The same procedure was repeated with additional samples.

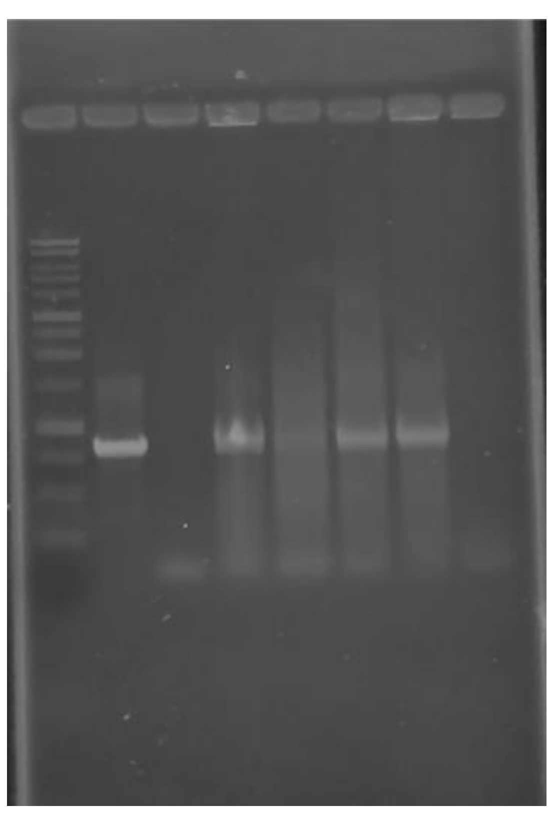
Mix Qiagen

H2O libre nucleasas	8 ul
Buffer 10x coral	2Ul
MgCl2	2ul
dnTPs	1ul
Ef1f 10	0.5ul
Ef1r 10	0.5ul
Q solution 5x y se usa a 1x	5Ul
DNA	5ul
Taq DNA	1ul

Total 25ul

buffer coral**

P.M	C+	C-	1	2	3	4	5



OBSERVATIONS

After noting variability in the performance of the **Roche Master Mix**, and considering that the **Qiagen Taq polymerase successfully amplified clinical samples extracted using the in-house DNA extraction method**, it was decided to **wait for a new Taq polymerase before continuing with further PCR assays**.

GEN5 DNA CONCENTRATIONS- ISOMIL- EXTRACTION METHOD

In the meantime, **Gen5 was used to determine the DNA concentration in clinical samples obtained through the in-house extraction method**, and the following results were obtained.

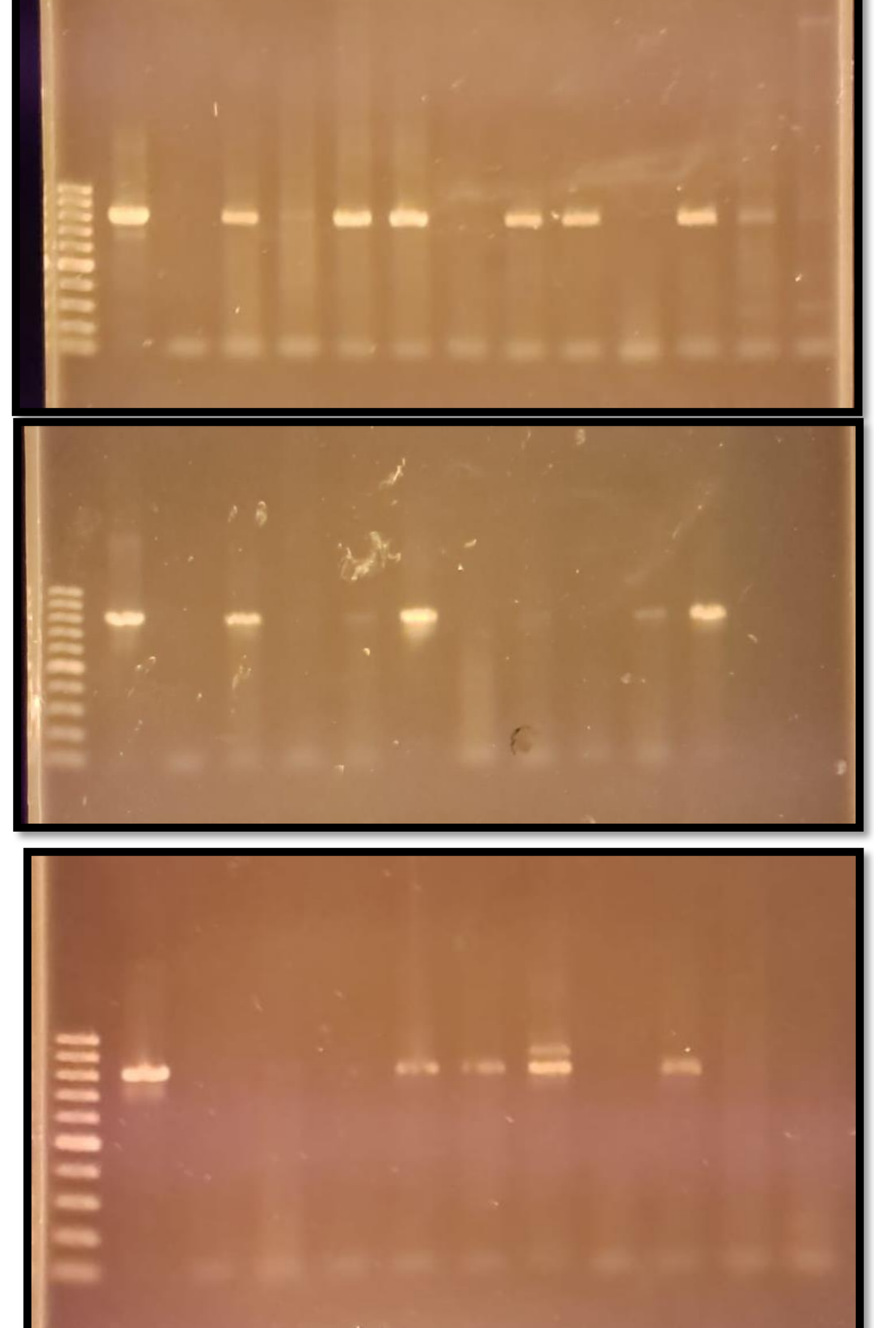
		2	3					2	3		
	A			260			A			260	
				280						280	
				260/280						260/280	
				ng/µL						ng/µL	
M-1	B	0.519	0.727	260	M-4	C1+	B	1.482	1.135	260	C2+
		0.336	0.533	280				0.798	0.913	280	
		1.546	1.364	260/280				1.857	1.243	260/280	
		519.343	**727.178**	ng/µL				**1481.957**	**1135.317**	ng/µL	
M-6	C	0.307	0.271	260	M-7	M19	C	0.272	0.562	260	M20
		0.221	0.215	280				0.199	0.431	280	
		1.392	1.263	260/280				1.363	1.304	260/280	
		307.368	**271.053**	ng/µL				**271.526**	**561.737**	ng/µL	
M-8	D	0.505	0.531	260	M-9	M21	D	0.291	0.15	260	M22
		0.322	0.456	280				0.201	0.133	280	
		1.568	1.164	260/280				1.45	1.126	260/280	
		505.15	**530.789**	ng/µL				**291.097**	**150.2**	ng/µL	
M-10	E	0.608	0.596	260	M-11	M23	E	0.382	0.334	260	M24
		0.507	0.364	280				0.311	0.304	280	
		1.2	1.636	260/280				1.23	1.099	260/280	
		608.199	**595.601**	ng/µL				**382.449**	**334.338**	ng/µL	
M-12	F	0.678	0.504	260	M-13	M25	F	0.336	0.577	260	M27
		0.464	0.36	280				0.274	0.456	280	
		1.461	1.402	260/280				1.226	1.267	260/280	
		678.24	**504.153**	ng/µL				**335.848**	**577.37**	ng/µL	
M-14	G	0.985	0.663	260	M-16	M28	G	0.26	0.447	260	M29
		0.81	0.446	280				0.229	0.338	280	
		1.216	1.485	260/280				1.134	1.321	260/280	
		984.82	**662.651**	ng/µL				**259.579**	**446.787**	ng/µL	
M-17	H	0.229	0.275	260	M-18	M30	H	0.546	0.003	260	
		0.191	0.211	280				0.399	0.001	280	
		1.199	1.302	260/280				1.369	1.963	260/280	
		229.468	**274.514**	ng/µL				**545.759**	**2.77**	ng/µL	

MARCH 2026 QIAGEN TAQ RESULTS

A new **Qiagen Taq polymerase kit** was obtained, and the PCR reaction mixture was prepared as follows:

- **H_2O:** 14.5 µL
- **Coral Buffer (10×):** 2.5 µL
- **$MgCl_2$:** 1 µL
- **dNTPs:** 0.5 µL
- **EF1F primer (10 mM):** 0.5 µL
- **EF1R primer (10 mM):** 0.5 µL
- **Q-solution:** 3 µL
- **Taq polymerase:** 0.5 µL
- **DNA template:** 2 µL

Total reaction volume: 25 µL.



Conclusion

PCR amplification was successfully obtained using Qiagen Taq polymerase, and the preliminary results were presented as a poster at a mycology conference. Molecular analyses are currently continuing using DNA extracted from fungal cultures in order to obtain clearer amplification and further confirm the presence of Fusarium spp.

This information is important because fungal infections, particularly those caused by species within the Fusarium solani species complex, can contribute to egg mortality and reduced hatching success in sea turtles. Improving our understanding of the presence and detection of Fusarium is therefore relevant for both wildlife health and conservation efforts.

In addition, Fusarium species are considered potentially zoonotic opportunistic pathogens, making it important to follow basic biosafety measures when handling samples. The use of gloves at all times and

proper laboratory hygiene practices is strongly recommended during sample processing and laboratory work.

Fusarium spp. EN CASCARONES DE TORTUGAS MARINAS (*Lepidochelys olivácea*) DE PUERTO VALLARTA, MÉXICO:

C. Segundo Zaragoza[1]*, D. Ríos Huerta[3], E. Maldonado Castro[1], M. González Hernández[2].

1. Unidad de Servicios de Diagnóstico y Constatación (USEDICO) del Centro de Enseñanza, Investigación y Extensión en Producción Animal en Altiplano (CEIEPAA), FMVZ.UNAM. 2. Facultad de Agronomía, Medicina Veterinaria y Zootecnia, Universidad Autónoma de San Luis Potosí México. 3. Programa Multidisciplinario de Posgrado en Ciencias Ambientales (PMPCA), Agenda Ambiental, Universidad Autónoma de San Luis Potosí (UASLP).

*c.segund@yahoo.com.mx. Financiamiento: DGAPA. UNAM. PAPIME PE208324 & Rufford 1117270

INTRODUCCIÓN

Fig. 1 *Lepidochelys olivácea* anidando

La **Sea Turtle Egg Fusariosis (STEF)** es una enfermedad emergente asociada con alta **mortalidad embrionaria** en tortugas marinas, principalmente causada por especies del complejo *Fusarium solani*, que incluye taxones con potencial **zoonótico oportunista** (1,2).

***Lepidochelys olivácea* (*fig. 1*)**, especie vulnerable que anida en el Pacífico mexicano, podría estar expuesta a infecciones fúngicas que afectan el **éxito de eclosión.**

En México, son limitados los estudios que integran diagnóstico micológico y molecular en playas de anidación.

OBJETIVO

Evaluar la presencia y viabilidad de *Fusarium spp.* en cascarones de crías no eclosionadas de *Lepidochelys olivácea* mediante aislamiento micológico e identificación molecular del gen TEF-1α.

MÉTODOS

Sitio de estudio: Campamento Boca de Tomates, Puerto Vallarta, Jalisco

Permiso **SBRA/DGVS/01065/25**

Coordenadas: 20°40′06.9″ N, 105°16′17.1″ O

Temporada Verano 2025

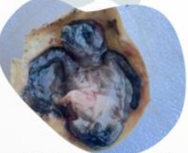


Fig 2. Cria no viable de *Lepidochelys olivácea*

Muestreo

10 nidos ex-situ

30 cascarones (crías no viables) con lesiones rosadas multifocales (fig 2 y 3).

Conservación nitrógeno líquido (-120°C) sin crioprotector

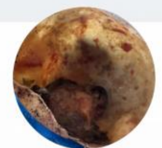


Fig 3. Cascarón con manchas rosas

Procesamiento micológico

A) Cascarón → KOH (72 h) → Observación de hifas (fig4)

Fig. 4 Hifas en muestra clínica

B) Agar dextrosa Sabouraud (SDA) →30°C→5 días

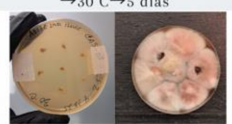


Fig. 5. SDA, día 1 y día 10

Identificación molecular

Muestra clínica (cascarón)

→ Extracción ADN (fenol-cloroformo-alcohol isoamílico)

→ PCR TEF-1α (350 pb)

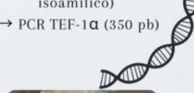


Fig.6 Extracción de DNA

RESULTADOS PRELIMINARES

11/20

Cultivos con crecimiento compatible a *Fusarium spp*

5/10 Muestras con ampificación especifica TEF-1α (fig. 7)

MPM C+ C- M1 M2 M3 M4 M5

400 pb
300 pb
200 pb
100 pb
← 350 pb

Fig 7. Gel de agarosa al 1.2% donde se observa amplificación del complejo *Fusarium solani (350pb)*

DISCUSIÓN Y CONCLUSIONES

1. Se detectó *Fusarium spp.* en cascarones no eclosionados de *Lepidochelys olivácea* mediante aislamiento y PCR (TEF-1α), aprox 350pb.
2. Los cascarones conservaron viabilidad fúngica tras almacenamiento a −120 °C sin crioprotector.
3. El diagnóstico directo, cultivo y PCR son útiles para el monitoreo micológico en playas de anidación.
4. Se requiere secuenciación para confirmar especies del complejo *Fusarium solani*.

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