Project Update: July 2024

Objective No. 1 Determine the diversity and genetic structure of the American Horseshoe Crab in its entire distribution in Mexico

Field expeditions

During the months of June and July 2023 we conducted two field expeditions to Laguna de Términos (Campeche state), the southernmost region in the distribution of *Limulus polyphemus* in the Gulf of Mexico. During the first field trip we conducted, with the support of the team of the Genetics Laboratory of the Universidad Juárez Autónoma de Tabasco (UJAT), a sounding with the inhabitants of Isla Aguada, Campeche, about the presence of this species. The link generated with the fishermen of the area was fundamental for the collection of the specimens. Within Laguna de Términos, more precisely in the so-called "Isla de los pájaros" we collected 32 specimens, from which we took morphometric measurements, sexed them and obtained a tissue sample for genetic studies (Fig. 1).

Fig. 1 Field work during the expeditions to Laguna de Términos, Campeche, México. © Heidi Beatriz Montejo Méndez, Elsi Beatriz Recino Reyes and Fabiola Briceño.

A third expedition (August 2023) was to area around Puerto Progreso, Yucatán, an extremely important region for the study and conservation of this species because it is an area where illegal fishing of adult specimens is carried out for use as bait in the octopus fishery. In this expedition we conducted night inspections in the wetlands of Puerto Progreso, Chelem and Chuburná. We

were able to collect and sample 22 specimens in Chuburná and 8 more in Chelem. In total, we included 62 more individuals to our study, thus increasing the representation to our study, thus increasing the representation of the distribution of *L. polyphemus* in Mexico (Fig. 2).

Fig. 2 Locations sampled within the Mexican distribution of *L. polyphemus*: Laguna de Términos (LT) and Champotón (CH), Campeche; Ría Celestún (RCL), Chuburná (CHU), Chelem (CHE) and Ría Lagartos (RL), Yucatán; Yum Balam (YB) and Sian Ka'an (SK), Quintana Roo.

Laboratory and genetic analysis

Genomic DNA extraction was carried out from muscle tissue by means of the universal salts protocol of Aljanabi and Martinez (1997) for most samples. In some samples where the previous method did not work, we use the NORGEN cell and tissue extraction kit, following its indications. The quality of genomic DNA was assessed by electrophoresis in agarose gels (Fig. 3).

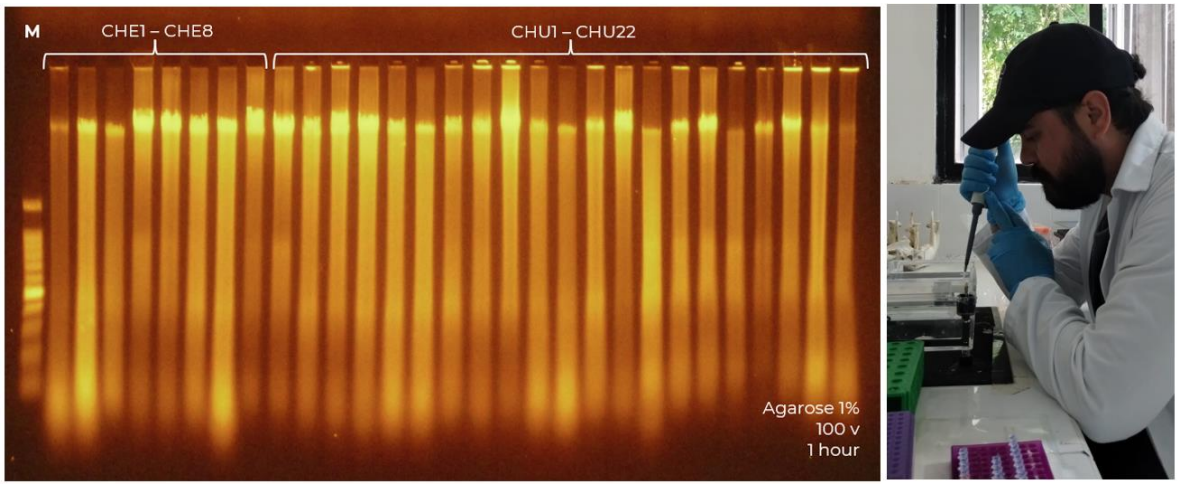

Fig. 3 Genomic DNA extracted from muscle tissue samples visualized on an agarose gel. © José Manuel García Enríquez.

To evaluate the diversity and genetic structure of *L. polyphemus* we amplified by PCR technique two types of genetic markers, microsatellites (SSRs) and Cytochrome oxidase subunit I (COI), of nuclear and mitochondrial origin, respectively. For a total of 154 individuals, we amplified five microsatellites (LpoD6, LpoD3, LpoA67, LpoA42 and LpoA40) through specific primers designed for this species (King and Eackles, 2004) and a 1190 bp fragment of COI from primers LCO1490 (Folmer et al., 1994) and Lim 582 (Pierce et al., 2000) (Fig. 4).

Fig. 4 Sample preparation for PCR amplification of SSRs and COI fragment. © Fabiola Corona.

Genotyping of the amplified products for SSRs is in progress and consists of visualization of the DNA banding pattern on high-definition agarose gels (agarose 1000, Invitrogen). We loaded 3 µl of the amplified products on a gel at a concentration of 3.4%. The size estimation of the amplified products was obtained through Bio-Vision v17.06 software using a 50 bp DNA ladder (Promega) (Fig. 5).

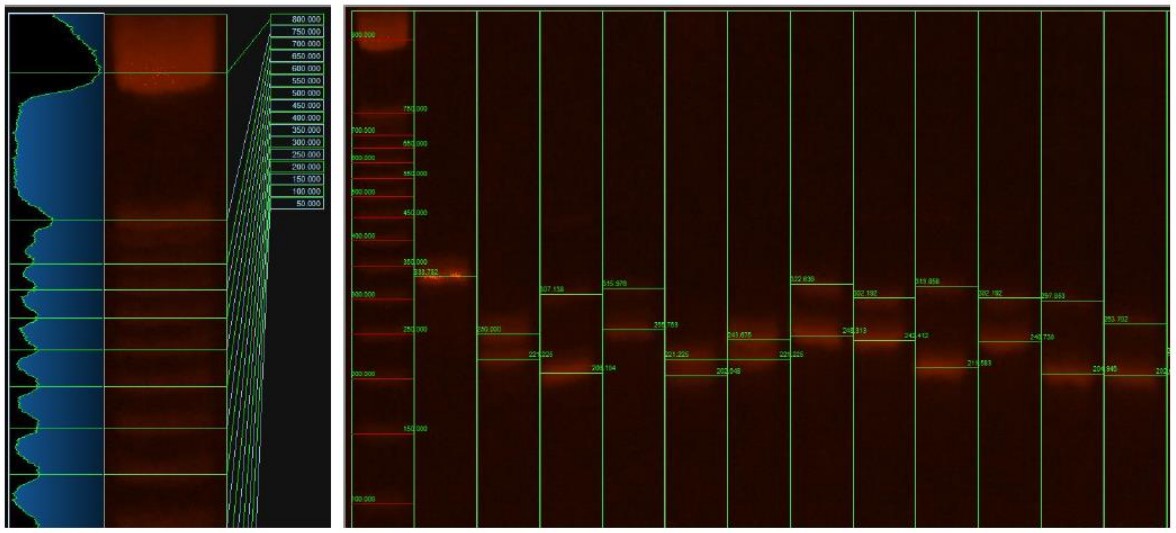

Fig. 5 Visualization of amplified SSRs and size estimation in base pairs using Bio-Vision v17.06.

The amplification products of the 1190 bp COI fragment were sequenced in both forward and reverse directions by the Genomic Services Laboratory of the CINVESTAV center. The electropherograms obtained were edited and assembled using the Geneious Prime v2023.0.4 software (Fig. 6).

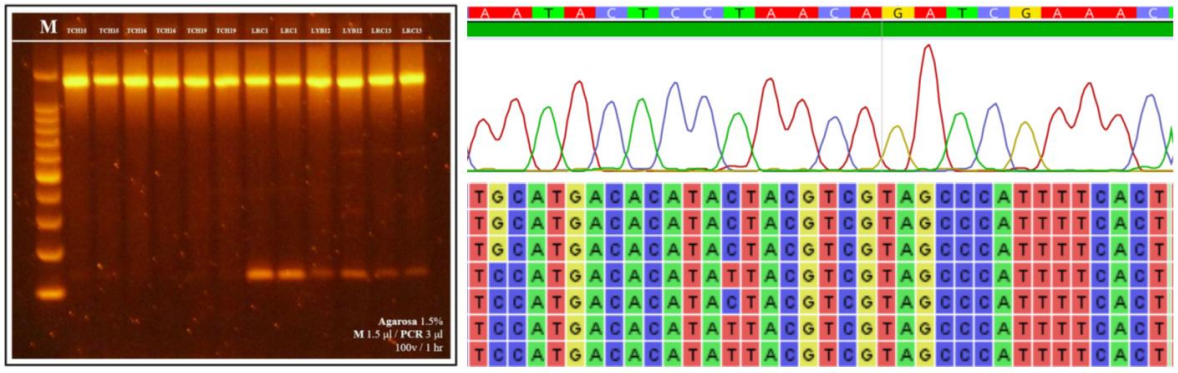

Fig. 6 Agarose gel visualization of the 1190 bp COI fragment; electropherogram resulting from sequencing and multiple alignment of DNA sequences. © José Manuel García Enríquez.

Preliminary results

We correctly amplified and sequenced the 1190 bp COI fragment for 154 individuals of *L. polyphemus* from eight localities representing the entire distribution of this species in Mexico. Our main results include the identification of 16 haplotypes arranged in a star-shaped haplotype network (Fig. 7). In addition, we obtained moderate overall values of haplotypic diversity (*h* = 0.447) and low nucleotide diversity (π = 0.00005). The localities of Chuburná, Chelem, Ría Lagartos and Sian Ka'an showed the lowest haplotypic diversity values (> h = 0.286), probably due to genetic erosion processes caused by anthropogenic exploitation, and therefore represent critical regions for conservation.

These results are reported in an article to be submitted to a peer-reviewed journal.

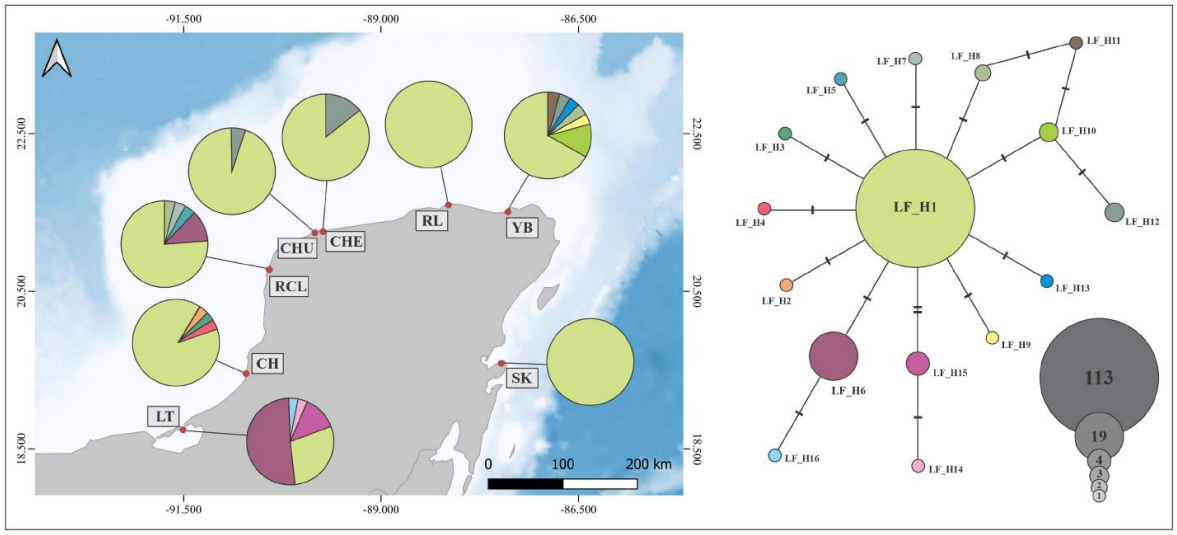

Fig. 7 Haplotype network and distribution of haplotypes in the different localities sampled.

Objective No. 2 Analize the GST and MT genes methylation patterns in populations of the American Horseshoe Crab

During June and July 2023, I had an academic stay at the Genetics Laboratory of the Universidad Juárez Autónoma de Tabasco, where I was trained in laboratory techniques and sample processing aimed at the analysis of DNA methylation patterns, which included DNA conversion using the sodium bisulfite method and the design of methylated/unmethylated primers specific for the Glutathione S-transferase 1 (GST) and Metallothionein 1 (MT) genes, involved in the response to stress caused by exposure to pollutants.

Design of methylated/unmethylated primers

To design specific primers it is necessary to have the information of the region to amplify (i.e., the DNA sequence). For this purpose, the reference genome of *L. polyphemus* was obtained from NCBI platform (https://www.ncbi.nlm.nih.gov/ [access code: GCF_000517525.1]). A search for the GST and MT genes was performed on the same platform (Fig. 8).

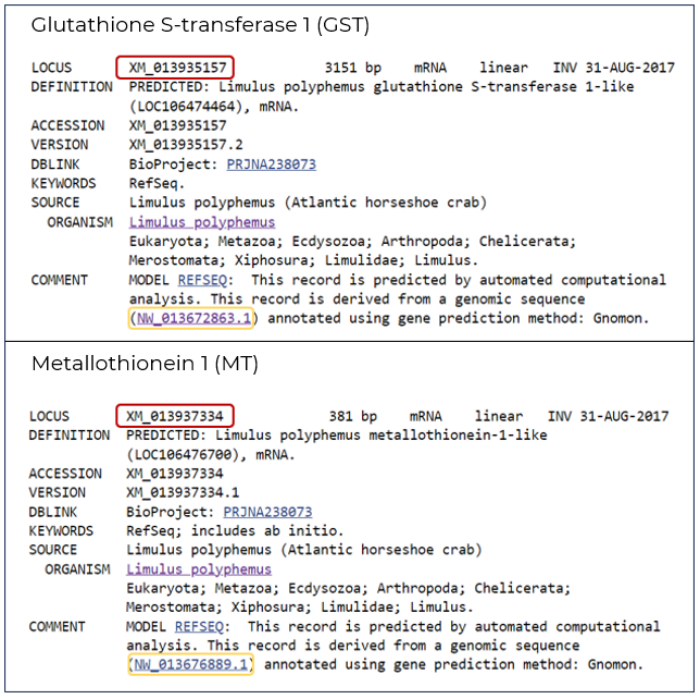

Fig. 8 Results of the search for GST and MT genes in the NCBI platform. In red the access codes to the locus from NCBI and in yellow the access to the contig of the reference genome where the gene is located.

Using the GENEIOUS PRIME 2023.0.04 program, contigs (i.e., overlapping DNA segments that together represent a consensus region) that included the genes of interest were extracted. Before locating the region where the genes are located within the contigs, it is important to recognize the structure of these genes, that is, how they are composed (e.g., UTR regions, promoter region, exons, introns). For this purpose, it was necessary to perform a search in the Ensambl Rapid Release platform (https://rapid.ensembl.org/index.html) using the accession codes of each gene. The results show that the GST gene is composed of two UTR regions (5' and 3'), three exons and two introns, whereas the MT genes is composed of a single exon (Fig. 9).

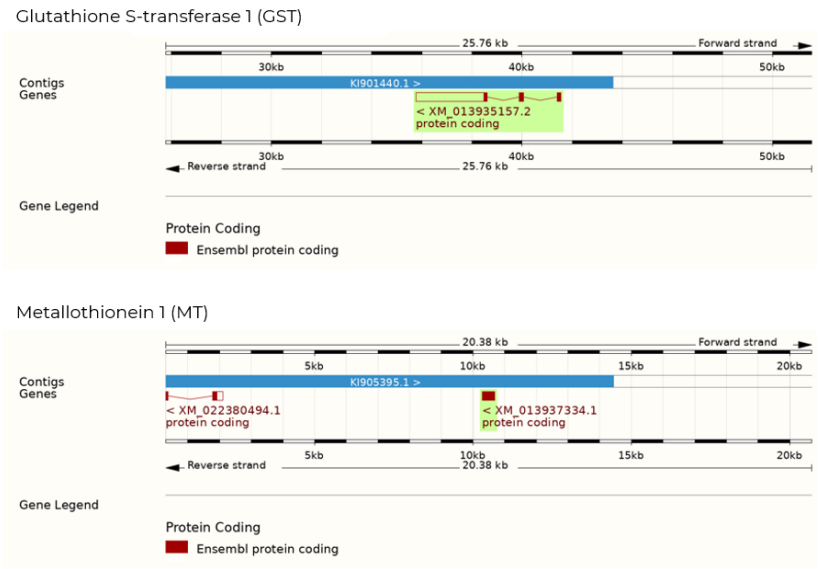

Fig. 9 Position and structure of GST and MT genes.

After recognizing the position and structure of both genes, the sequences of each of them were aligned with their respective contigs. Subsequently, a DNA fragment was selected that included at least 300 bp before the UTR region and 300 bp after it, since the promoter region should be found in this section. From these DNA fragments, the search for CpG islands and the design of specific primer was performed using the METHPRIMER program (Li and Dahiya, 2002). In both genes the program detected a CpG island (Fig. 10) therefore the primers were designed in such a way that they encompassed part of these islands. These primers were sent for synthesis to a private company.

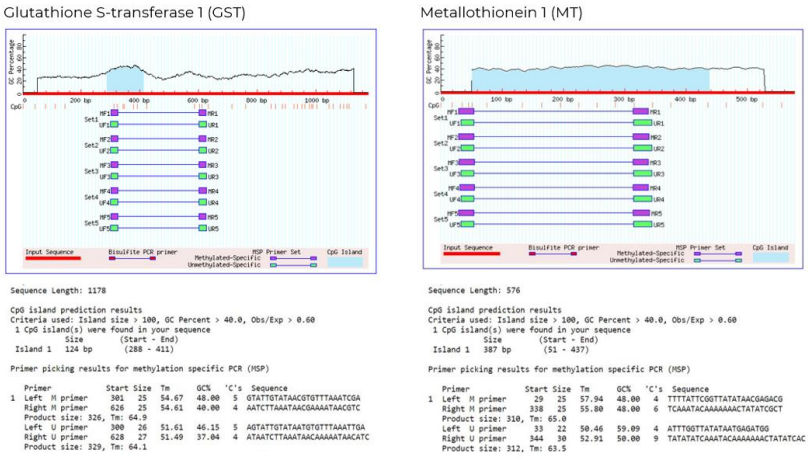

Fig. 10 CpG islands and methylated/unmethylated primers for the GST and MT genes of *L. polyphemus*.

Sodium bisulfite method

A first conversion test was performed using the bisulfite method on 10 genomic DNA samples, corresponding to individuals from five different locations sampled. The EZ DNA METHYLATION™ kit was used for this purpose.

Standardization of PCR amplification parameters for both primers (methylated/unmethylated) is currently underway. Once this is done, the samples will be amplified, sequenced and analyzed to determine the methylation levels of both genes in individuals from different localities that present different degrees of anthropogenic pressure, particularly pollution.

Objective No. 3 Environmental Education Campaigns

For the Environmental Education activities, we generate different elements or support products designed for the local communities:

(1) Infographics that have been shared through social media (Fig. 11); (2) an informative brochure addressing generalities (e. g. origin of the species, ecology, exploitation and main risks, both globally and locally (Fig. 12); and (3) material for outreach activities (Fig. 13)

Additionally, and with the purpose of approaching and analyzing the social perception of this species, we generated a questionnaire based on three main axes: knowledge, attitudes and practices. Through it we conducted interviews that will allow us to recognize the context of each region towards the species, from local knowledge about species to its cultural use or exploitation. So far, we have conducted 60 interviews in the regions of the Gulf of Mexico (Champotón, Campeche) and the north coast of the Yucatán Peninsula (Chuburná, Chelem, Puerto Progreso and Chicxulub). After the interviews, we offer the participants an informative brochure and discuss the importance of the conservation of this species in its Mexican distribution (Fig. 14).

Fig. 11 Infographic about the species L. polyphemus shared on social media (https://www.facebook.com/photo/?fbid=969372708522978&set=a.546758494117737).

Fig. 12 Front and inside view of *L. polyphemus* information brochure.

Fig. 13 Environmental education activities as part of the annual "ECOSUR a puertas abiertas (EAPA)" event on June 2024. © César Raziel Lucio Palacio, José Manuel García Enríquez and Salima Machkour M'Rabet.

Fig. 14 Interviews with fishermen and local community members in Champotón, Campeche (Gulf of Mexico). © Jacob Huerta.

Other activities

Project logo

We designed a logo to represent our conservation project towards *L. polyphemus* (Fig. 15). This logo is used in the different products generated (e.g., shirts for our team, environmental education material, among others), together with the Rufford Foundation logo.

Fig. 15 Logo for *L. polyphemus* conservation project in Mexico.

Presentation of preliminary results

Our preliminary genetics results were recently shared through an oral presentation at the 53th Annual Meeting of The American Arachnological Society, from June 24 to 27, in Chetumal, Quintana Roo, Mexico (Fig. 16).

Fig. 16 Oral presentation made during the 53th Annual Meeting of The American Arachnological Society. © Jacob Huerta.

Reference

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