

Protein Phosphatase type 1 identification in different embryonic development stages from *Kinosternon scorpioides***Identificação da Proteína Fosfatase do Tipo 1 (PP1) em diferentes estágios do desenvolvimento embrionário de *Kinosternon scorpioides*****Short title: PP1 identification in *K. scorpioides***

DOI:10.34117/bjdv6n6-592

Recebimento dos originais: 08/05/2020

Aceitação para publicação: 26/06/2020

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ABSTRACT

The genetic mechanisms involved in testudines' embryonic development are still little known. Many of those processes can be clarified by means of identification and validation studies of genes during the embryonic development. Thus, we aim to identify the presence of PP1 through its expression in different embryonic stages from the semi-aquatic *Kinosternon scorpioides*, a wild fauna Amazonian belonging species. Embryo/foetus samples from different stages of development were utilized, 27, obtained from eggs submitted to two different controlled temperatures (26 °C e 30 °C) in artificial hatchers from the scientific breeding ground for the species. The RNA was extracted from embryonic samples and a reverse transcription in cDNA was also done and the PCR products were sequenced by Sanger techniques. The amplification by qualitative PCR from PP1 demonstrated positive results in all of the specimens, obtaining uniformity in bands profile, confirmed by amplified cDNA concentration from the different embryonic stages. Therefore, the data here reported allows the validation of PP1 as a basal expression gene for molecular studies from the *Kinosternon* genus.

Keyword: mud turtle, protein phosphatase-1, *K. scorpioides*, embryogenesis.

RESUMO

Os mecanismos genéticos envolvidos no desenvolvimento embrionário são ainda pouco conhecidos para os quelônios. Muitos desses processos podem ser esclarecidos através de estudos de validação e identificação de genes durante o desenvolvimento embrionário. Assim, buscamos identificar a presença da PP1 por meio de sua expressão em diferentes estágios embrionários da tartaruga semiaquática *K. scorpioides*, uma espécie pertencente à fauna silvestre amazônica. Utilizamos 27 amostras de embriões/fetos de diferentes fases de desenvolvimento, obtidos de ovos submetidos a duas temperaturas controladas (26 °C e 30 °C) em chocadeiras artificiais provenientes do criadouro científico para a espécie. O RNA foi extraído de amostras embrionárias e retrotranscrito em cDNA e os produtos de PCR foram sequenciados pelas técnicas de Sanger. A amplificação por PCR qualitativo da PP1 mostrou resultados positivos em todos os espécimes, obtendo uniformidade no perfil de bandas, confirmada a partir da análise da concentração de cDNA amplificado dos diferentes estágios embrionários. Portanto, os dados aqui reportados permitiram validar a PP1 como gene de expressão basal para uso em estudos moleculares no gênero *Kinosternon*.

Palavras-chave: tartaruga do lodo, proteína fosfatase – 1, *K. scorpioides*, embriogênese.

1 INTRODUCTION

Kinosternon scorpioides (Linnaeus, 1766) it is a reptile belonging to the Kinosternidae Family and Testudines Order. Widely geographically spread, composes the set of wild Amazonian fauna species, being registered from Costa Rica to Northern Argentina and Brazil. This species possesses a semi-aquatic habit and dwells in swampy areas, however during the breeding season might utilize dry land (CABRERA; COLANTONIO, 1997; CASTRO, 2006).

Currently, regarding its environmental, economic and cultural importance, it has been the focus of many studies, whether they are experimental, in breeding ground or in the wild. It is known that efficient conservation development projects and the species' biology comprehension are still limited as a result of the little knowledge concerning Temperature-Dependent Sexual Differentiation (TSD) processes and the species' reproduction becomes susceptible to climate modifications (RODRIGUES et al., 2017).

To comprehend the TSD, it is necessary to investigate its molecular mechanisms, specially the genic expression pattern of the involved proteins. One of the steps for this process is choosing a basal expression gene as a reference. In this scope, for the reptiles, studies are limited to *Trachemys scripta*, a native North American species (CHOJNOWSKI; BRAUN, 2012; MATSUMOTO; CREWS, 2012; BIESER et al., 2013; BIESER; WIBBELS, 2014; REBELO et al., 2015).

Facing this reality, the Phosphatase Protein type 1 (PP1) has been identified in different vertebrate groups, and utilized as a normalizer reference gene in quantitative PCR reactions. The PP1 belongs to an important protein group from the Serine/Threonine phosphatases that are divided in 6 subtypes: PP1, PP2A, PP2B, PP4, PP5 e PP7. They are involved in many biological mechanisms, for instance, glycogen metabolism, in muscular contraction processes, neuronal activity, cellular division, protein synthesis, membrane receptors regulation, DNA damage, circadian cycle and RNA Splicing, among others (INGEBRITSEN; COHEN, 1983; FONG et al., 2000; MICKEY et al., 2001; KUMAR et al., 2002; CELEUMANS; BOLLEN, 2004; SCHUTZ et al., 2011; RAMOS et al., 2019).

Thereby, focusing to identify a constant basal expression gene capable to be utilized as a template in neotropical testudines embryonic development, in this study, it was aimed to identify PP1 presence by its expression in different embryonic development stages of the semi-aquatic turtle *K. scorpioides*.

2 METHODOLOGY

2.1 THE ANIMALS AND ETHICAL ASPECTS IN THE RESEARCH

The eggs gathered during oviposition were obtained from adult animals kept in the Scientific Breeding Ground of Veterinary Medicine Course from Universidade Estadual do Maranhão (UEMA), Licenced (1899339/2008) by the Brazilian Institute of Environment and Renewable Natural Resources of Maranhão – IBAMA – MA. The specimens were maintained and accompanied by video monitoring *in loco* and in all the procedures that were done, the recommended animal well-being principals from the Animal Ethics and Experimentation Committee of the Veterinary Medicine Course UEMA (CEEA/UEMA: 034/2010) were strictly followed.

2.2 SAMPLES COLLECTION AND EXPERIMENTAL DESIGN

In the scientific breeding round, a breeding stock of 112 animals was monitored (81 females and 31 males) distributed in 5 stalls. A total of 144 eggs were collected during 12 months and stored in artificial hatchers in 26 °C e 30 °C. The temperature difference was considered to confirm, when possible, macroscopically the gender of the individuals.

For the molecular study, 3 samples from each embryonic stage were withheld in regular 7 days intervals in a 3 months period. Unfertilized eggs were excluded from the study. For the collection of embryos/foetuses, the egg shell was carefully ruptured aided by surgical forceps with its content deposited underneath a Petri dish. Animal in later life stages were included: a 15 weeks old sample and adult, from which the DNAs were extracted for comparison.

2.3 RNA-DNA EXTRACTION AND QUANTIFICATION

The RNA was extracted from embryos/foetus using Trizol Reagent® (Invitrogen Corporation, Carlsbad, CA, EUA), according to manufacturer specifications. For DNA extraction the Medrano et al. (1990) was performed. The data related to the embryonic period and used samples quantities are expressed in Table 1. For the extraction of 40-100 g of sample tissue (the embryos/foetus were macerated and a part of them was used) was homogenized in 1 ml of Trizol. The extracted material was stored in a temperature of -80 °C. Samples were diluted 50 times with pure water and concentration reads were performed in the spectrophotometer. RNA purity was determined regarding the $A_{260/280}$ proportion, considering values between 1,8 and 2,0 as appropriate.

Table 1 – Experimental outline to obtain RNA/DNA samples from the *K. scorpioides* specimens of the study

Nucleic Acid	Period (weeks)	Total (samples)
RNA	2 ^a – 9 ^a (1/3 of the embryonic development)	27
RNA	15 ^a	1
DNA	adult	1
Total		29

2.4 SYNTHESIS OF cDNA AND PP1 AMPLIFICATION

A reverse transcription was performed in the total RNA in cDNA, using the commercial SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen®) kit, to a 20 µL final reaction volume.

For the synthesis, 3 µL of DEPC water were utilized, 1,0 µL OF Random Hexamer (50 ng / µL), 1,0 µL of annealing tampon, 2,0 µL of RNase OUT (40U / µL) and 1,0 µL of 2 × First-Strand Reaction Mix. The cDNA synthesis conditions used were the following: 65 °C per min, ice incubation for 1 min, 25 °C for 10 min and 50 °C for 50 min.

For PP1 gene amplification, the following reagents were utilized for each DNA sample (cDNA and genomic DNA): 0,5 µL of GoTaq® Colorless Master Mix 1 × 2 ng from each primer (2 µM), 1,5 µL of Mg2 + (25 mM) and 1,0 uL of cDNA (approximately 500 µg / µL). The final volume was 10 µL. All of the samples were processed in a thermal cycler (PCR Gene-Mate series®), with a protocol that consisted in 5 min in 50 °C, followed by 40 amplification cycles of 2 min in 95 °C, annealing temperature of 65 °C for 15 s, and, finally, 72 °C for 15 s and 72 °C for 3 min. The primers'

annealing temperature (60 °C) was standardized according to previous descriptions from Ramsey et al. (2007), whose primers are found described in Table 2.

Table 2 - Primers utilized in qualitative PCR

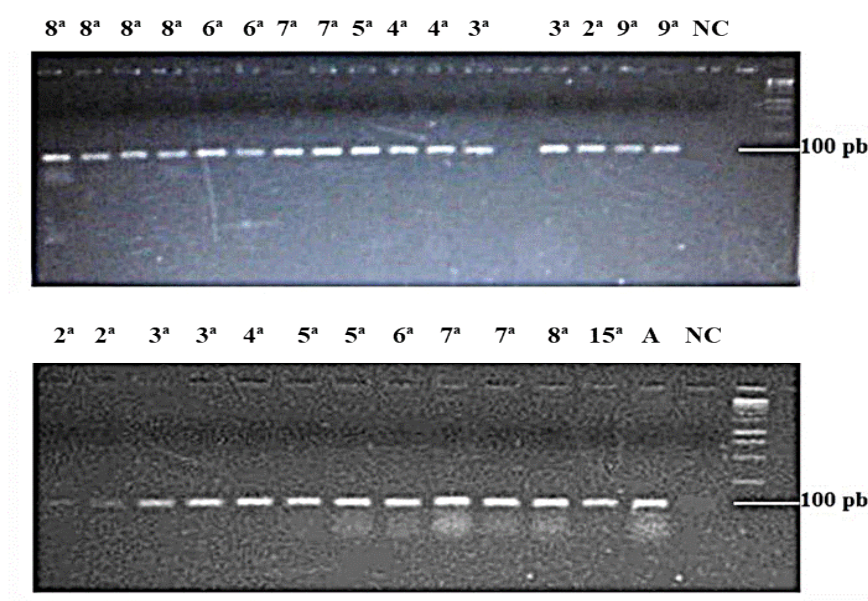
Gene	Primer	Sequence	Reference
PP1	PP1F	5'-CAGCARACCCTGAGAACTTCTTCCTG-3'	Ramsey et al. (2007)
	PP1R	5'-GCGCCTCTTGCACTCATCAT-3'	

The amplifications were confirmed in horizontal electrophoresis using 2% stained with Ethidium bromide agarose gel and visualized/photographed in an ultraviolet transilluminator. Amplification products were quantified by spectrophotometer. For verification, 3 PCR products samples from the experiment were sequenced utilizing Sanger's techniques. The chromatograms were visually verified in the Chromas Lite Version 2.1.1 (Technelysium Pty Ltd. Queensland, Australia) and compared with the data bank available in the NCBI Genbank through Blast algorithm.

3 RESULTS AND DISCUSSION

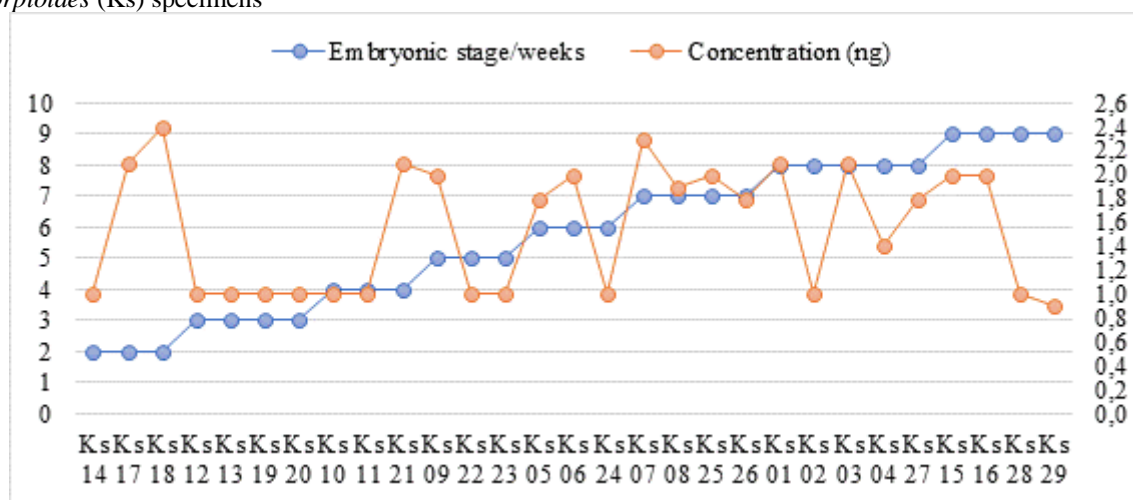
The PP1 amplification by qualitative PCR presented positive results in all of the sampled individuals of the *K. scorpioides* species (Figure 1), not only in the reactions performed with cDNAs, but also with genomic DNA, validating the primers' crossed amplification described for *T. scripta* (RAMSEY et al., 2007) which were used as the basis of this study. This technique has been widely used in biological investigations, above all for specific animal identification by the DNA (NASCIMENTO et al., 2010).

Figure 1- PP1 amplification in *K. scorpioides* specimens in different embryonic stages and in adults (A). The number correspond to the embryonic stages in weeks of the analysed in the study. NC – Negative Control. Molecular Weight Marker – 100 pb



The presence of this amplification product was verified by DNA concentration analysis of DNA, varying from 0,9 to 2,4 ng for different embryonic stages (Figure 2). With the advance of the embryonic stages it is discernible an increase in the amplified DNA concentration. Even though this growth cannot be explained as an increase of the genic expression rates/levels in each stage, it is understood as an possible expression indicative of a potential differential expression during the embryonic development, once that in the first weeks, the PP1 expression was basal and tended to increase in the later levels of embryogenesis, having optimal concentration conditions in individual from 7 to 8 weeks of development. The quantification of the samples by the spectrophotometer, however, verifies amplification products in all the phases studied, therefore, registering the PP1 presence. According to Barford (2010), among the diverse phosphatase proteins from the PPP family found in eukaryotic and prokaryotic cells, PP1 is the main one, being in the majority of vertebrate groups, presenting important functions in physiological processes from the organisms, such as glycogen metabolism regulation, cell division, etc.

Figure 2 – Relation between the total amplified (ng) cDNA/DNA concentration and the embryonic stage from different *K. scorpioides* (Ks) specimens



According to Aoyama et al. (2003), PP1 is part of an important group of proteins belonging to the Serine/Threonine group. This protein plays important roles related to many essential physiologic factors of the vital cycle, operation and development of the organisms. Among these factors, regulatory, metabolic and functional are also added (MATSUMOTO et al., 2013; CHEN et al., 2019; MATHIEU et al., 2019; ARIÑO et al., 2019; NILSSON, 2019).

Regarding the obtained genic sequences, we highlight that *K. scorpioides* has little genetic information available in scientific data bases, and here we describe the first PP1 sequences for the genus, contributing for comparative parameters for further studies. As Antunes et al. (2014) points

out, the data description is important and offer secure information concerning the genetic knowledge and, above all the organisms' genomes.

The cDNA and DNA (both approximately 100 pb) presented 100% of identity with other PP1 fragments in many vertebrate groups, among which we highlight the reptiles and birds (Table 3), displaying high gene conservation between different taxonomic groups. Thereby, considering that PP1 has been utilized as a normalizer reference gene in many organisms, mainly in the vertebrate group, we validated its use as a reference gene in molecular studies for *Kinosternon*.

Table 3 – Identity percentage of the PP1 fragments sequenced in this study with other vertebrate groups available in GenBank through Blast algorithm

PP1 sequence obtained in the study (<i>K. scorpioides</i>)							
PP1 - CTGCCGCGGCCTCCGAGGRGGCAACTCGTGGTTGCCACGCAGCAGGAAGA AGTTCTCAGGGTCTGCTGA							
GenBank	Region	Species	Class	Order	Score	E-value	Identity
XM_006112965.2	PPP1CA/ mRNA	<i>Pelodiscus sinensis</i>	Reptilia	Testudines	73.1	1e-09	100%
XM_010727979.1	PPP1CA/ mRNA	<i>Meleagris gallopavo</i>	Ave	Galliformes	63.9	6e-07	100%
XM_032794984.1	Catalytic subunit- mRNA	<i>Chelonoidis abingdonii</i>	Reptilia	Testudines	89.8	1e-14	100%
XM_030561026.1	Catalytic subunit alfa - mRNA	<i>Gopherus evgoodei</i>	Reptilia	Testudines	89.8	1e-14	100%
XM_024220006.3	Catalytic subunit alfa - mRNA	<i>Terrapene carolina</i>	Reptilia	Testudines	89.8	1e-14	100%
XM_005280533.3	Catalytic subunit mRNA	<i>Chrysemys picta bellii</i>	Reptilia	Testudines	89.8	1e-14	100%
DQ848991.1	mRNA gama	<i>Trachemys scripta</i>	Reptilia	Testudines	89.8	1e-14	100%

With this work, we included *K. scorpioides* among the organisms PP1 presents itself as a basal expression protein. The validation of PP1 gene as a reference makes possible conducting molecular studies, not only regarding Temperature-Dependent Sexual Differentiation, but also other metabolic and regulatory processes of *Kinosternon* genus, amplifying the range of important papers about neotropical testudines reproduction. Such pieces of information will allow researchers to elaborate applicable strategies for the management and conservation of the species in their natural habitat, also amplifying the knowledge status from this and other reptile species.

4 CONCLUSION

In this work, the first descriptions about PP1 in *K. scorpioides* were presented. The protein presence in different embryonic development stages of the species was identified and validated and

we indicated it as a reference gene for embryonic development and genetic mechanisms studies for *Kinosternon* genus.

To sum up, the results obtained in this investigation give opportunity for new hypothesis testing, therefore amplifying the biologic knowledge of the species, providing valuable information about the mechanisms orientated to PP1's functionality in animals.

ACKNOWLEDGMENTS

The authors thank the Fundação de Apoio à Pesquisa e Desenvolvimento Científico do Maranhão (FAPEMA), the sponsorship from Projeto REBAX (Processo REBAX 03634/13 – Edital 030/2013); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Programa de Pós-Graduação em Ciência Animal (PPGCA) of Universidade Estadual do Maranhão (UEMA).

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