Latin American Journal of Aquatic Research, 54(5): 801-814, 2026
DOI: 10.3856/vol54-issue5-fulltext-3660

Research Article

In silico analysis of arginine and creatine kinases, exploring the coexistence of phosphagens in the cannonball jellyfish *Stomolophus* sp.2

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ABSTRACT. The cannonball jellyfish *Stomolophus* sp.2 is distributed in the southern region of the Gulf of California. At high temperatures, it shows metabolic responses that produce large amounts of adenosine triphosphate (ATP) to cope with thermal stress. In addition to mitochondria, phosphagens are suggested as one mechanism for rapid ATP production in this species. Recently, two transcripts encoding the phosphagen-kinase homologs, arginine kinase (AK) and creatine kinase (CK), were confirmed in the jellyfish transcriptome as part of the species' ability to synthesize ATP. This study identifies and characterizes both enzymes to understand the molecular adaptations of *Stomolophus* sp.2 to environmental challenges, and explores the evolution of this family of enzymes in the animal kingdom. The transcripts and deduced proteins from jellyfish AK and CK were analyzed and confirmed through bioinformatic analysis. Conserved domains in both proteins and their predicted structures confirmed their identity. Phylogenetic trees were built to explore these proteins' evolutionary origin in early animal history. Results revealed that the AK and CK sequences of jellyfish share conserved phosphagen-kinase signatures, N- and C-terminal domains, and a specific active site. Phylogeny suggests a divergence event in later clades, emphasizing the conservation and evolution of these enzymes. This study provides new evidence on the presence of the two phosphagen enzymes in the jellyfish; these findings expand our understanding of the evolution and molecular adaptation of phosphagen enzymes in the animal kingdom, highlighting the biological plasticity of *Stomolophus* sp.2 to face climate change.

Keywords: bioinformatics; nucleotide; phosphagens; phylogeny; transcriptome; bioenergetics

INTRODUCTION

The knowledge of the evolutionary history of the Animal phyla is the result of an arduous scientific effort that integrates data from different disciplines and tools, including phylogenetic, biogeographic, geological, and fossil evidence, to understand the earliest groups of organisms, such as cnidarians (jellyfish, sea anemones, and corals) (Technau & Steele 2011, Gershwin, 2016). To date, biochemical and physiological responses in living species have advanced understanding of their molecular adaptations to continuously changing environmental conditions (Rossnerova et al. 2020, Somero 2022, Xu & Wang 2022). The earliest jellyfish fossils date to the Ediacaran, 585 million years ago, and some morphological changes have been minimal

Associate Editor: Débora Torrealba

throughout their evolutionary history (Gershwin 2016). Among cnidarians, medusozoans include pelagic adult stages called jellyfish, whose long evolutionary history has allowed them to acquire the biochemical and physiological adaptations that suggest a high degree of plasticity, allowing them to produce massive aggregations known as "blooms" (D'Ambra et al. 2021).

As one of the early metazoans in the sea, cnidarians have retained some ancestral genes, many of them later lost in animal species (Technau et al. 2005), but also have gained some biological innovations that include the acquisition of complex multicellularity, an asexual reproduction phase in their metagenic life cycle that provides for alternative reproduction phases (López-Martínez et al. 2022), diploidy (Diupotex-Chong et al. 2009) and a genome expansion providing most of the gene families from bilaterians (Kortschak et al. 2003, Robin et al. 2021, Santander et al. 2022).

In the Gulf of California, the cannonball jellyfish *Stomolophus* sp.2 (previously described as *Stomolophus meleagris*) is distributed along the southern region (Getino-Mamet et al. 2017, 2019, Gómez-Daglio & Dawson 2017). This species has increased in population abundance over the last few decades (López-Martínez & Álvarez-Tello 2013).

The increasing abundance of *Stomolophus* sp.2 correlates with its ability to compensate for continuously changing environmental conditions, as seen in various jellyfish species (Purcell 2012, Williams 2015). Recent evidence suggests that seawater temperature is one of the factors determining jellyfish biochemical adaptations, including the extensive transformation of chemical energy into adenosine triphosphate (ATP) reserves (Nevárez-López et al. 2020). Moreover, the jellyfish mitochondrial electron transport chain is adapted to support high energy budgets to meet the cell's basic needs (Sastré-Velazquez 2020, Nevárez-López et al. 2024). *Stomolophus* sp.2 has, as its transcriptome confirms, transcripts encoding two kinases that participate in the two phosphagen systems: arginine kinase (AK) and creatine kinase (CK) (Gamero-Mora et al. 2025). Although it has been suggested that CK is primarily found in vertebrates, and AK is mainly found in invertebrates such as insects, crustaceans, and unicellular organisms (Auger et al. 2021), some animal groups are known to possess both kinases, CK and AK, such as poriferans (Ellington 2000, Perović-Ottstadt et al. 2005), sipunculids, brachiopods, annelids, echinoderms, hemichordates, tunicates and cephalochordates (Van Thoai et al. 1972, Robin & Guillou 1980, Ellington 2001); all plesiomorphic metazoans of the animal evolutionary line (Ellington 2001).

The two phosphagen systems are found in metazoans as alternative energy-producing pathways that use AK and CK to hydrolyze the high-energy bonds of arginine- and creatine-phosphate, which are stored in muscle in most species (Kotlyar et al. 2000, Jubrias et al. 2001, García-Orozco et al. 2007). Researchers first described phosphagen compounds in the 1920s (Vasconcellos et al. 2024). In animals, eight phosphagen systems are known; the enzymes AK and CK are found in most animal phyla, and researchers discovered both in the 1920s in the muscle of invertebrates and vertebrates, respectively. Therefore, from its inception, it was believed that CK was exclusive to vertebrates (Vasconcellos et al. 2024), until CK was later found in various invertebrate phyla, among which the following stand out: Porifera, Brachiopoda, Phoronida, Annelida, Echinodermata, Hemichordata and Chordata (Ennor & Morrison 1958, Robin 1964, 1974, Van Thoai 1968, Watts, 1968, 1971, 1975, Van Thoai et al. 1972, Ellington 2001) Despite this, to date there are no records of these energy production pathways in cnidarians, and the enzymes catalyzing the on-off kinase reactions to allow the rapid delivery of large ATP quantities during intense activity to maintain cellular energy levels (Lygate 2024).

This study aims to characterize the transcripts, deduced proteins, and predicted structures of the two phosphagen enzymes expressed in the jellyfish *Stomolophus* sp.2 umbrella, evaluate their phylogenetic relationships, and better understand their origin and assess a possible divergence event in the evolutionary history of phosphagens. These results will also provide basic information to understand jellyfish energy metabolism better.

MATERIALS AND METHODS

The *Stomolophus* sp.2 transcripts of AK and CK

The nucleotide sequences of one AK and eight CK from the genome of jellyfish *Aurelia* sp.1 (Gold et al. 2019; GenBank Bioproject: PRJNA490213) were used as a reference to find both transcripts in the *Stomolophus* sp.2 transcriptome (Gamero-Mora et al. 2025; GenBank PRJNA851814). High identity percentages were detected for four AK and seven CK-B unigenes of *Stomolophus* sp.2, and the deduced proteins from each transcript were analyzed. All sequences were individually compared with the nucleotide and protein databases using the BLAST-N and BLAST-P algorithms (Altschul et al. 1990), and the online tool PROSITE (https://prosite.expasy.org/) to confirm their identity as phosphagen kinases (Sigrist et al. 2013).

A consensus sequence was generated for AK and CK type-B (CK-B) transcripts, along with their deduced protein sequences, using BioEdit v7.2.5 software. The transcripts and proteins were used as references for searching for enzymes from other species using BLAST-N in the Core nucleotide database (core_nt) and BLAST-P in the Non-redundant protein sequences (nr) database. Additionally, multiple alignment analysis for each enzyme using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo?stype=dna) was performed.

The 3D predictive structural model of jellyfish arginine and creatine kinases

The 3D models of both deduced proteins, AK and CK-B, were generated using homology comparison and domain coordinates from the AK model of *Anthopleura japonica* (PDB code 4RF9) and the CK-B model of *Homo sapiens* (PDB code 1I0E) as templates. The predictive model coordinates were built using the Phyre2 server (http://www.sbg.bio.ic.ac.uk/~phyre2; Kelley et al. 2015). The PyMOL Molecular Graphics System v1.7.5.0 software was used to visualize each enzyme model.

The predicted three-dimensional structures were confirmed by comparing the resulting jellyfish AK and CK-B models with specific proteins previously established by structure and identity in other species using the DALI server (http://ekhidna2.biocenter.helsinki.fi/dali/; Holm 2023). The identities of the PDB proteins that resulted in high similarity to each of the jellyfish models were analyzed in terms of percentage of identity and RMSD value.

Phylogenetic analysis of arginine and creatine kinases (AK and CK) from *Stomolophus* sp.2

A total of 16 nucleotide sequences for AK and 24 nucleotide sequences for CK-B were included in the multiple alignment and phylogenetic analyses generated in Clustal X (Table 1; Larkin et al. 2007). The software PAUP4 v4.0a169 was used to construct phylogenetic trees, and the relationships among nucleotide sequences of enzymes from different species were estimated using maximum likelihood (ML; Whelan & Goldman 2001) approximation. The nucleotide substitution model GTR and a G distribution were selected based on likelihood, using the Akaike corrected information criterion in jModelTest version 2 (Darriba et al. 2012). Nodal support by non-parametric bootstrapping was estimated with 1,000 pseudoreplicates. In addition, Bayesian inference was performed using MrBayes software version 3.2.7 (Ronquist et al. 2012) with two independent runs starting from random trees in which the Metropolis-Hastings Markov Chain Monte Carlo (MC3) method included four chains (three heated and one cold) running simultaneously for 10,000,000 generations. Default settings were used, and we excluded trees and model parameter values recorded in the pre-convergence "burn-in" phases of the two runs from the calculation of parameter values and trees. Final BI trees were edited using FigTree 1.4.2 (Rambaut 2014).

RESULTS

The *Stomolophus* sp.2 transcripts of AK and CK

The consensus sequences from four AK and seven CK-B unigenes in the *Stomolophus* sp.2 transcriptome (Gamero-Mora et al. 2025) and the blast-N analysis showed high identity percentages above 65% with *Aurelia aurita* sequences. The deduced proteins were confirmed as CK-B and AK, respectively. The CK-B nucleotide sequence (GenBank acc. no. PV754314) is 1,416 bp long, including a 5'-UTR region of 63 bp (-63 to -1), an ATG start codon at position 1, and a coding region consisting of 1,146 bp. The TGA stop codon was detected at position 1,144, and the 3'-UTR region of 207 bp includes a polyadenylation signal AATAAA at position 1,387. The coding sequence was detected in the open reading frame (ORF) 1 and consists of a deduced protein of 381 amino acids (aa) with a calculated molecular weight of 42.661 kDa and a calculated isoelectric point (pI) of 5.27 (Fig. 1)

The AK consensus sequence (GenBank acc. no. PV749638) resulted in a sequence of 1,725 bp, which includes a 5'-UTR region of 181 bp (-181 to -1), an ATG start codon at position 1, a TAA stop codon at position 1,132, and a 3'-UTR region of 410 bp. The 1,134 bp CDS in ORF 2 encodes a deduced protein of 377 aa with a calculated molecular weight of 43.424 kDa and a calculated pI of 6.11 (Fig. 2).

The 3D predictive structural model of jellyfish arginine and creatine kinases

The predictive 3D structure of the jellyfish CK-B was built using the human crystallographic structure as a template (PDB 1I0E; Shen et al. 2001). It retains the globular conformation characteristic of CKs, with a 100% confidence level, 100% coverage of the deduced amino acid sequence, and an identity percentage of 79% (Fig. 3a). The protein structure is composed of 42% α-helices, 14% β-sheets, and 17% loops. Conserved amino acids in the phosphate-nucleotide binding sites, along with phosphagen kinase signatures,

Table 1. Summary of sequences included in the multiple alignment and phylogenetic analyses. AK: arginine kinase, and CK-B: creatine kinase type B.

Enzyme	Database / Origin	Specie	GenBank accession no. / ID
AK	This study	*Stomolophus* sp.2	AK1
			AK2
			AK-consensus
	GenBank	*Suberites ficus*	DQ786183.1
		Nematostella vectensis	AB665524.1
		Anthopleura japonica	AB008014.1
		Stylophora pistillata	XM_022938063.1
		Bolinopsis microptera	XM_063834067.1
	BioProject: GHXY01.1	*Beroe forskalii*	351338.1
			351343.1
			351332.1
			351333.1
	Bioproject: GHXS01.1	*Hormiphora californiensis*	001779.1
			01001777.1
			001778.1
	Bioproject: GKLW01.1	*Bolinopsis microptera*	027732.1
CK-B	This study	*Stomolophus* sp.2	CK1
			CK2
			CK3
			CK4
			CK5
			CK6
			CK8
			CK-consensus
	GenBank	*Suberites ficus*	DQ786185.1
		Actinia tenebrosa	XM_031714028.1
		Nematostella vectensis	AB665526.1
		Stylophora pistillata	XM_022926331.1
		Orbicella faveolata	XM_020770048.1
		Acropora millepora	XM_029339991.2
		Bolinopsis microptera	XM_063834339.1
	BioProject: GHXY01.1	*Beroe forskalii*	288276.1
			325096.1
			325095.1
	Bioproject: GFAT01.1	*Mnemiopsis leidyi*	027122.1
			017517.1
	BioProject: GKLW01.1	*Bolinopsis microptera*	049514.1
			049515.1
			049519.1
			049517.1

are located in a cleft that keeps them close together (Fig. 3b).

This predictive model depicts the CK-B monomer, which is active as a homodimer or as a heterodimer with a CK-M monomer (Grossman & Sellers 1998). Therefore, the structural model shares specific features that confirm that the cannonball jellyfish *Stomolophus* sp.2 possesses the characteristic enzyme of the CK phosphagen system, which is reported mainly in vertebrates. To confirm these findings, the CK-B predictive model was blasted in the Dali server, and the RMSD value of 0.631 confirmed that the model is very similar to the crystallographic structure of *Gallus gallus* CK-B (PDB: 1QH4). However, the crystallographic structure of CK-B from *Stomolophus* sp.2 will help confirm the enzyme's structure.

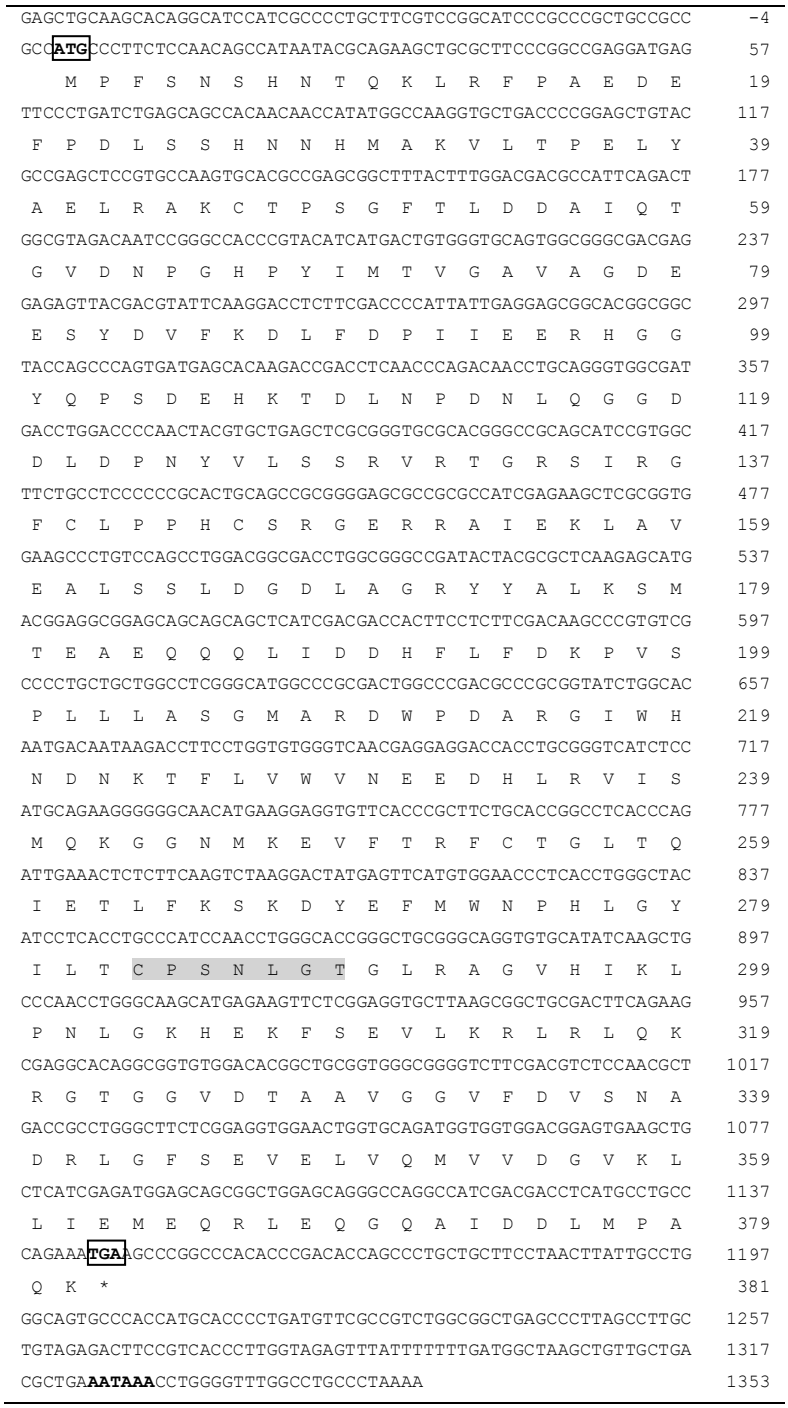


Figure 1. The nucleotide and deduced amino acid sequences of the creatine kinase type B from the jellyfish *Stomolophus* sp.2. Start and end codons are framed; the characteristic phosphagen kinase signature is shaded in grey.

The predictive model of *Stomolophus* sp.2 AK was built using the crystallographic structure of the anemone *A. japonica* as a template (code 4RF9; Wang et al. 2015). This predictive model of jellyfish AK shows a typical globular conformation with 100% confidence, 100% coverage, and 48% identity (Fig. 4). The protein structure contains 40% α-helices, 16% β-sheets, and 15% loops. As in the CK-B model, the nucleotide phosphate-binding site amino acids and the phosphagen kinase signature are positioned close

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                                                                C   -181
TTTGAGCAGCTAGCTCAAATATAACTTGCTAACTTCTTACGATATTCTAAGAGCAATTTT   -180
CTGTACTCTGTTCATCTTTGGTTGAAGCGGGGGGTTTAGAGAATTTACAGCAAACCGGGG   -120
GTGTATTTTTCAGATGTCTTAAACGAGAAAGACCGGTTCAATTTAATTTGCCGAGAAGAG    -60
ATGGAGCCGGTGGACACTAAGCAAGCCTTGAGCACTGAGGAAATCCCACAAGGAACCTTC     60
 M  E  P  V  D  T  K  Q  A  L  S  T  E  E  I  P  Q  G  T  F      20
ATGGGCCCCCATCTTGATGGTCCAGAGGCTCTCCGCTACCTTCCGTTTTTCCCAACTGGA    120
 M  G  P  H  L  D  G  P  E  A  L  R  Y  L  P  F  F  P  T  G      40
ACCAATTCTCTTCTTAGTAAGTGCCTAACGAAGGATGTTTGGGAACAGTGCAAAGACAAA    180
 T  N  S  L  L  S  K  C  L  T  K  D  V  W  E  Q  C  K  D  K      60
AACGACAAATATGGATATAGCTTTAAGCAGGCAATCTTCTCTGGATGTAAGTGGCCTGAG    240
 N  D  K  Y  G  Y  S  F  K  Q  A  I  F  S  G  C  K  W  P  E      80
TCTAGAATTGGTGTTTACGCTGGCTCCTATGATTCATACAATGCATTCGCTCCTCTGTTT    300
 S  R  I  G  V  Y  A  G  S  Y  D  S  Y  N  A  F  A  P  L  F     100
GATAAGATCATCCATACGTATCATGAAAAAAGCCCCAAAGACAAGCACATATCTAACATG    360
 D  K  I  I  H  T  Y  H  E  K  S  P  K  D  K  H  I  S  N  M     120
GATTACACTCGGCTAAACTGCCCTGATTTTCCTGATGATGAGGACAAAATGATAACATCT    420
 D  Y  T  R  L  N  C  P  D  F  P  D  D  E  D  K  M  I  T  S     140
ACCAGAATAAGACTTGCAAGAAACCTTGACCAATACCCTCTTGGAACTGCTGTCACTTTC    480
 T  R  I  R  L  A  R  N  L  D  Q  Y  P  L  G  T  A  V  T  F     160
TTAGAAAGAAAAAGAATTGAAGAAAAGATTGAAAATATCCTTGAAAAGTTTACTGGAGAG    540
 L  E  R  K  R  I  E  E  K  I  E  N  I  L  E  K  F  T  G  E     180
CTCAAAGGGAAATATTACTCCCTTTCAAATATGACTGATGCCGAGAGACAGCAGCTTATT    600
 L  K  G  K  Y  Y  S  L  S  N  M  T  D  A  E  R  Q  Q  L  I     200
TCTGAACACATTCTTTTCAAAGGTGGTGACCAATATCTTGAATCCTGTGGTCTTGAAAGA    660
 S  E  H  I  L  F  K  G  G  D  Q  Y  L  E  S  C  G  L  E  R     220
GACTGGCCAAATAGCAGAGGAATCTTTTACAATTACAGGAAGACCTTCCTTGTATGGGTT    720
 D  W  P  N  S  R  G  I  F  Y  N  Y  R  K  T  F  L  V  W  V     240
AATGAAGAGGACCACCTGAGAATCATTTCAATGCAGAATGGCTCCAACATTTGGCAGGTT    780
 N  E  E  D  H  L  R  I  I  S  M  Q  N  G  S  N  I  W  Q  V     260
TTCAAGAGACTAGCTGAAGCTACAGCTTTAATCGAGAAAGAGGCTACATTTGCATACGAT    840
 F  K  R  L  A  E  A  T  A  L  I  E  K  E  A  T  F  A  Y  D     280
GAGCACATTGGATATATCACATCTTGTCCGACCAACCTGGGGACAGGACTAAGAGCTTCT    900
 E  H  I  G  Y  I  T  S  C  P  T  N  L  G  T  G  L  R  A  S     300
GTTCACATCAAGCTTCCTAAACTGGCCAAGAATAAAGACCAGCTCCAGGTCATTGCTGAT    960
 V  H  I  K  L  P  K  L  A  K  N  K  D  Q  L  Q  V  I  A  D     320
AAATTTCATGTTCAGATCAGAGGAATTGACGGAGAGCATAGCAAGACCGAGAGTGAGGTG   1020
 K  F  H  V  Q  I  R  G  I  D  G  E  H  S  K  T  E  S  E  V     340
TTTGATATTTCTAACTTAAGAAGGTTTGGAAGATCTGAAGTCGACCTCGTCCAGGATATG   1080
 F  D  I  S  N  L  R  R  F  G  R  S  E  V  D  L  V  Q  D  M     360
TATGACGGTGTCAAAGCCATAATTTGTGCTGAAAAGAAGTTGCAATTTGCATAATCAAAA   1140
 Y  D  G  V  K  A  I  I  C  A  E  K  K  L  Q  F  A  *           377
ACGTGTAAACTCGTGAAGGCGCAGGCGTCTTCATAGCTTTGAATGTTTCACTTGTAGTTT   1200
CAGTGCTAATGTAAATTAATATAATTGAAAGAAAATGTAATTAATTAAAGCTGTAGAGAA   1260
AATCTAACAAGAGTAGAATCTTTAGAACCAAATATCACATCGAGGTAAAATAGTGGATAT   1320
GATAAGTGCAATTTCGAGACATATTGCTAGCCAACTGAATAATGTAGTATGCCATCGCTG   1380
CAATTTGCGAATGATTTGTGTGCTTTTGCAGCAATAGATGGAATCATTGAAGCACCTCCA   1440
ATCAAAACACGCTGCATCCAATGCCATTGCAACCCAGGGGGGGGGGGTAATCATTAACTT   1500
TCAATTAGGGGGTCCATTCCAAGATTTGGGGTCTTAAATCTTAC                   1544
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Figure 2. The nucleotide and deduced amino acid sequences of the arginine kinase from the jellyfish *Stomolophus* sp.2. Start and end codons are framed; the characteristic phosphagen kinase signature is shaded in grey.

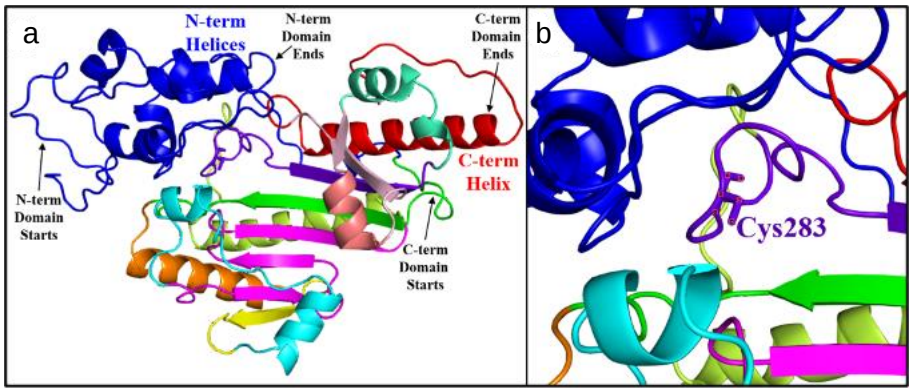


Figure 3. The predicted 3D structure of creatine kinase type-B from *Stomolophus* sp.2. a) 3D structure and b) active site. The N-terminal domain PS51509 (residues 11-98, blue). The C-terminal domain PS51510 (residues 125-367, starts in green and ends in red). The nucleotide-phosphate binding sites (residues in the 128-132, 292-296 positions). The nucleotide-phosphate binding amino acids (residues 191 and 236). The phosphagen kinase signature PS00112 (residues 283-289, purple). Look at Cysteine 283 residue, which participates in the nucleotide-phosphate transfer reaction.

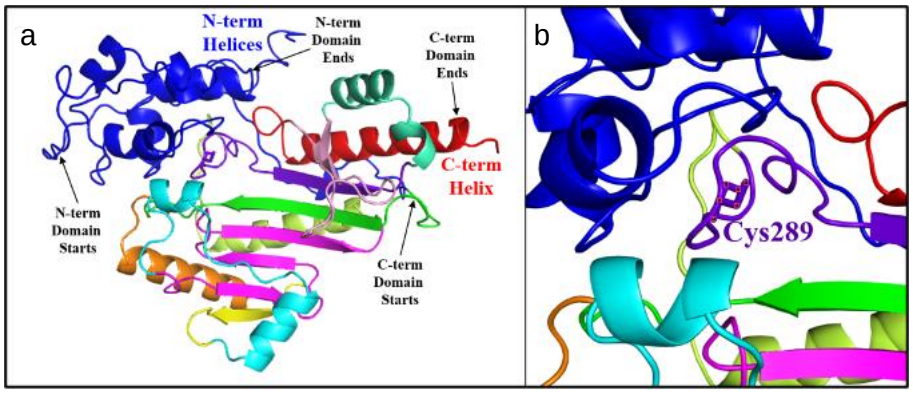


Figure 4. Predicted structural model of arginine kinase from *Stomolophus* sp.2. a) 3D structure, and b) active site. The N-terminal domain PS51509 (residues 26-109, blue). The C-terminal domain PS51510 (residues 137-374, starts in green and ends in red). The nucleotide-phosphate binding sites (residues 140-144, 298-302, and 327-332). The nucleotide-phosphate binding amino acids (residues 203 and 247). The phosphagen kinase signature PS00112 (residues 289-295, purple). Look at the cysteine residue 289, which participates in the nucleotide-phosphate transfer reaction.

together, creating a cleft where catalysis occurs upon substrate binding.

The jellyfish AK predicted structure shares the conserved characteristics and signatures of the arginine-phosphate/arginine-kinase system. A DALI comparison yielded an RMSD of 0.889 and an AK of the anemone *A. japonica* (PDB: 4RF8). These data confirmed AK presence in the cannonball jellyfish. As previously suggested, the crystallographic structure of the AK from *Stomolophus* sp.2 will help confirm the enzyme's structure.

Phylogenetic analysis of arginine and creatine kinases (AK and CK) from *Stomolophus* sp.2

The phylogenetic analysis of CK-B included 24 nucleotide sequences from three species of Ctenophora, five species of Anthozoa, and a member of the Porifera plesiomorphic taxa as the outgroup (Fig. 5). The *Stomolophus* sp.2 CK-B consensus sequence and six of the seven CK-B sequences obtained from the transcriptome, with a length of 1,146 bp, are included as an internal group. The topology recovers Porifera as the outgroup and two main clades: one for Cnidaria (Anthozoa and *Stomolophus*) and the other for the diver-

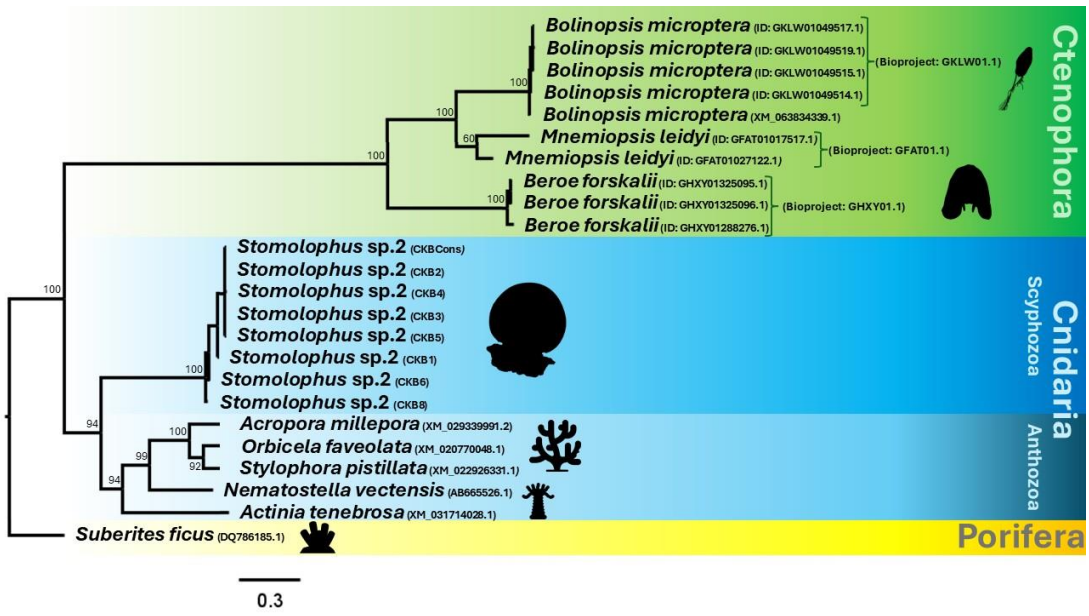


Figure 5. Recovered phylogenetic tree of creatine kinase type-B nucleotide sequences constructed under Bayesian inference (MrBayes3.2). CKBCons: consensus sequence; CKB1-8: sequences found in this study. Numbers in nodes indicate posterior probabilities. GenBank numbers and ID numbers continue the scientific names.

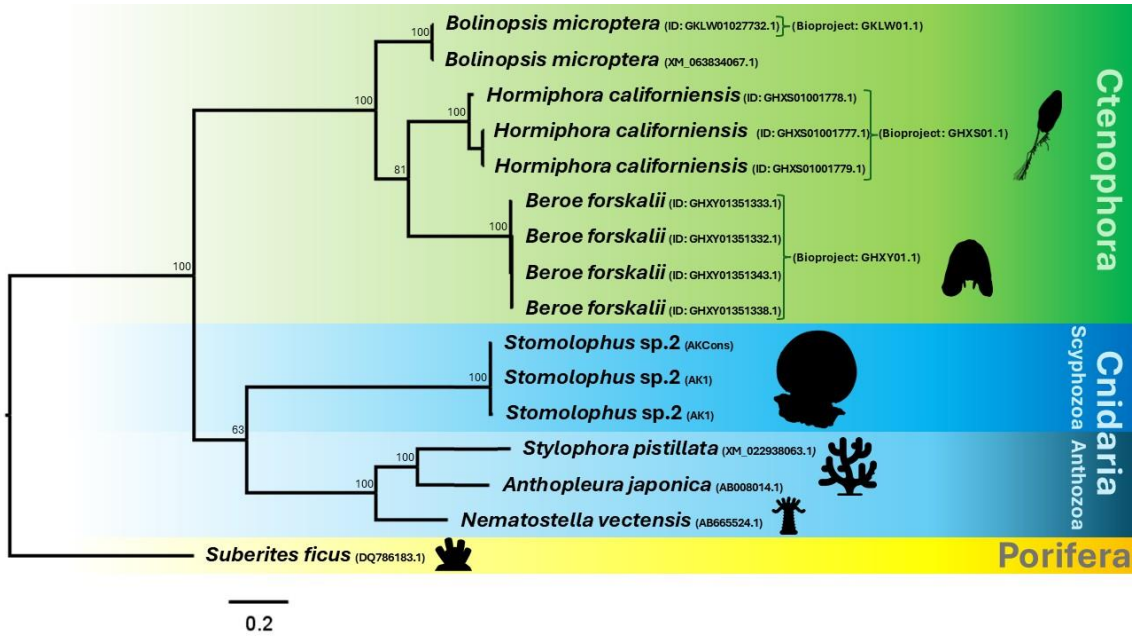


Figure 6. Phylogenetic tree of arginine kinase nucleotide sequences recovered by Bayesian inference (MrBayes 3.2). AKCons: consensus sequence; AK1-2: sequences found in this study. Numbers in nodes indicate posterior probabilities. GenBank and accession numbers follow the scientific names.

gent Ctenophora. Monophyly is observed across the tree with high nodal support (posterior probability).

The phylogenetic analysis of AK recovered a topology similar to CK-B, with well-supported monophyly. We used 16 nucleotide sequences from eight species of Ctenophora, Cnidaria (Anthozoa and *Stomolophus*), and Porifera for the analysis, as follows: two of the four AK sequences were obtained from the transcript-tome

of *Stomolophus* sp.2 (the remaining two sequences were discarded because they proved to be short AK regions that contributed little information). The AK consensus (Fig. 6) is 1,134 bp long and is also included as an internal group. Porifera remains plesiomorphic as a sister to the main clades of Cnidaria (Anthozoa and *Stomolophus*) and Ctenophora.

DISCUSSION

Transcripts and predictive 3D models of AK and CK-B

The search of the transcriptome of the cannonball jellyfish *Stomolophus* sp.2, reported by Gamero-Mora et al. (2025), identified two transcripts encoding AK and CK (typical of phosphagen metabolism). The sequences of both proteins were characterized by an informatics approach to confirm their identity. Multiple alignments were generated based on the amino acid sequences of AK and CK, and different characteristics of both enzymes were identified.

Analysis of the AK and CK amino acid sequences (GenBank PV749638 and PV754314, respectively) revealed that both sequences possess the PS00112 signature, a key feature within the active site of a phosphagen kinase enzyme, characterized by the 8-residue pattern "C-P-x(0,1)-[ST]-N-[ILV]-G-T" (the initial cysteine at position 1 of this signature is a key amino acid in the transferase reaction; the position 3 represented as "x(0.1)-" indicates that "x" can be substituted by any amino acid "x(1)-" or even deleted "x(0)-" at that signature position; the amino acids within brackets at positions 4 and 6 represent the specific options found at those positions) which are located at amino acid positions 289-295 and 283-289 of AK and CK, respectively. In addition, the presence of the N-terminal domain of phosphagen kinase (PS51509) was identified in regions 26-109 of AK and 11-98 of CK. According to what is reported for this domain, alpha-helices are the secondary structures that predominate in this domain (Bush et al. 2011); this agrees with the results obtained in the structural models. Additionally, the typical C-terminal domain of phosphagen kinase (PS51510) is located in regions 137-374 and 125-367 of AK and CK, respectively (Conejo et al. 2007, Tanaka et al. 2007, Andrews et al. 2008, Lim et al. 2010).

Furthermore, the alignment results identify the guanidino specificity region (GS region) in both enzymes, which folds as a flexible loop in both 3D models within the N-terminal domain of the AK predictive model in the 74-89 region. This region gives essential capacity to the N-terminal domain since its appropriate folding positions the Glu80 residue (E) and the Gln221 residue (Q) of the C-terminal domain close to each other in space, since both interact with each other, forming a hydrogen bond that keeps the enzyme stabilized after binding to the arginine molecule (Uda et al. 2006, 2012). Furthermore, in the GS region of AK in most organisms, a Gly residue interacts with the arginine substrate, but in the AK of *Stomolophus* sp.2, Arg82 replaces it; this does not create a conflict during catalysis because the Gly backbone interacts with the carboxyl end of the arginine substrate (Zhou et al. 1998), a function that can be carried out by several different amino acid residues. On the other hand, in the case of the *Stomolophus* sp.2 CK enzyme, the GS region is located in the 59-78 region, where we have an insertion of three specific amino acids (P67, Y68, and I69) that are necessary to accept the creatine substrate, a larger compound than arginine (Vasconcellos et al. 2024), which is also where we find the His66 residue that is suggested to form a hydrogen bond with the Asp326 residue so that both amino acids intervene in the interaction of N- and C-terminal domains, respectively (Uda et al. 2012).

Phosphagen kinases have been reported to have nucleotide-binding sites, which were also found during the bioinformatic analysis performed. Both enzymes have three nucleotide-phosphate binding sites where they act to produce energy. These sites are located at positions 140-144, 298-302 and 322-327 of the AK sequence, and at positions 128-132, 292-296 and 320-325 of the CK sequence of *Stomolophus* sp.2. Both, their three nucleotide-binding sites are located within the C-terminal domain, that coincides with reports for essential sites for enzyme catalytic activity (Forstner et al. 1999, Merceron et al. 2015). Furthermore, two nucleotide-phosphate (ATP)- binding amino acids are highlighted, both located within the C-terminal domain of both *Stomolophus* sp.2 enzymes. It should be noted that in both cases they are the same amino acids, only varying in position in the sequence, being for AK: His203 and Arg247; while for CK: His191 and Arg236. It has been reported that phosphagen kinases from different species use amino acid residues to bind to a substrate and catalyze the phosphate group transfer reaction from the residues in question (Lim et al. 2010). Therefore, the deduced amino acid sequences and their resulting structural models have the specific characteristics that allow us to confirm that the cannonball jellyfish has the two phosphagen kinase enzymes that are notable for being present in the vast majority of animal kingdom phyla; however, the crystallographic structure of AK and CK is necessary to obtain for more detailed studies.

Phylogenetic analysis

Confirmation of the presence of both phosphagen enzymes in the jellyfish *Stomolophus* sp.2 agrees with the phylogenetic signal for each, given the closely related groups, and generates similar topologies in this study for both, consistent with what has been reported in the literature. Porifera, as a plesiomorphic taxon in the cladogram (outgroup), supports the sister monophyletic clades Cnidaria and Ctenophora (Nielsen 2012, Gershwin 2016), which share a common ancestor that inherited both phragmoplasts in the evolutionary lineage. Subclass Medusozoa is represented by Scyphozoa (*Stomolophus*) and Anthozoa, represented by Octocoralia (*Acropora*, *Orbicella* and *Stylophora*) and Actiniaria (*Nematostella* and *Actinia*). Conversely, outside Cnidaria, Ctenophora diverges into the classes Nuda (*Beroe*) and Tentaculata (*Bolinopsis* and *Mnemiopsis*). The existence of the creatine system in *Stomolophus* sp.2 in our study, and the presence of the arginine system as alternative pathways for rapidly obtaining energy under stress conditions, apparently throughout Cnidaria (Uda et al. 2012, Bonilla et al. 2021, Vasconcellos et al. 2024, Fung et al. 2025), confirm the persistence of both systems in animal evolutionary history since the origin of eumetazoans. Furthermore, Conejo et al. (2007) indicate that two AK genes in choanoflagellates are extensively similar to the CK and AK genes of sponges, postulating a possible evolutionary trend in the emergence of both phosphagen systems.

Based on the above, different hypotheses can be generated to explain the presence of both enzymes, using *Stomolophus* sp.2 as a model cnidarian with notable early metazoan characteristics. Proposal that the phosphagen enzyme family was generated from gene duplication and fusion events from an ancestral enzyme, as well as these enzyme lineages changing among themselves until generating isoforms, such as the case of the CK enzyme, or even being selected by a specific clade, as apparently occurs in the divergence of only CK system in vertebrates, separating from AK, which is considered its ancestral gene (Suzuki et al. 1998, 1999, Uda et al. 2006, Ellington & Suzuki 2007, Conejo et al. 2007, Vasconcellos et al. 2024). Even though there are other less common phosphagen enzymes such as glycocyamine kinase (GK), taurocyamine kinase (TK), hypotaurocyamine kinase (HTK), worm kinase (LK), and ophelin kinase (OK), which can be found within the Spiralia clade, which includes mollusks, annelids, platyhelminthes, polychaetes, and other invertebrate phyla (Ellington 2001).

Our AK and CK enzyme computer analysis, including other Scyphozoans (*Rhopilema*, *Aurelia*, and *Nemopilema*) not shown here, results in a high degree of variation, apparently supporting a large number of mutations in the short term in these genes (according to evolutionary times), resulting in new or alternate genes, as is the case with the GS region among all the different phosphagen enzymes. A direct relationship has been reported between the length of the GS region and the size of the substrate catalyzed by the phosphagen enzyme in question (Vasconcellos et al. 2024). Kang (2025) emphasizes high structural variability in this enzyme family, mentioning that vertebrate CK forms a distinct clade among animals, while AK and other related invertebrate phosphagen kinase enzymes present diverse structural adaptations, such as a different domain architecture or an active site mobility for each enzyme, which can be explained by diverse evolutionary pressures and a high degree of genetic plasticity. The presence of both phosphagen systems in jellyfish represents adaptive traits that have contributed to their survival throughout their evolutionary history. A detailed study of these systems, detected in our research, may help answer questions about their survival mechanisms.

CONCLUSIONS

We confirm the presence of both phosphagen enzymes in the jellyfish *Stomolophus* sp.2. Our characterization of the Ak and Ck phosphagen systems confirms the existence of molecular properties to fulfill the function of producing rapid response energy under stress conditions, and also suggests the origin of phosphagen enzymes as a lineage from Porifera to Chaetognata.

Credit author contribution

D. Sastré-Velásquez: investigation, methodology, data curation, software, formal analysis, writing - original draft, writing-review & editing and visualization; E. De La Re-Vega: funding acquisition, conceptualization, methodology, data curation, formal analysis, visualization, writing-review & editing and project administration; A. Muhlia-Almazán: funding acquisition, methodology, data curation, conceptualization, formal analysis, visualization, writing - original draft and writing-review & editing; A. Varela-Romero: formal analysis, visualization and writing-review & editing; M. Rodríguez-Armenta: writing-review & editing; A. Fernández-Giménez: investigation and writing-review & editing.

Conflict of interest

The authors declare no known competing financial interests or personal relationships that could have influenced the work reported in this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGMENTS

We thank the Mexican National Council for Science and Technology (CONACYT) [grant Fronteras de la Ciencia, 171862] and Muhlia-Almazán. In addition, thanks to the team of Dr. De La Re-Vega, Dr. Muhlia-Almazán, Dr. Rodríguez-Armenta, Dr. Varela-Romero, and Dr. Fernández-Jiménez, for their constant willingness and support throughout the work carried out; this would not have been possible without their guidance.

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Received: July 1, 2026; Accepted: September 29, 2026

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