© 2021 Journal of Pharmacy & Pharmacognosy Research, 9 (1), 1-12, 2021
ISSN 0719-4250
http://jppres.com/jppres

Original Article

Variation of cytochrome oxidase-I gene and venom proteins of *Rhopalurus junceus* populations in the *Moanicum* sector: Pharmacological implications

[Variación del gen citocromo oxidasa-I y proteínas del veneno de poblaciones de *Rhopalurus junceus* en el sector *Moanicum*: Implicaciones farmacológicas]

Rodolfo Rodríguez-Ravelo[1,a], Ariel Ruíz-Urquiola[2,a], Lourival D. Possani-Postay[3], Humberto J. Morris-Quevedo[4], Magdelaine Rodríguez-Ravelo[5], Georgina Espinosa-López[6*]

[1]Center for Mountain Development, Ministry of Science, Technology and Environment, El Salvador, Guantánamo, Cuba.
[2]Museum für Naturkunde, Leibniz Institute for Research on Evolution and Biodiversity, Humboldt University, 10115 Berlin, Germany.
[3]Department of Molecular Medicine and Bioprocesses, Institute of Biotechnology, National Autonomous University of Mexico, Cuernavaca 62210, Mexico.
[4]Center of Studies for Industrial Biotechnology (CEBI), Universidad de Oriente, Santiago de Cuba 5, CP 90500, Cuba.
[5]Department of Pediatry, Pediatric Hospital "Pedro A. Pérez", Guantánamo, Cuba.
[6]Department of Biochemistry, Faculty of Biology, University of Havana, 25 Street # 455, Vedado, Havana 4, CP 10400, Cuba.
[a]These two authors contributed equally.
*E-mail: georgina@fbio.uh.cu

Abstract

Context: In Cuba, the venom of the endemic scorpion *Rhopalurus junceus* has been used in traditional medicine for cancer treatment. The genetic variability in populations from distinct origin and differences in the composition of venom –a little explored topic- could influence the pharmacological effects of products used in medical practice.

Aims: To characterize the populations of *R. junceus* from six different districts of the *Moanicum* sector in northeastern Cuba, taking into account the variations in the sequences of cytochrome oxidase subunit I gene (COI) and peptide masses of venom.

Methods: For the genetic characterization, a 658 bp gene fragment of COI was amplified by PCR. The genetic structure of populations was analyzed hierarchically, using an analysis of molecular variance (AMOVA). In proteomic analysis, molecular masses of venom constituents were arranged in increasing order, and different components were considered when masses differed by 2 Da.

Results: *R. junceus* species in the six districts studied in the *Moanicum* sector belong to different populations. The sequences of COI gene showed high levels of genetic variability, recovering 47 haplotypes (87% unique). The composition of the venom was also significantly different between the districts. The most abundant components presented masses of 3-6 kDa (K^+-channel specific peptides) and 6,1-10 kDa (Na^+-channel specific peptides).

Conclusions: Populations of *R. junceus* showed high genetic differentiation and a broad intraspecific variation in the venom peptides (3-10 kDa) in six districts of *Moanicum* sector of Cuba. This richness in scorpion varieties with different toxins profile should be considered for pharmacological applications.

Keywords: cytochrome oxidase subunit I; molecular variance; *Rhopalurus junceus*; scorpion venom.

Resumen

Contexto: En Cuba, el veneno del escorpión endémico *Rhopalurus junceus* se ha utilizado en la medicina tradicional para el tratamiento del cáncer. La variabilidad genética en poblaciones de distintos orígenes y las diferencias en la composición del veneno -tema poco explorado- podrían influir en los efectos farmacológicos de productos utilizados en la práctica médica.

Objetivos: Caracterizar poblaciones de *R. junceus* de seis distritos diferentes del sector *Moanicum* en el nordeste de Cuba, teniendo en cuenta las variaciones en las secuencias del gen citocromo oxidasa subunidad I (COI) y las masas peptídicas del veneno.

Métodos: Se amplificó un fragmento de COI de 658 pb por PCR. La estructura genética de las poblaciones se analizó jerárquicamente, utilizando un análisis de varianza molecular (AMOVA). En el análisis proteómico, las masas moleculares de los péptidos del veneno se organizaron en orden creciente, y se consideraron componentes diferentes cuando diferían en 2 Da.

Resultados: Los seis distritos estudiados en el sector *Moanicum* pertenecen a poblaciones diferentes. Las secuencias del gen COI mostraron altos niveles de variabilidad genética, recuperando 47 haplotipos (87% únicos). La composición del veneno también fue significativamente diferente entre los distritos. Los componentes más abundantes presentaron masas de 3-6 kDa (péptidos específicos del canal K^+) y 6,1-10 kDa (específicos del canal Na^+).

Conclusiones: Las poblaciones de *R. junceus* mostraron alta diferenciación genética y variación intraespecífica en los péptidos del veneno (3-10 kDa) en seis distritos del sector *Moanicum*. Esta riqueza de escorpiones con diferentes perfiles de toxinas debe considerarse para las aplicaciones farmacológicas.

Palabras Clave: citocromo oxidasa subunidad I; *Rhopalurus junceus*; varianza molecular; veneno de escorpión.

ARTICLE INFO
Received: June 30, 2020.
Received in revised form: September 11, 2020.
Accepted: September 12, 2020.
Available Online: September 15, 2020.

INTRODUCTION

The *Moanicum* phytogeographic sector in the northeastern region of Cuba presents a complex geomorphological evolution with a predominance of mountains, intramontane valleys and a dense hydrographic network (Borhidi, 1996). These natural components favor the richness of habitats, and a high level of endemism (Mancina and Cruz, 2017).

The Cuban scorpion *Rhopalurus junceus* is an endemic species belonging to *Buthidae* family, whose venom has been used for a long time in Cuban traditional medicine for treatment of some illnesses, including cancer (http://escozul-cuba.com/). Previous reports have shown various aspects of *R. junceus* venom, such as: electrophoretic and chromatographic separation (Hernández-Betancourt et al., 2009), biochemical/molecular characterization (García-Gómez et al., 2011), a comparative proteomic analysis (Rodríguez-Ravelo et al., 2015), the cytotoxic effect against epithelial cancer cell lines (Díaz-García et al., 2013) and the synergistic effect of the venom combined with conventional cytostatics in cervical cancer cell line HeLa (Yglesias-Rivera et al., 2019).

Research on scorpion venoms has been largely restricted to a few species within the medically important *Buthidae* family, most of the studies deal with scorpions from the North Africa area, continental America, China, India and the Caucasian regions, but far less is known about scorpion from other regions, including the Caribbean Islands. Natural selection and the ecological environment where these animals live are considered major facts that should define the substantial biodiversity of pharmacologically active components contained in animal venoms (Rodríguez-Ravelo et al., 2013).

The genetic variability displayed by scorpion populations from distinct origin and differences in venom composition (absence or presence of certain constituents) are scarcely studied. This investigation was aimed to analyze both genetic variation and peptide masses differentiation of *R. junceus* venom in six districts of *Moanicum* sector of Cuba. For the genetic characterization, the gene encoding cytochrome oxidase subunit I was chosen in agreement with its frequently use in scorpion populations molecular studies (Ojanguren-Affiltro et al., 2016; Suranse et al., 2019). To the best of our knowledge, there is no evidence about the genetic differentiation and the intraspecific variation in the venom peptides of *R. junceus* in Cuba using these molecular approaches. Scorpion varieties with different toxins profile should be considered for pharmacological applications.

MATERIAL AND METHODS

Reagents

Taq polymerase was purchased from New England Lab Fisher Scientific and nucleotides from Promega PCR Nucleotide Mix 25 mM (Fisher BioReagent). The primers were designed according to Folmer et al. (1994) and synthetized in the DNA Laboratory of the Museum für Naturkunde, Leibniz Institute for Research on Evolution and Biodiversity, Humboldt University (Germany). The rest of the reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Genetic characterization of the populations of *Rhopalurus junceus*

For the genetic characterization, 107 scorpions from six districts of the *Moanicum* sector were used, including 24 from *Nipense*, 11 from *Cristalense*, 20 from *Moaënse*, 17 from *Baracoënse*, 15 from *Purialense* and 20 from *Yaterense*. Adult animals were identified according to Teruel and Armas (2006).

Muscles were taken from the right leg III and preserved in ethanol (96%) at 4ºC. Extraction of total DNA was performed according to the BioSprint 96 DNA Plant protocol for plant tissues in the BioSprint 96 workstation (USA).

A 658 bp fragment of the cytochrome oxidase subunit I (COI) gene was amplified with the pair of universal primers LCO 1490 (5'GGTCAACAAATCATAAAGATATTGG) and HCO 2198

(5'TAAACTTCAGGGTGACCAAAAAATCA). Polymerase chain reaction (PCR) was performed in 25 µL of reaction volume containing 0.2 µL of Taq DNA polymerase (5 000 U/mL), 2.5 µL of 10X ThermoPol PCR Buffer (1x contains 20 mM Tris-HCl, 10 mM $(NH_4)_2SO4$, 10 mM KCl, 2 mM $MgSO_4$ and 0.1% Triton® X-100 pH 8.8); 0.5 µL of dNTP (10 mM of each nucleotide), 1 µL of each primer (LCO 1490- 55 nmol; HCO 2198- 68 nmol) for 1 µL of genomic DNA (0.5-500 ng) in bidistilled water. The process was carried out in an automatic thermocycler (ThermalCycler 2720).

The nucleotide sequences of the COI gene fragment were aligned for identifying polymorphic sites using the ClustalW algorithm available in the Mega v.5 program (Tamura et al., 2011). The haplotypic indexes (Hd), nucleotide diversity (π) and selective neutrality were calculated by the "Tajima's D" and "Fu's Fs". All these parameters were determined with the Arlequin program v.3.1 (Excoffier et al., 2005).

Haplotypic frequencies between districts were compared through the χ^2 independence test corrected with the Bonferroni procedure with 1000 simulations, using the CHIRXC program (Zaykin and Pudovkin, 1993). The genetic structure of populations in the districts was analyzed hierarchically, by means of an analysis of molecular variance (AMOVA) using the Arlequin program v. 3.1 (Excoffier et al., 2005).

Four hypothetical scenes were established, based on the statistically significant differences found between pairs of districts through the χ^2 test and the genetic differentiation index (Fst). The proposed scenarios were: 1) the entire *Moanicum* sector constitutes a panmicta population, 2) the *Cristalense* and *Yaterense* districts are a demographic unit with respect to *Nipense*, *Moaënse*, *Baracoënse* and *Purialense*, 3) the *Purialense* and *Yaterense* districts are a demographic unit with respect to *Nipense*, *Moaënse*, *Cristalense* and *Baracoënse,* and 4) the *Purialense*, *Cristalense* and *Yaterense* districts are a demographic unit with respect to those of *Nipense*, *Moaënse* and *Baracoënse*.

Proteomic characterization of *Rhopalurus junceus* venom

Venom from scorpions was obtained by electrical stimulation. This method does not damage animal integrity and allows the best yields compared to other methods (Tobassum et al., 2018). Proteomic characterization was performed based on previously results of Rodríguez-Ravelo et al. (2013). Molecular mass data for the *R. junceus* venom from each district were determined on the LCQFleetThermo Fisher Scientific Inc. (San Jose, CA, USA) mass spectrometer. Peptidic masses were arranged in an increasing order and different components were considered when molecular masses differed by 2 Da. Molecular mass ranges were established as follows: <3 kDa, 3-6 kDa, 6.1-10 kDa and >10 kDa. "Unique" molecular masses were those appeared only in one district, and "common" those found in more than one district within each evaluated interval.

The contribution of molecular masses by districts were determined through the χ^2 independence test with 1000 Monte Carlo simulations with the CHIRXC program (Zaykin and Pudovkin, 1993).

RESULTS

Variation of the cytochrome oxidase I gene of *R. junceus* in the *Moanicum* sector

The analysis of the 658 bp sequence of COI gene in the six districts of the *Moanicum* sector detected 134 variable sites, of which 96 were parsimoniously informative and 38 constituted point mutations. On average in all populations, the most frequent base was thymine and the one with lesser frequency was cytosine (Table 1).

The translation of the nucleotide sequences into polypeptides allowed the identification of 115 variable sites, of which 27 were point mutations. Ten of these 27 mutations represented *"miss sense"* mutations in which one amino acid was replaced by other of different chemical nature.

Table 1. Nucleotide composition of a COI fragment of *R. junceus*.

District	A (%)	T (%)	C (%)	G (%)
Nipense	18.37	44.36	13.54	23.73
Cristalense	18.01	44.00	13.78	24.21
Moaënse	17.64	44.33	13.64	24.39
Baracoënse	17.48	43.97	14.03	24.51
Purialense	17.92	43.92	13.90	24.26
Yaterense	18.01	44.02	13.76	24.22
Total	**17.81**	**44.05**	**13.82**	**24.39**

A: Adenine; T: Thymine; C: Cytosine; G: Guanine; COI: Cytochrome oxidase I.

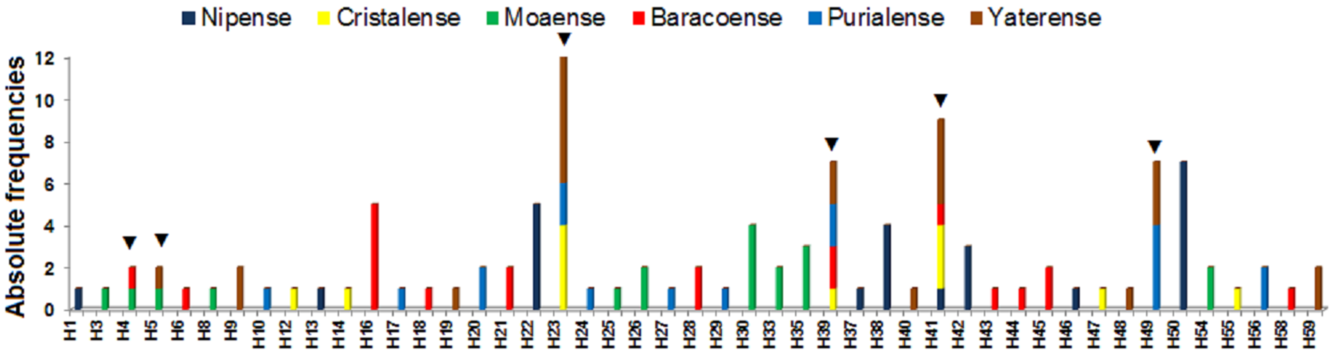


Figure 1. Geographical distribution of haplotypic frequencies of *Rhopalurus junceus* in the six districts of *Moanicum* sector.

Haplotypes were represented in different colors according to phytogeographic districts. The triangles (▼) indicate those that are shared between the districts.

The sequencing of 658 bases of COI gene in *R. junceus* in the six districts revealed high levels of genetic variability, recovering 47 haplotypes with 41 (87%) of unique haplotypes. Only six haplotypes are shared in two districts, and the most shared haplotypes were h39 and h41 present in four districts (Fig. 1). Thus, the values of haplotypic diversity (Hd) and nucleotide diversity (π) were high for the *Moanicum* sector, in general, and for each of the districts, in particular (Table 2).

The Tajima D and Fs values of Fu tests (Table 3) for each district and for the entire population, did not show significant differences from zero (D = p>0.05 and Fu = p>0.02). The population of the *Moanicum* sector and their districts were found under neutral equilibrium, where most of the evolutionary changes are due to genetic drift.

The *R. junceus* populations in the phytogeographic districts of the *Moanicum* sector were genetically structured since the χ^2 values of the haplotypic frequencies between districts (Table 4) and the significance of the Fst values (Table 5) are generally statistically significant. The districts *Cristalense-Yaterense* and *Purialense-Yaterense* represented an exception, because of individuals exchange between these pairs of districts.

The hierarchical analysis of molecular variance under the four hypothetical scenarios considered in this study (Table 6) showed that the populations of the *Cristalense* and *Yaterense* districts, as well as, *Yaterense* and *Purialense* did not exhibit differences between- or within the groups. The scenario that grouped *Cristalense*, *Yaterense* and *Purialense* did not present differences between- and within the groups either. This fact clarifies the similarities observed in both Fst and χ^2 values between the three districts: *Cristalense*, *Yaterense* and *Purialense*.

Table 2. Haplotypic diversity (Hd), nucleotide diversity (π) and average number of different nucleotides (K) per population in *R. junceus*.

District	**n**	**Hd ± SD**	**π ± SD**	**K**
Nipense	24	0.86 ± 0.04	0.019 ± 0.01	4.76
Cristalense	12	0.86 ± 0.08	0.048 ± 0.03	6.15
Moaënse	18	0.92 ± 0.04	0.013 ± 0.01	8.46
Baracoënse	20	0.93 ± 0.04	0.028 ± 0.01	11.74
Purialense	17	0.93 ± 0.04	0.054 ± 0.03	9.30
Yaterense	23	0.89 ± 0.04	0.045 ± 0.02	6.18
Total	**114**	**0.91**	**0.038**	**26.96**

n: Sample number; SD: Standard deviation.

Table 3. Neutrality test of Tajima (D) and Fu´s (Fs) values for *R. junceus* populations with COI marker.

District	**Tajima D**	**P**	**Fu's F_S**	**P**
Nipense	-0.214	0.458	4.599	0.953
Cristalense	1.352	0.943	6.020	0.983
Moaënse	1.495	0.947	0.545	0.624
Baracoënse	-0.316	0.419	1.946	0.802
Purialense	0.723	0.806	5.739	0.984
Yaterense	2.050	0.998	8.999	0.998

Table 4. Probability of the null hypothesis -no differences between the pairs of compared districts of the *Moanicum* sector for *R. junceus*.

χ^2 (d.f.) $p<0.05$	***Nipense***	***Cristalense***	***Moaënse***	***Baracoënse***	***Purialense***	***Yaterense***
Nipense	0	32.7 (14)	42.0 (18)	41.9 (19)	41.0 (18)	43.80 (17)
Cristalense	<0.001	0	30 (16)	28.80 (17)	20.75 (14)	**13.78 (13)**
Moaënse	<0.01	<0.001	0	35.99 (20)	35 (19)	38.97 (18)
Baracoënse	<0.01	=0.002	<0.001	0	37 (21)	39.78 (20)
Purialense	<0.05	=0.023	<0.001	< 0.001	0	**22.75 (16)**
Yaterense	<0.01	**=0.43**	<0.001	<0.001	**=0.05**	0

Calculated χ^2 values; d.f.: Degrees of freedom; values in bold: Comparisons that do not show significant statistical differences.

Table 5. Genetic differentiation values (Fst) by pairs of districts in the *Moanicum* sector using the *R. junceus* COI gene.

F_{st} $p<0.05$	*Nipense*	*Cristalense*	*Moaënse*	*Baracoënse*	*Purialense*	*Yaterense*
Nipense	0	0.290	0.622	0.534	0.316	0.303
Cristalense	***	0	0.320	0.150	-0.035	-0.050
Moaënse	**	***	0	0.184	0.246	0.340
Baracoënse	**	***	**	0	0.108	0.203
Purialense	*	**	***	***	0	-0.001
Yaterense	**	ns	***	***	ns	0

Probability value of Fst (p= 0.05 *, p= 0.01**, p= 0.001 ***, ns= no significant).

Table 6. Hierarchical analysis of molecular variance under four hypothetical scenarios of geographic structure in populations of *R. junceus* in the *Moanicum* sector.

Hypothetical scenarios	Between groups (% total; p)	Between groups, inside the scenario (% total; p)	Within the groups (% total; p)
(I) The six districts constitute a demographic unit	26.1; p<0.001		73,9; p=0.0
(II) *Cristalense* + *Yaterense* as a demographic unit	30.12; p=0.07	-2.1%; p=0.81	72,8; p<0.001
(III) *Purialense* + *Yaterense* as a demographic unit	25.8; p=0.19	1.6%; p=0.39	72.5; p<0.001
(IV) *Cristalense* +*Yaterense* + *Purialense* as a demographic unit	30.1; p=0.05	-0.4; p=0.74	70.3; p<0.001

Variation in the peptide components of the venom of *R. junceus* in the *Moanicum* sector

In the analysis of the 153 venom components of the six districts of the *Moanicum* sector, it was found a relative abundance (63%) of unique peptides, regardless the molecular mass interval (Fig. 2). However, common molecular masses between at least two phytogeographic districts were more frequent in the 6.1 - 10 kDa molecular mass range.

The contribution of different phytogeographic origins to venom components grouped by molecular mass intervals was independent within each district, except in the *Baracoënse* district (Fig. 3). Molecular masses between 6.1 and 10 kDa were more frequent in the venom of scorpions than the rest of the intervals in all the districts. It was followed by the 3 - 6 kDa range, and lowest in the intervals of less than 3 kDa and greater than 10 kDa. In particular, the contributions of molecular masses lower than 3 kDa, and between 3 to 6 kDa, were similar in the *Nipense* district.

The components corresponding to molecular masses greater than 10 kDa were infrequent in the venom, and only belong to three phytogeographic origins of the center (*Yaterense*) and northwest (*Moaënse* and *Baracoënse*) of the *Moanicum* sector. In contrast, these components were extremely frequent in the *Baracoënse* district (Fig. 3). However, components minor than 3 kDa were found in most districts, except in *Baracoënse*, and were highly frequent in the *Nipense*.

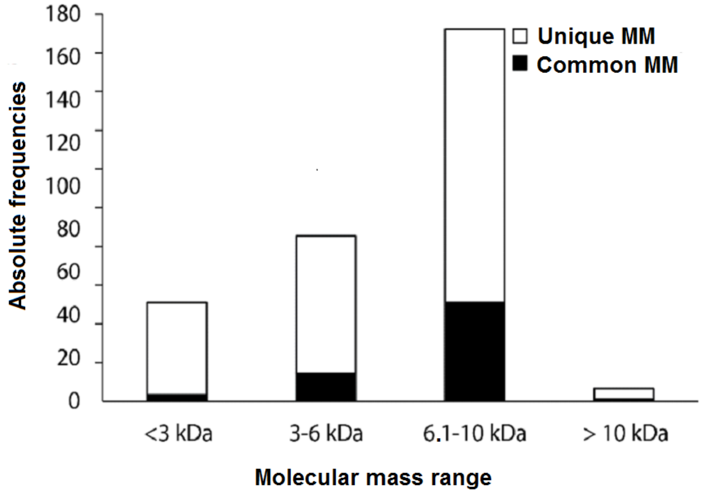
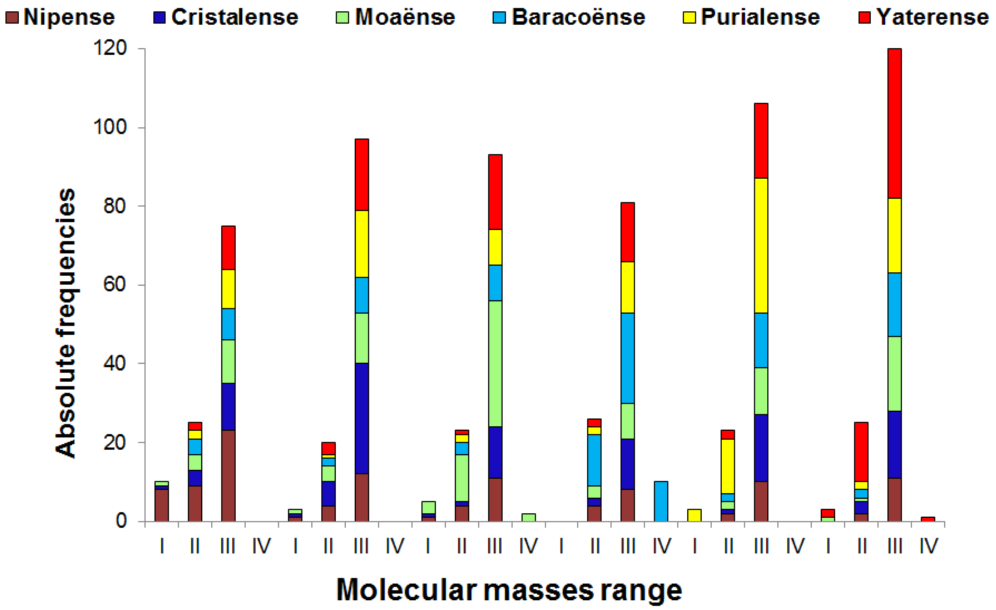


Figure 2. Frequency distribution of the unique and common peptide components of the *Rhopalurus junceus* venom.

MM: Molecular Masses.



A) $11.68_{(10)}$;0.32; $5.82_{(10)}$ 0.88; $12.44_{(15)}$ 0.49; $23.53_{(10)}$ 0.001; $12.29_{(15)}$ 0.25; $12.65_{(15)}$ 0.62

B) I (<3 kDa) 75.34(25) <0.001
II (3.1-6 kDa) 118.47(25) <0.001
III (6.1-10 kDa) 92.84(25) <0.001
IV (>10 kDa) Different

Figure 3. Contribution of the districts in the *Moanicum* sector to venom components of *Rhopalurus junceus* according to the molecular mass intervals of peptides. **A)** Contribution according to the molecular mass intervals in each district; **B)** Contribution according to the molecular mass intervals between the districts.

The results of the statistical analyses are represented as χ^2 (d.f., p) where g.l, the degrees of freedom, and p the probability. Molecular mass intervals are designated by Roman numerals.

In the *Baracoënse* district, the contribution of different origins to the grouped components was highly dependent. Unlike the rest of the districts, the proportion between molecular mass intervals, that comprise components between 3 and up to more than 10 kDa, was less evident. The *Cristalense*, *Purialense*, and *Yaterense* districts share similar amounts of peptides with molecular masses between 6.1 - 10 kDa with each other, and also with the *Baracoënse*.

However, when comparing the groups of components by molecular mass intervals for the different districts, the interactions between them are highly significant (Fig. 3). For the components corresponding to molecular masses less than 3 kDa, the contributions of each district were greater. These components were very frequent in the *Nipense* district in relation to the rest of the districts, On the other hand, in the *Cristalense* district it was an equal contribution of these components *per se* and with the adjacent *Nipense* and *Moaënse* districts. In the *Baracoënse*, *Purialense* and *Yaterense* districts, the molecular masses less than 3 kDa were unique to districts.

The venom components with molecular masses between 3 and 6 kDa were represented in all the districts. In the *Cristalense*, the contribution was similar to the *Nipense* district ($\chi^2_{(5)}$ = 3.00; 0.74) and it was no different from that of the other contributing districts: *Moaënse* ($\chi^2_{(5)}$ = 8.94; 0.13) and *Baracoënse* ($\chi^2_{(5)}$ = 10.13; 0.07), in contrast to the *Purialense* ($\chi^2_{(5)}$ = 16.24; 0.005) and *Yaterense* ($\chi^2_{(5)}$ = 11.38; 0.03).

The *Nipense* district did not differ either in the contributions of the northern districts *Moaënse* ($\chi^2_{(5)}$ = 8.13; 0.14) and *Baracoënse* ($\chi^2_{(5)}$ = 7.48; 0.20), but it contrast with the southern *Purialense* ($\chi^2_{(5)}$ = 16.53; 0.002) and *Yaterense* ($\chi^2_{(5)}$ = 17.01; 0.001). The contributions of the *Moaënse*, *Baracoënse*, *Purialense* and *Yaterense* districts was significantly variable to each other for this range of molecular masses.

The molecular masses corresponding to components between 6.1 - 10 kDa from each of the districts are also more frequent within the district *per se* (Fig. 3). However, in the *Baracoënse* district the distribution of the venom components of the district itself was about twice with respect the contribution in the rest of the districts. The *Yaterense* district had a representation of molecular masses for this interval similar to *Cristalense* ($\chi^2_{(5)}$ = 10.75; 0.06), *Baracoënse* ($\chi^2_{(5)}$ = 9.74; 0.07) and *Purialense* ($\chi^2_{(5)}$ = 11.52; 0.05). This similarity was manifested only between the pairs of *Purialense* and *Cristalense* districts ($\chi^2_{(5)}$ = 9.31; 0.10) and *Purialense* with *Baracoënse* ($\chi^2_{(5)}$ = 10.06; 0.07).

The components of molecular masses greater than 10 kDa were represented only in three districts and were exclusive to them and they were very frequent in the *Baracoënse* district (Fig. 3).

DISCUSSION

Scorpion venom is very complex, so its variation cannot be unequivocally attributed to a few factors. In this study, three of the five terrestrial ecoregions of importance for conservation in the *Moanicum* sector according to the World Wide Fund for Nature (WWF) were recognized: wet and dry forests (*Baracoënse* , *Purialense* and *Yaterense*), the pine forests (*Nipense* and *Moaënse*), and the xeromorphic scrub (*Cristalense*). These are areas with particular ecological, climatic and geomorphological characteristics, having a high richness of endemic species of flora and fauna (Mancina and Cruz-Flores, 2017).

The collection of *R. junceus* scorpions was carried out in an area of 150 km long and 90 km wide (13 500 km^2) in the *Moanicum* sector. Therefore, the variation in the sequences of the COI gene, and that of the peptide masses of venom components could be the result of the effect of both abiotic and biotic factors on *R. junceus* populations. These, in turn, could influence the feeding strategies of scorpions, and potentially their exposure to predation, a poorly studied source of venom variation (Gangur et al., 2017).

Populations of the scorpion *R. junceus* in the *Moanicum* sector displayed a relatively high and homogeneous haplotypic diversity (Hd) (Table 2, Fig. 1). These results are in agreement with values reported by Yamashita and Rhoads (2013) in *Cen-*

truroides vittatus populations from the United States (Hd = 0.86 to 1.00) and Suranse et al. (2019) in *H. tamulus* species (Hd = 0.94) using the same gene.

The evaluation of nucleotide diversity (π) is another way of measuring genetic diversity. A value of 0.038 (3.8%) was obtained in this study for the COI gene of *R. junceus*, closer to those found in other invertebrates, such as 0.0039 - 0.0180 in *Centruroides vittatus* populations from the United States (Yamashita and Rhoads, 2013) and 0.049 for the scorpion *Paruroctonus boreus* (Miller et al., 2014).

The high haplotypic diversity and nucleotide diversity obtained in the *R. junceus* populations of the *Moanicum* sector are consistent with other *Buthidae* taxa from Africa and Europe. Similar to our study, Suranse et al. (2017; 2019) found that changes in allele frequencies were not statistically significant from neutrality. These values of Hd and π could be attributed to the geographical barrier effect between the mentioned populations.

A plausible explanation for the similarity found between the populations of *Cristalense*, *Yaterense* and *Purialense* would arise from the dual role of rivers as a contact barrier, and also as a possible dispersal corridor in rainy periods for adjacent populations, thus producing a discrete gene flow. The marked influence of rivers on genetic diversity has been considered for other scorpion species (Koç et al., 2017).

Under stable demographic conditions, stability in genetic patterns is expected according to the balance between the effect of genetic drift and migration. In this study, the populations of *Cristalense*, *Moaënse*, *Purialense* and *Yaterense* are significantly and positively distant from the "D" statistic (Table 3). This phenomenon could be attributed to the fact that such populations have recently suffered a population bottleneck, in which case the value of π would be little affected (unless this situation is prolonged in time and intensity). However, the value of k (sites of segregation) would be substantially reduced, giving a positive "D".

The genetic structure analysis of *R. junceus* revealed that there are six populations in the *Moanicum* sector in eastern Cuba, suggesting that the differences are due to variations between the populations (districts). However, it does not occur between the *Cristalense-Yaterense-Purialense* districts. In this sense, we are faced with a non-panmictic population in which mating is not random and there is no genetic exchange (Table 5). Likewise, the Fst fixation indexes showed a significant high variation between districts (Table 6) resulting in a selective geographic distribution pattern. This pattern coincides with similar studies of genetic structuring obtained in populations of *Centruroides vittatus* in the United States with values of Fst from 0.86 to 1.00 (Yamashita and Rhoads, 2013). Mountainous systems, similar to the *Moanicum* sector play an important role in shaping the current distribution of various types of genetic data, such as the genetic structure of scorpions (Suranse et al., 2017) and phenetic data like venom peptide composition (Suranse et al., 2019).

The composition of the *Rhopalurus junceus* venom is significantly different between the districts since the highest percentage of peptides found in the venom are unique (Fig. 2). This marked peptidomic differentiation between *R. junceus* populations, known as intraspecific variation, has been studied in different scorpion species such as *Centruroides sculpturatus* from two different regions of Arizona (Carcamo-Noriega et al., 2018), *Parabuthus granulatus*, *Uroplectes otjimbinguensis*, *Uroplectes planimanus* and a scorpionid, *Opistophthal muscarinatus* (Schaffrath et al., 2018). Thus, data from proteomics studies have confirmed the close relationship of scorpion venom composition to environmental and biological factors, which play a crucial role in the quantity and diversity of components.

Most of the *R. junceus* venom peptides were found in the 6-10 kDa mass range. The histogram provided by King and Hardy (2013) indicated a bimodal mass distribution for scorpion venom where most of the peptides were within the 3.5 -

4.0 or 6.5 - 7.5 kDa range. The *R. junceus* peptide mass profile agrees with this general trend considering that one third of its peptides were in the 3 - 6 kDa range. Consistent with the main distribution of 6.1 - 10 kDa, 30% of the *R. junceus* venom peptides were in the range of <3 kDa. According to previous studies of peptide bioactivity, 3 - 4 kDa venom peptides are expected to be blockers of potassium and chloride ion channels, those of 6 - 10 kDa are blockers of sodium ion channels, while the 1 - 2 kDa components can be peptides without disulfide bonds that typically show antimicrobial activity (Cid-Uribe et al., 2017).

Other species of the *Buthidae* family and particularly, the congeneric species *Rhopalurus princeps,* had more than 42% of the components in the molecular mass range of 6.0 - 8.0 kDa, which also correspond to peptides that affect Na^+ channels (Mikaelian et al., 2020). The prevalence of these components that supposedly modulate Na^+ channels could have some practical pharmacological implications, since tumor cells use ion channels to support their proliferation, adhesion and invasion processes. Ion channel expression is also altered, dysregulated, or overexpressed in various types of cancers (Litan and Langhans, 2015).

The second components of *R. junceus* venom in abundance were the modulating components of K^+ channels (3.0 - 4.0 Kda). These could have some kind of pharmacological effect since different types and subtypes of K^+ channels have been overexpressed/ dysregulated in cancers, including glioblastoma, breast, colon, and prostate cancer and lymphoma (Comes et al., 2015). Several studies have shown that the complete venom of *Androctonus crassicauda*, *Centruroides margaritatus*, *Leiurus quinquestriatus*, *Rhopalurus princeps*, and *Rhopalurus junceus* reduced the proliferation of human breast, colorectal, lung, cervical, and laryngeal cancer cells (Ramírez et al., 2018; Mikaelian et al., 2020).

These findings on the *"venomics"* of *R. junceus* populations from the *Moanicum* sector open new opportunities for developing novel peptide-based anticancer drugs. Therefore, we strongly recommend a differentiated inclusion of its venom in the anti-tumor activity tests, since those studies have been carried out so far with samples from other Cuban regions (Díaz-García et al., 2013; 2017; 2019). The intraspecific peptidomic and genetic variations found in *R. junceus* should also be consider in further studies. This variability in venom composition supposes that an expected pharmacological effect would not be reached with scorpions collected from different areas of Cuba, in general, and from the *Moanicum* sector, in particular. The most illustrative case of the biomedical implications of this variation was observed with the divergence of *R. junceus* venom components patented by Díaz-García et al. (2012), whose molecular masses were not confirmed in the peptides fingerprint obtained by Rodríguez-Ravelo et al. (2013; 2015). In this sense, Zancolli et al. (2019) stated that the proteomics of individual snake toxins was invariably associated with the presence or absence of the coding genes.

CONCLUSIONS

Overall, these findings indicate that populations of *R. junceus* showed high diversity and genetic differentiation, as well as a broad intraspecific variation in the venom peptides with molecular masses from 3 to 10 kDa in six districts of *Moanicum* sector of Cuba. This richness in scorpion varieties with different toxins profile should be considered for future pharmacological applications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Authors thank Dr. Thomas von Rintelen, Coordinator of the DNA Laboratory of the Museum für Naturkunde, Leibniz Institute for Research on Evolution and Biodiversity, Humboldt University, by his support in the analysis of mtDNA sequences. This work was also conducted with support from the National Council of Science and Technology, Mexican Government and the National Autonomous University of Mexico, Grant Number IN200113.

REFERENCES

Borhidi A (1996) Phytogeography and vegetation ecology of Cuba. 2edn revised and enlarged edition. Budapest: Akademiai Kiadó.

Carcamo-Noriega EN, Olamendi-Portugal T, Restano-Cassulini R, Rowe A, Uribe-Romero SJ, Becerril B, Possani LD (2018) Intraspecific variation of *Centruroides sculpturatus* scorpion venom from two regions of Arizona. Arch Biochem Biophys 638: 52–57.

Cid-Uribe JI, Jiménez-Vargas JM, Zamudio FZ, Possani LD (2017) Comparative proteomic analysis of female and male venoms from the Mexican scorpion *Centruroides limpidus*: Novel components found. Toxicon 125: 91–98.

Comes N, Serrano-Albarrás A, Capera J, Serrano.Novillo C, Condom E, Ramón y Cajal S, Ferreres JC, Felipe A (2015) Involvement of potassium channels in the progression of cancer to a more malignant phenotype. Biochim Biophys Acta 1848: 2477–2492.

Díaz-García A, Fraga-Castro JA, González-Marrero I, Guevara Orellana I, Medina-Gali RM, Pérez-Capote MR, Garlobo YR, Coipel JR, Rodriguez Torres CC (2012) Peptides from the venom of the *Rhopalurus junceus* scorpion and pharmaceutical composition. WIPO (PCT) WO2012041261A2.

Díaz-García A, Morier-Díaz L, Frion-Herrera Y, Rodríguez-Sánchez H, Caballero-Lorenzo Y, Fraga-Castro J (2013) *In vitro* anticancer effect of venom from Cuban scorpion *Rhopalurus junceus* against a panel of human cancer cell lines. J Venom Res 4: 5–12.

Díaz-García A, Ruiz-Fuentes J, Rodríguez-Sánchez H, Fraga-Castro J (2017) *Rhopalurus junceus* scorpion venom induces apoptosis in the triple negative human breast cancer cell line MDA-MB-231. J Venom Res 8: 9–13.

Díaz-García A, Pimentel-González G, Basaco-Bernabeu T, Rodríguez-Aurrecochea JC, Rodríguez-Sánchez H, Sánchez-Monzón I, Hernández-Gómez M, Rodríguez-Torres C, Rodríguez-Capote MR, Guevara-Orellanes I (2019) Pharmacokinetics and biodistribution of *Rhopalurus junceus* scorpion venom in tumor-bearing mice after intravenous and oral administration. Iran Biomed J 23(4): 287–296.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.11: An integrated software package for population genetics data analysis. Evol Bioinform Online 1: 47–50.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3(5): 294–299.

Gangur AN, Seymour JE, Liddell MJ, Wilson D, Smout MJ, Northfield TD (2017) When is overkill optimal? Tritrophic interactions reveal new insights into venom evolution. Theor Ecol 11: 141–149.

García-Gómez BI, Coronas FIV, Restano-Cassulini R, Rodríguez RR, Possani LD (2011) Biochemical and molecular characterization of the venom from the Cuban scorpion *Rhopalurus junceus*. Toxicon 58: 18–27.

Hernández-Betancourt O, Compte AO, Quesada-Leiva L, Rodríguez-Pargas A (2009) Caracterización electroforética y cromatográfica del veneno del alacrán *Rhopalurus junceus*. Arch Med Camagüey 3: 1–8.

King GF, Hardy MC (2013) Spider-venom peptides: structure, pharmacology, and potential for control of insect pests. Ann Rev Entomol 58: 475–496.

Koç H, Sipahi H, Yagmur EA (2017) Genetic diversity within *Scorpio maurus* (Scorpiones: Scorpionidae) from Turkey. North-Western J Zool 13: 161–164.

Litan A, Langhans SA (2015) Cancer as a channelopathy: ion channels and pumps in tumor development and progression. Front Cell Neurosci 9: 86.

Mancina CA, Cruz-Flores DD (2017) Diversidad biológica terrestre de Cuba. In: Diversidad biológica de Cuba: presentación (Mancina CA, Cruz-Flores D (eds.), La Habana: Editorial AMA, pp. 1–5.

Mikaelian AG, Traboulay E, Zhang XM, Yeritsyan E, Pedersen PL, Ko YH, Matalka KZ (2020) Pleiotropic anticancer properties of scorpion venom peptides: *Rhopalurus princeps* venom as an anticancer agent. Drug Des De Ther 14: 881–893.

Miller AL, Makowsky RA, Formanowicz DR, Prendini L, Cox CL (2014) Cryptic genetic diversity and complex phylogeography of the boreal North American scorpion, *Paruroctonus boreus* (Vaejovidae). Mol Phylogenet Evol 71: 298–307.

Ojanguren-Affilastro AA, Mattoni CI, Ochoa JA, Ramírez MJ, Ceccarelli S, Prendini L (2016) Phylogeny, species delimitation and convergence in the South American Bothriurid scorpion genus *Brachistosternus* Pocock 1893: integrating morphology, nuclear and mitochondrial DNAq. Mol Phylogenet Evol 94: 159–170.

Ramírez A, Vera E, Gamboa-Domínguez A, Lambert P, Gariglio P, Camacho J (2018) Calcium-activated potassium channels as potential early markers of human cervical cancer. Oncol Lett 15(5): 7249–7254.

Rodríguez-Ravelo R, Coronas FIV, Zamudio FZ, González-Morales L, López GE, Urquiola AR, Possani LD (2013) The Cuban scorpion *Rhopalurus junceus* (Scorpiones, Buthidae): component variations in venom samples collected in different geographical areas. J Venom Anim Toxins 19: 13.

Rodríguez-Ravelo R, Batista CVF, Coronas FIV, Zamudio FZ, Hernández-Orihuela L, Espinosa-López G, Ruiz-Urquiola A, Possani LD (2015) Comparative proteomic analysis of male and female venoms from the Cuban scorpion *Rhopalurus junceus*. Toxicon 107: 327–334.

Schaffrath S, Prendini L, Predel R (2018) Intraspecific venom variation in southern African scorpion species of the genera *Parabuthus*, *Uroplectes* and *Opistophthalmus* (Scorpiones: Buthidae, Scorpionidae). Toxicon 144: 83–90.

Suranse V, Sawant NS, Paripatyadar SV, Krutha K, Paingankar MS, Padhye AD (2017) First molecular phylogeny of scorpions of the family Buthidae from India. Mitochondr DNA Part A 28: 606–611.

Suranse V, Sawant NS, Bastawade DB, Dahanukar N (2019) Haplotype diversity in medically important red scorpion (Scorpiones: Buthidae: *Hottentotta tamulus*) from India. J Genet 98:17.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739

Teruel R, Armas LF (2006) Un nuevo *Rhopalurus* Thorell 1876 (Scorpiones: Buthidae) de Cuba oriental. Bol Soc Entomol Aragon 39: 175–179.

Tobassum S, Tahir H, Zahid M, Gardner Q, Ahsan M (2018) Effect of milking method, diet, and temperature on venom production in scorpions. J Insect Sci 18(4): 19.

Yamashita T, Rhoads DD (2013) Species delimitation and morphological divergence in the scorpion *Centruroides vittatus* (Say, 1821): Insights from phylogeography. PLoS ONE 8: e68282.

Yglesias-Rivera A, Rodríguez-Sánchez H, Díaz-García A (2019) Synergistic effect of *Rhopalurus junceus* scorpion venom combined with conventional cytostatics in cervical cancer cell line HeLa. J Pharm Pharmacogn Res 7: 67–76.

Zancolli G, Calvete JJ, Cardwell MD, Greene HW, Hayes WK, Hegarty MJ, Hans-Werner, H, Holycross AT, Lannutti DI, Mulley JF, Sanz L, Travis ZD, WhorleyWüster JR, WüsterW (2019) When one phenotype is not enough–divergent evolutionary trajectories govern venom variation in a widespread rattlesnake species. P Roy Soc B: Biol Sci 286: 1898.

Zaykin DV, Pudovkin AI (1993) Programs to estimate significance of Chi-2 values using pseudo-probability tests. J Hered 84: 152–160.

AUTHOR CONTRIBUTION:

Contribution	Rodríguez-Ravelo R	Ruiz-Urquiola A	Possani-Postay LD	Morris-Quevedo HJ	Rodríguez-Ravelo M	Espinosa-López G
Concepts or ideas	x	x	x	x		x
Design	x	x	x	x		x
Definition of intellectual content	x	x				x
Literature search	x	x			x	
Experimental studies	x	x				
Data acquisition	x				x	
Data analysis	x				x	x
Statistical analysis	x	x			x	x
Manuscript preparation	x			x		x
Manuscript editing	x			x		x
Manuscript review	x	x	x	x	x	x

Citation Format: Rodríguez-Ravelo R, Ruiz-Urquiola A, Possani-Postay LD, Morris-Quevedo HJ, Rodríguez-Ravelo M, Espinosa-López G (2021) Variation of cytochrome oxidase-I gene and venom proteins of *Rhopalurus junceus* populations in the *Moanicum* sector: Pharmacological implications. J Pharm Pharmacogn Res 9(1): 1–12.