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ARTICLE

Proteomic analysis of the venom of the predatory ant *Pachycondyla striata* (Hymenoptera: Formicidae)

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Abstract

The ants use their venom for predation, defense, and communication. The venom of these insects is rich in peptides and proteins, and compared with other animal venoms, ant venoms remain poorly explored. The objective of this study was to evaluate the protein content of the venom in the Ponerinae ant *Pachycondyla striata*. Venom samples were collected by manual gland reservoir dissection, and samples were submitted to two-dimensional gel electrophoresis and separation by ion-exchange and reverse-phase high-performance liquid chromatography followed by mass spectrometry using tanden matrix-assisted laser desorption/ionization with time-of-flight (MALDI-TOF/TOF) mass spectrometry and electrospray ionization-quadrupole with time-of-flight (ESI-Q/TOF) mass spectrometry for obtaining amino acid sequence. Spectra obtained were searched against the NCBInr and SwissProt database. Additional analysis was performed using PEAKS Studio 7.0 (Sequencing de novo). The venom of *P. striata* has a complex mixture of proteins from which 43 were identified. Within the identified proteins are classical venom proteins (phospholipase A, hyaluronidase, and aminopeptidase N), allergenic proteins (different venom allergens), and bioactive peptides (U10-ctenitoxin Pn1a). Venom allergens are among the most expressed proteins, suggesting that *P. striata* venom has high allergenic potential. This study discusses the possible functions of the proteins identified in the venom of *P. striata*.

KEYWORDS
MALDI-TOF/TOF, peptides, proteins, Q-TOF, toxin, venom

1 | INTRODUCTION

The venom of the animals can be broadly defined as a secretion produced by specialized glands that when injected into a target organism are able to disrupt normal physiological or biochemical processes, facilitate feeding, or defense by the producing animal (Casewell, Wuster, Vonk, Harrison, & Fry, 2013).

Venoms arose at different times and through different lineages of evolutionary history (Casewell et al., 2013), and is currently limited to a few animal groups (Escoubas, Quinton, & Nicholson, 2008) including insects.

The venom of bees, wasps, and ants (Hymenoptera) is a complex mixture of hydrocarbons, salts, sugars, biogenic amines, alkaloids, free amino acids, formic acid, proteins, peptides, and other bioactive compounds (Aili et al., 2014; Santos, Pieroni, Menegasso, Pinto, & Palma, 2011; Touchard et al. 2014; Touchard, Dejean, Escoubas, & Orivel, 2015). Pioneering studies have demonstrated that the venoms of stinging ants are mostly composed of small peptides, similarly to other venomous animals (Aili et al., 2014; Touchard et al., 2014, 2015, 2016b). However, a wide variety of proteins has also been described in the venom of different species of ants (Touchard et al., 2016a).

Ants employ venoms for a variety of purposes including predation and defense against predators and competitors, defense against microbial pathogens, social communication (Orivel & Dejean, 2001; Touchard et al., 2016a), and as herbicides (Touchard et al., 2016a). However, ant venoms remain almost unexplored in comparison with other animals (Aili et al., 2017; Rifflet et al., 2012; Touchard et al., 2014). The species of ants with the most well-characterized venoms are the fire ant *Solenopsis invicta* and *Solenopsis saevissima* and *Myrmecia pilosula* (Santos et al., 2011; Touchard et al., 2016a).

Peptides with neurotoxic activity have been identified in the venom of different species of ants. Poneratoxin, ectatomin, and poneritoxin are substances that affect ion channels (Piek et al., 1991; Pluzhnikov et al., 1999; Touchard et al., 2016a), and were isolated from the venom of *Paraponera clavata* (Paraponerinae), *Ectatomma tuberculatum* (Ectatomminae), and *Anochetus emarginatus* (Ponerinae), respectively. Additionally, the venom of ants has myotoxin in *S. invicta* (Pinto et al., 2012), phospholipase (PL) A and B in *S. invicta* (Baer et al., 1979; Pinto et al., 2012), *Pogonomyrmex badius* (Schmidt & Blum, 1978), and *Myrmecia pyriformis* (Lewis, Day, & De La Lande, 1968), and hyaluronidase, lipases, acid phosphatase, and esterase in *P. badius* (Schmidt & Blum, 1978) that induce cell necrosis. The presence of bioactive peptides indicates that the venom of ants may be a potential source for design of new therapeutic drugs.

In Ponerinae ants, studies of protein content in the venom were carried out in *Pseudoneoponera insularis* (former *Pachycondyla insularis*) (Maschwitz, Jessen, & Maschwitz, 1981), *Dinoponera grandis* (Leluk, Schmidt, & Jones, 1989), *Odontoponera transversa* (Leluk et al., 1989), *Neoponera goeldii* (former *Pachycondyla goeldii*) (Orivel & Dejean, 2001), *Dinoponera australis* (Johnson, Copello, Evans, & Suarez, 2010), *Dinoponera quadriceps* (Cologna et al., 2013), *Neoponera villosa* (former *Pachycondyla villosa*) (Pessoa et al., 2016), and *Anochetus emarginatus* (Touchard et al., 2016b). Although some of the venom components of ants have been isolated and characterized (Orivel & Dejean, 2001; Morgan et al., 2003; Szolajska et al., 2004; Wiese et al., 2006), there are few data on its composition, chemical, and pharmacological properties.

Pachycondyla striata (Ponerinae) has a distinguished behavior, producing a foamy substance from terminal abdominal region, as a defense mechanism. The venom, released as a foam-like substance (Mackay & Mackay, 2010), has been also reported in *Pachycondyla tridentata* and *Pseudoneoponera insularis* (former *P. insularis*) (Maschwitz et al., 1981). However the venom compounds in this ant remains unknown. Thus, the objective of this study was evaluate the protein composition of the venom in the predatory ant *P. striata*, collected in the state of Minas Gerais, Brazil.

2 | MATERIALS AND METHODS

2.1 | Study sites and sample preparation

Fifteen nests of *P. striata* were collected in Viçosa (20°48′ S 42°51′ W), state of Minas Gerais, Brazil. A specimen of each colony was taxonomically identified and deposited in the Collection of Mimercologia/CEPEC/CEPLAC in Ilhéus, state of Bahia, Brazil.

Eighty workers from different colonies of *P. striata* were cryoanesthetized and the venom reservoir, without the venom gland, transferred to 10% w/v protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA). Each gland was gently disrupted with tweezers and the sample was centrifuged at 13,700 × *g* for 15 min at 4°C and the supernatant was collected. Each sample had 80 venom reservoir randomly collected from the 15 nests. The amount of protein in the extract was determined by the bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin as standard.

2.2 | Two-dimensional gel electrophoresis

The immobilized pH gradient (IPG) strips 7 cm, pH 3–10 linear, (GE-Healthcare, Milwaukee, Wisconsin, USA) were rehydrated for 10 h in 125 μl of rehydration solution containing 80 μg of protein, 2% v/v IPG buffer pH 3–10, 40 mM dithiothreitol (DTT), and DeStreak solution (GE-Healthcare). The first dimension gel was carried out in IPGphor Ettan III equipment with linear mode voltage (300 V for 12 h, 1,000 V for 30 min, 5,000 V for 2 h, 5000 V for 1 h, and 200 V for 1 h). After isoelectric focusing, the IPG strips were equilibrated in equilibration solution (6 M urea, 75 mM Tris-HCl pH 8.8, 29% v/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), and 0.002% w/v bromophenol blue), containing 1% DTT w/v and 2.5% w/v iodoacetamide at different times, under agitation for 15 min each. The second dimension gel was carried out in a vertical electrophoresis Mini Protean II apparatus (Bio Rad, Hercules, California, USA), by SDS-PAGE using 14% acrylamide gels, at 80 V until the bromophenol blue front had reached the end of the gel. Gels were stained with colloidal Coomassie blue solution (8% w/v ammonium sulfate, 0.8% v/v phosphoric acid, 0.08% w/v Coomassie blue G-250, and 20% v/v methanol for 36 h and stored in 5% v/v acetic acid solution. Three samples were used for gel electrophoresis, representing a triplicate.

The gel scanning was performed using Image Scanner III equipment (GE-Healthcare) in 16-bit transparency mode using red-blue and blue-green channels and with a resolution of 600 dpi. The images were analyzed using Image Master 2D Platinum 7.0 software (GE Healthcare).

2.2.1 | Separation by ion-exchange and reverse-phase high-performance liquid chromatography

Another set of 80 venom reservoir were used, from which 2 ml of the crude venom containing 1,500 μg of proteins were fractionated by ion-exchange chromatography (Mono-Q 5/50GL with quaternary ammonium ligand; GE Healthcare). The chromatographic separation was carried out using high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, Massachusetts, USA) system. Elution of peptides was performed using a flow rate of 1 ml/min and monitored by following the absorbance at 220 nm. Solvent A was 50 mM sodium phosphate, pH 7.4, and solvent B was 50 mM sodium phosphate and 1 M NaCl. The linear gradient employed was 5–95% B in 80 min and proteins of the venom were separated into eight fractions, which were collected every 10 min. The fractions recovered after ion-exchange chromatography separations were then fractionated by reverse phase on a C18 column (Protein & Peptide 218TP54; Vydac) in HPLC system. Elution of peptides was performed using a flow rate of 1 ml/min and monitored by following the absorbance at 220 and 280 nm. Solvent A was 0.1% trifluoroacetic acid (TFA) and solvent B was 80% acetonitrile (ACN) and 0.1% TFA. The linear gradient employed was 5–95% B in 65 min and proteins of the venom were separated into 130 fractions; these were collected every 30 sec.

2.3 | Samples digestion

The spots obtained from two-dimensional gels were excised and dehydrated with 50% v/v ACN and 20 mM ammonium bicarbonate, pH 8.0. After dehydration, the gels pieces were incubated sequentially with 65 mM DTT for 30 min at 56°C and 200 mM iodoacetamide for 30 min in the dark at room temperature, followed by washes in 100 mM ammonium bicarbonate, pH 8.0, and 100% v/v ACN. The gels pieces were treated with 10% v/v ACN and 40 mM ammonium bicarbonate containing trypsin enzyme (25 ng/μl) (Sequencing Grade Modified Trypsin®, Promega, Madison, Wisconsin, USA) and incubated at 37°C for 16 h. Peptides were extracted from the gel pieces by incubating then with 5% v/v formic acid and 50% v/v ACN. The solution with the peptides was dried in the Speedvac and resuspended in 10 μl of

0.1% v/v TFA. The samples were desalted using ZipTip pipette tips with C18 resin (Millipore, Billerica, Massachusetts, USA) according to the manufacturer's instructions, and then vacuum dried.

The samples from fractionation by reverse-phase chromatography were subjected to in-solution trypsin digestion. The fractions were dried in the Speedvac and resuspended in 100 mM ammonium bicarbonate. After solubilized, the samples were reduced in 200 mM DTT for 1 h at room temperature and then alkylated with 100 mM iodoacetamide under the same conditions, in the dark. To neutralize the alkylation process, 200 mM DDT (20 μl) was added in the sample for 1 h at room temperature. Nonautolytic trypsin was added to protein sample (1 μg per sample) for 18 h at 37°C and dried in the Speedvac. The samples were desalted under the same conditions described above. Both peptides samples from in-gel and in-solution digestion were analyzed by tandem mass spectrometry using matrix-assisted laser desorption/ionization with time-of-flight analyser (MALDI-TOF/TOF).

A third strategy was employed, a shotgun liquid chromatography-tandem mass spectrometry (LCMS/MS) approach, analyzing the peptides created from crude venom following in-solution digestion. For this methodology, a lyophilized aliquot of crude venom of *P. striata* containing 500 μg of protein was solubilized in 100 mM ammonium bicarbonate and submitted to reduction and alkylation under the same conditions described earlier. Nonautolytic trypsin was added to protein sample (1:50; 1 μg trypsin: 50 μg protein) for 18 h at 37°C and then dried in the Speedvac. The peptides were resuspended in 0.1% v/v formic acid and analyzed using liquid chromatography coupled to mass spectrometry (ESI-Q/TOF).

3 | MASS SPECTROMETRY ANALYSIS

3.1 | MALDI-TOF/TOF

Peptides obtained from in-gel and in-solution (following reverse-phase chromatography) digestion were solubilized in 0.1% v/v TFA and mixed with matrix [5 mg/ml 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxy-cinnamic acid (mixture 1:1)] prepared in 50% v/v ACN and 0.1% v/v TFA. Peptides were applied to steel plate MTP Anchor Chip TM TF 600/384 (Bruker Daltonics, Bremen, Germany) and submitted to analysis on an Ultraflex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics).

The spectra were acquired with the mass spectrometer operating in reflector mode (LPPepMix) for MS with mass range 500–5,000 Da and MS/MS spectra were acquired in LIFT mode (40–1,878 Da).

3.2 | ESI-Q/TOF

Aliquots (30 μl) of crude venom submitted to in-solution digestion containing 1 and 10 μg of protein were separated by liquid chromatography (reverse phase–HPLC) (Shimadzu Corporation, Tokyo, Japan) coupled to the mass spectrometer (micrOTOF QII, Bruker Daltonics) equipped with microelectrospray ionization source. The liquid chromatography was performed using a total flow of 100 μl/min, with UV detection (200–280 nm) and column temperature controlled (35°C). The spectrum was obtained from a detection range of 50–3000 *m/z* in positive mode. For MS/MS analyses, ions with greater intensity were isolated as a precursor ion and generated fragment ions induced dissociation with collision energy 10 eV. Argon gas was used as collision gas.

3.3 | Protein identification

The mass spectra obtained from MALDI-TOF/TOF were processed using FlexAnalysis 3.3 software (Bruker Daltonics) and peaks known to be contaminants were removed. The resulting mass list was submitted to identification using the software MASCOT (Matrix Science Ltd, UK; Peptide Mass Fingerprint, PMF, and MS/MS Ion Search). Spectra from MALDI-TOF/TOF and ESI-Q-TOF were searched against the NCBInr and SwissProt database with mass tolerance of 0.5 and 0.1 Da and 50 ppm error. Additional analysis was performed using PEAKS Studio 7.0 (Bioinformatic Solutions

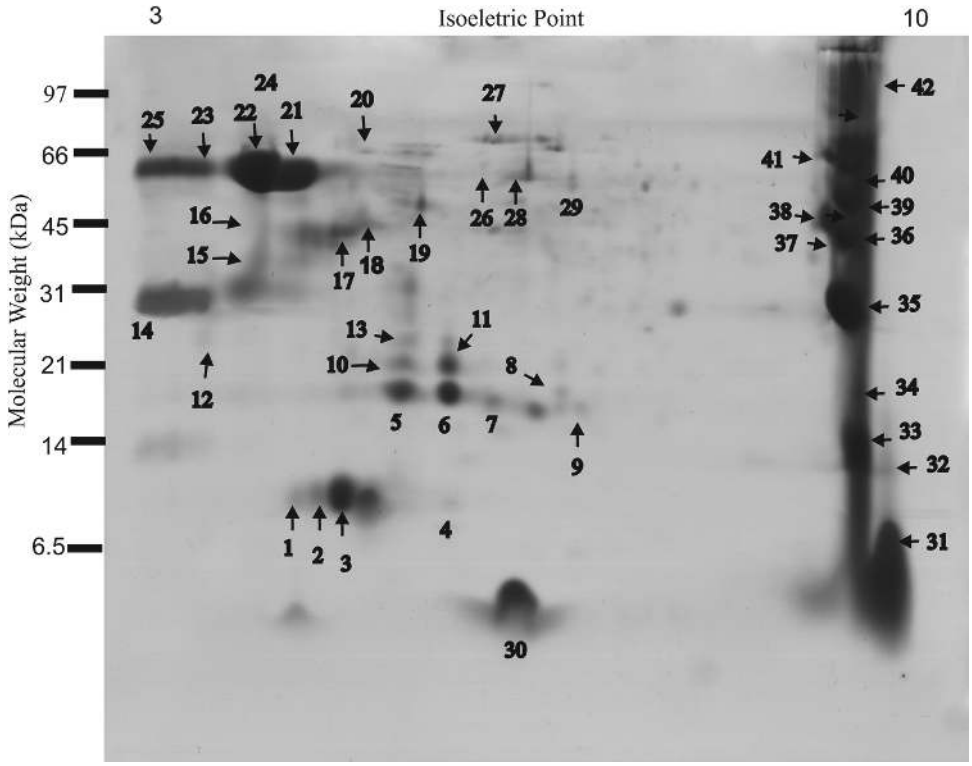

FIGURE 1 Two-dimensional polyacrylamide gel electrophoresis (14%) of the venom proteins of *Pachycondyla striata* (Ponerinae), stained in colloidal Comassie blue solution, showing the proteins identified in MALDI-TOF/TOF (numbers) shown in Table 1

Inc., Waterloo, Canada) and the following search parameters were used: no restrictions on protein molecular weight; one tryptic missed cleave allowed; parent mass error tolerance 0.2 Da; and fragment mass errors tolerance 0.5 Da. In the data of both MALDI-TOF/TOF and ESI-Q-TOF analyses, carbamidomethylation of cysteine and oxidation of methionine were used as fixed and variable modifications, respectively. Ant (Formicidae), "proteins from animal venoms" (cone snails, snakes, insect, spider, amphibians), and Metazoa (Animals) ware selected as taxon for entry into databases. Significant result was considered when the score of the suggested protein was higher than the score calculated by the MASCOT software ($P < 0.05$ and $P < 0.01$). A false discovery rate (FDR) of 0% was used.

MS/MS spectra from MALDI-TOF-TOF were submitted to de novo sequencing using the program Biotools 3.2 (Bruker Daltonics) and MS/MS spectra from ESI-Q-TOF and MALDI-TOF-TOF using the program PEAKS Studio 7.0 (Bioinformatics Solutions Inc.), with average local confidence score (ALC) $\geq$ 70%. The peptides sequences predicted by these programs were submitted to MS Blast research database (http://genetics.bwh.harvard.edu/msblast/) and the sequences that presented significant alignments with proteins derived from ants or animal venom databases were considered as positive identifications.

4 | RESULTS

Patterns of venoms from three different 2D gels showed a high similarity among them with high scatter plot correlation coefficient (>86%). These gels showed the presence of 145 "spots" (protein) with molecular masses and isoelectric points ranged from 3.8 to 138.7 kDa and 3.27 to 9.69, respectively (Figure 1). The MS1 and MS2 spectra (Supporting Information Figure 1) from the 2D gel samples resulted in the identification of 42 proteins (Table 1), with 26 from

TABLE 1 Protein identification from 2-D gels of *Pachycondyla striata* venom with MALDI-TOF/TOF analysis

Spot	Accession Code	Protein	Exp MW/*pI*	Cal MW/*pI*	Score/Total Score	Query Cover	Peptides Sequences
1	W4VV29_ATTCE	Uncharacterized protein[a]	73.82/10.4	9.26/ 4.5		1	QASHEERLEPM(+15.99)DR
2	W4VV29_ATTCE	Uncharacterized protein[a]	73.82/10.4	9.5/ 4.7		1	QASHEERLEPM(+15.99)DR
3	W4VV29_ATTCE	Uncharacterized protein[a]	73.82/10.4	9.38/ 4.9		1	QASHEERLEPM(+15.99)DR
4	G3TM62_LOXAF	Uncharacterized protein[c]	26.25/5.9	8.54/ 5.9		8	LGEHNIEVLEGNEQFIKAAK
5	A0A026VRZ6_CERBI	Ecdysteroid-regulated 16 kDa protein[(a,b,c)]	14.15/5.0	19.68/5.5		9	NSFDVLPLYPR
6	W4VV29_ATTCE	Uncharacterized protein[a]	73.82/10.4	19.47/ 5.8		1	QASHEERLEPM(+15.99)DR
	E2B8I3_HARSA	Putative uncharacterized protein[d]	18.5/6.5	19.47/ 5.8	83/83	91	WLSGHTPDGEPYVR
	E2B8I3_HARSA	Putative uncharacterized protein[e]	18.5/6.5	19.47/ 5.8	83/83	91	SGHTPDGEPFVR
7	W4VV29_ATTCE	Uncharacterized protein[a]	73.82/10.4	18.65/ 6.2		1	QASHEERLEPM(+15.99)DR
8	E9IRW0_SOLIN	Putative uncharacterized[a]	125.0/6.4	19.68/ 6.8		2	IEESSFQEADLLNLGMLDNT
9	F4 × 1A7_ACREC	Putative uncharacterized protein[a]	22.06/10.5	14.87/ 6.9		1	GEVQHGRNVAGGTEC(+57.02)TLNVAR
10	W4VV29_ATTCE	Uncharacterized protein[a]	73.82/10.4	22.85/ 5.5		2	QASHEERLEPM(+15.99)DR
11	W4VV29_ATTCE	Uncharacterized protein[a]	73.82/10.4	22.50/6.1		1	QASHEERLEPM(+15.99)DR
12	E9IPW8_SOLIN	Putative uncharacterized protein[a]	25.61/8.0	26.06/ 3.8		1	ALLRLSRDVTSTR
13	V8P8L6_OPHHA	Uncharacterized protein[b]	100.3/9.0	25.27/ 5.5		1	VSAVRQLGPNR
14	W4VT65_ATTCE	Uncharacterized protein[a]	70.20/9.2	30.52/3.2		1	AVERIHEIIR
15	W4WBM1_ATTCE	Uncharacterized protein[a]	40.42/6.9	36.6/ 4.2		5	PAPVAARDVSTIQQQMLDER
16	W4WBM1_ATTCE	Uncharacterized protein[a]	40.42/6.9	43.03/4.3		5	PAPVAARDVSTIQQQMLDER
17	W4WED9_ATTCE	Uncharacterized protein[a]	61.67/9.7	43.74/ 5.1		4	RSVINSPTTHHTIQSIILDLYRNK
18	W4WBM1_ATTCE	Uncharacterized protein[a]	40.42/6.9	46.0/ 5.4		5	PAPVAARDVSTIQQQMLDER
19	W4WBM1_ATTCE	Uncharacterized protein[a]	40.42/6.9	48.69/5.6		5	PAPVAARDVSTIQQQMLDER
20	E2C0G4_HARSA	Gamma-aminobutyric acid type B receptor subunit 2[a]	87.84/9.5	70.67/5.1		3	GTVAFSSQPDLEPDDPRQSLADLYK
21	F4WC18_ACREC	Putative uncharacterized[a]	12.92/5.0	57.65/4.6		10	ALQAGLGPLGQK

(Continues)

TABLE 1 (Continued)

Spot	Accession Code	Protein	Exp MW/*pI*	Cal MW/*pI*	Score/Total Score	Query Cover	Peptides Sequences
	E9J6C8_SOLIN	Putative uncharacterized protein[e]	116.9/8.9	57.65/4.6	53/ 106	85	BNCTPNVSRWR-BZVATDVSWGNLR-BAR ATDVSWGNLR-BVFRDFHCFZZDZ-BHDHZ FHCFZDZZ-BSDATCGDPNFDMVNPTSGR- BSDATCGDNPFDMVNPTSGR-BSDATCGD NPFDLVDNTSGRBMAANFDPNFDMVNPTS GR
22	W4VW88_ATTCE	Uncharacterized protein[a]	61.70/5.2	58.63/4.2		3	SPSVFVQTYEEGVK
23	E9J676_SOLIN	Putative uncharacterized protein[a]	17.92/4.1	61.68/3.7		0	YLADDSC(+57.02)ENKDLAIDSR
24	F4WU18_ACREC	Replicase polyprotein 1a[a]	30.10/4.1	60.63/4.1		6	DDEDHVSIEWLSAIDSR
25	E2AQX5_CAMFO	Valacyclovir hydrolase[e]	56.61/9.1	62.0/3.5	52/104	70	BCCECNPPPDSAFADNFVRBCCECPNPPD SAFADNFVRBCCECNPPPDSAFADRFGR-
26	T1DAG5_CROHD	Ribosomal protein S29, mitochondrial[a]	45.28/9.0	63.09/6.1		0	GAIVATLSQTGAPSTLR
27	TX35A_PHONI	U10-ctenitoxin-Pn1a[f]	8.8/ 5.2	79.67/6.1	66/ 63	43	RSCKEDRNGCCRLYTCNCWYPTPGDQW CKCQLW
28	W4WDX0_ATTCE	Uncharacterized protein[a]	28.21/9.3	58.63/6.5		0	KLSALFAGIVNQLLR
29	WNT7A_AOTTR	Protein Wnt-7a[f]	39.0/9.0	54.80/6.9	26/20		CLGHLFLSLGMVYLR + Oxidation (M)
30	B6EWW5_GLOBR	Aminopeptidase N[b]	112.1/5.4	3.8/4.5		2	TGSPEDWDFVWEMFR
	EAI_01132	Aminopeptidase N[g]	9.9/8.2	3.8/4.5	23/22	24	QAPEIFEALDKLLPDNTYR
31	B0WA81_CULQU	Venom allergen 5[a]	28.37/8.8	7.38/9.6		3	IVDLHNKLR
32	A0A0K2TDB3_LEPSM	Venom allergen 3-like isoform 1[e]	26.41/8.6	11.7/9.6	50/175	70	BDPYPZFPZ-BDPYPZCLR-BFDMNZZAR- BTYESTALPZ-BSSLGLHPZLR-BSTVGLHP ZLR-BLDVZLHNZLR-BAPZPGAWDDELNG LAZR-BAZPEDWDNELAMVNPZ-BAZPPFW DNELHFAERBAZPDEWTZELHFAER-BNDN MLWTZELADLGZR-BNDNMLWTZELADG LZR-BNDNMLMRZELADLGZR-BNGHYVF VVSGSDPGGZNPGR-BWHYDLVVSGTTNR SNPGR-BNCEYFNYGSYNGPSGYNFYEAZ -BNCEYFNYGSPGNYSGYNFYEAZ-BZDCY FNYGSPGNYSGYNFYEAZ-BTMEYFLYGSP GNYSGYNFYVTZ-BTMEYFLYGSYNGPSG YNFYVTZ-BZLZYNPVLGSYNGPSGYNFYE AZ-BZLZYNPVVASYNGPSGYNFYEAZ-BZ LZYPNVVASYNGPSGYNFYEAZ

(Continues)

TABLE 1 (Continued)

Spot	Accession Code	Protein	Exp MW/*pI*	Cal MW/*pI*	Score/Total Score	Query Cover	Peptides Sequences
33	A0A026WVP5_CERBI	Pyruvate dehydrogenase E1component subunit alpha[a]	43.71/8.5	13.46/9.3		4	ERILNANLVTPEEIK
34	E3PQY5_9HYME	Venom protein A1YI24CM3[e]	21.72/9.4	14.40/9.4	64/64	66	BVEDPVWDNELAMPGZR-
35	VA3_SOLIN	Venom allergen 3[a]	23.86/8.7	29.25/9.2		8	MPNLTWDPELATIAQR
	D4P8F3_SOLSV	Allergen Sol i III[f]	15.70/9.7	29.25/9.2	56/28		MPNLTWDPELATIAQR
36	B0WA81_CULQU	Venom allergen 5[a]	28.37/8.8	39.01/9.3		3	IVDLHNKLR
37	A0A026WZP1_CERBI	Hyaluronidase[a]	36.32/9.1	40.01/9.3		3	QNWASLEPYK
	E9IQ14_SOLIN	Hyaluronidase[b]	39.87/9.0	40.01/9.3		3	QNWASLEPYK
	E2ADC1_CAMFO	Hyaluronidase[e]	39.97/8.4	40.01/9.3	712/712	100	MSYLVFLSSLGATTPEAGNPQQFDVYWNVPSF ICHKYGVKFENLKNFGIHQNANDEFRGE
	E2ADC1_CAMFO	Hyaluronidase[g]	39.97/8.4	40.01/9.3			QNWASLEPYK
38	V8NWW7_OPHHA	Hamartin[b]	63.25/6.2	44.45/9.0		2	LSASPLHNQLR
39	CA426_CONBU	Conotoxin[g]	6.56/10.8	51.22/9.3	93/93	70	LVVLATTVVSFRSNRASDGRKIAVNKRRREL VVPPGKLRECCGRVG
40	A0A0K2TDB3_LEPSM	Venom allergen 3-like isoform 1[e]	44.11/8.6	54.22/9.3	48/ 179	83	BMYYHPZLR-BMYNWPZLR-BVPMZLHNZ LR-BPVMZLHNZLR-BCALZNHLGDHZ-BV VPLNWDNELNEGNPZ-BVVPLNWDNELNE GPNZ-BPTPLNWDNELNEGNPZ-BZTDLWD NELAMPLVZ-BZTDLLWDNELAMLPVZ-B ZTDLLWDNELAMPPDZ-BERGFPWTZELA DEVPZ-BSWSEPWTZELADEVPZ-BMNPED WTZELADEPVZ-BZEWRWDNELADGGDP Z-BEZWRWDNELADGGDPZ-BSAVWRWD NELADGLZR-BFAEYVPANEYYYNSGWYG AHZ-BFAEYPVANEYYYNSGWYGAHZ-BF AEYVPANEYYYNSGWYGHAZ-BFAEYFNY GSPGNYSGDGWSDEPZ-BFAEYFNYGSYN GSPGDGWSDEPZ-BSMEYFNYGSPGNYEN FZFGHAZ-

(Continues)

TABLE 1 (Continued)

Spot	Accession Code	Protein	Exp MW/*pI*	Cal MW/*pI*	Score/Total Score	Query Cover	Peptides Sequences
41	B0W489_CULQU	Venom allergen[e]	21.91/7.4	67.71/9.1		66	BZTDPEWDNELATLLLZ-BNDDLLWDNEL ATLLLZ-BZTDEPWDNELATLLLZ-BTCRL LWDNELAMLPVZ-BDFPLNWDNELAMLPV Z-BMMPLNWDNELAMLPVZ-BFAEYVPAN EYYYNSGWYGHAZ-BFAEYVPANEYYYN SMZFGHAZ-BFAEYPVANEYYYNSMZFGH AZ-
42	W2TQ82_NECAM	Uncharacterized protein[c]	9.50/7.8	138.7/9.3		11	QIVDLHNQLR
	A0A0K2U2R7_LEPSM	Venom allergen 3[e]	38.13/8.7	138.7/9.3	56/ 198	61	BNVNZLHNZLR-BNRGZLHNZLRBGRNZL HNZLR-BMDLPNWDNELAMLAPR-BDML PNWDNELAMLAPR-BDMPLNWDNELAML APR-BDMNLLWDNELAMPDPZ-BNMDEPW DNELAMPLAR-BNMDEPWDNELAMLPAR -BCCNNPAWTZELAMPWR-BCCPMPAWTZ ELAMPWR-BPEHTHWDDELADLGER-BZR PZPVANEYYYNSMZAEVGGZ-BZRPZPVA NEYYYNSMZAEVNZ-BNCEYZPREYYYNS MZAEVNZ-BFAEYFNYGSPGNYSGYNFPG HAZ-BFAEYFNYGSYNGPSGYNFPGHAZBF AEYFNYGSPGNYSGYNFPGAHZ-

[a]Identification assisted by Peaks software, version 7.0, database Formicidae; result validation with false discovery rate (FDR = 0.0);
[b]identification assisted by Peaks software, version 7.0, database venom; result validation with false discovery rate (FDR = 0.0);
[c]identification assisted by Peaks software, version 7.0, database metazoa; result validation with false discovery rate (FDR = 0.0);
[d]identification by sequencing "de novo," assisted by Peaks software, version 7.0, average local confidence score (ALC ≥ 70%) and MS blast;
[e]identification by sequencing "de novo," assisted by BioTools software, version 3.2, desired sequences score ≥ 60, and MS blast;
[f]identification by peptide mass fingerprint (PMF);
[g]identification by MS/MS ions search.

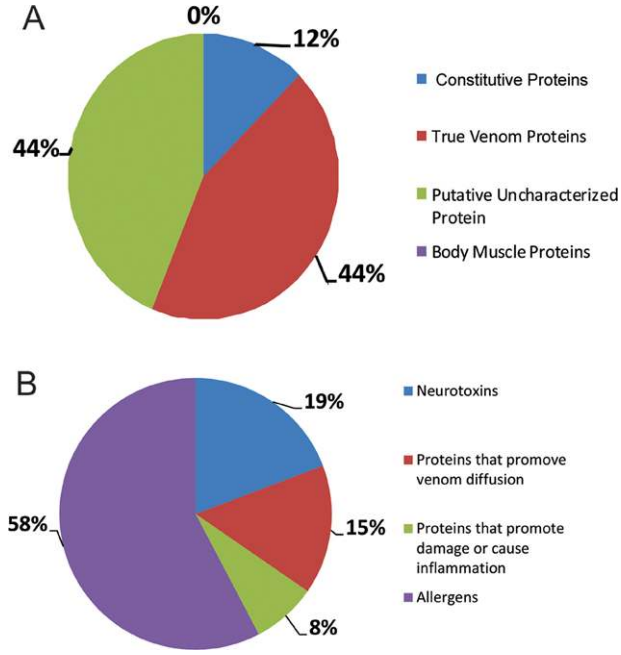

FIGURE 2 Overall classification of the proteins from the venom of the ant *Pachycondyla striata* (Ponerinae) based on functional protein groups. (A) Functional protein groups. (B) True venom components

ants (*Cerapachys biroi*, *Harpegnathos saltator*, *S. invicta*, *Acromyrmex echinatior*, *S. saevissima*, and *Camponotus floridanus*). Twenty two of the identified proteins were proteins inferred from the genome (*spots* 1–4, 6–19, 21–23, and 28). The spots 22 (Putative uncharacterized protein), 35 (venom allergen 3), 31 and 36 (Venom allergen 5), and 39 (Conotoxin) were the most abundant venom components, together they were almost 40% of the total volume of venom.

The MS/MS spectra of venom fractions obtained in reverse-phase HPLC, resulted in the identification of the protein Retroelement polyprotein from *Lasius niger* (Hymenoptera: Formicidae) and three venom allergens from of *S. invicta*, *Pachycondyla chinensis* (Hymenoptera: Formicidae), and *Bombyx mori* (Lepidoptera: Bombycidae), besides three unidentified proteins from *S. invicta* and *Atta cephalotes* (Hymenoptera: Formicidae) (Table 2).

A total of five proteins were identified through shotgun analysis, with four proteins (venom PLA1 2; hypothetical protein EAG_14340; hypothetical protein EAI_17579, coiled-coil-domain-containing protein 39) found in ants and one (basic PLA2 vurtoxin) from *Vipera renardi* (Squamata: Viperidae) (Table 2).

All proteins identified were classified into four different functional categories: constitutive cell proteins, true venom proteins, putative uncharacterized protein (Tables 3 and 4), and body muscle proteins (Figure 2A). The true venom proteins were classified into other four functional categories: neurotoxins, proteins that play a role in venom diffusion, proteins that cause damage or inflammation, and allergens, to simplify this data (Figure 2B).

5 | DISCUSSION

This is the first characterization of the venom proteins of *P. striata*. Our data show that the venom of *P. striata* is highly diverse, similar to the venom of other ants that have been characterized (Aili et al., 2014, 2017; Bouzid et al., 2014; Touchard et al., 2014). The combination of the three fractionation and analysis methods allowed the identification of 43 proteins in the venom of *P. striata*. Despite the good quality of the spectra obtained were found match to an entry in the database. Neurotoxins, proteins that promote venom diffusion or modulate prey defense mechanisms, proteins causing tissue damage or inflammation, and proteins that stimulate the production and/or antibodies reactions, are in

TABLE 2 Proteins identified in *Pachycondyla striata* venom from reverse-phase chromatography on HPLC and MALDI-TOF/TOF analysis, and using shotgun strategy and ESI-Q/TOF

Access Code	Protein	Organism	Score/ Total Score	Query Cover (%)	FDR[g]	ALC[h]	Peptide Sequence
A0A0J7K739_LASNI[e]	Retroelement polyprotein[c]	*Lasius niger*		2	0.0		PFEIISIDTIGGFGGSR
C0ITL3.1	Venom allergen[a]	*Pachycondyla chinensis*	75/116	90			MPDLLWDNELA
E9J797_SOLIN[e]	Putative uncharacterized protein[c]	*Solenopsis invicta*		6	0.0		QILSLLR
C0ITL3.1[e]	Venom allergen 5[a,b]	*P. chinensis*	70/102	72			WDNELATLAZR
			101/101	87		87	MPDLLWDNELATLAQR
XP_004927517.1[e]	Predicted: Venom allergen[a]	*Bombyx mori*	72/105	53			MDLLWDNELATLAZR
E9IVS5_SOLIN[e]	Putative uncharacterized protein[c]	*S. invicta*		9	0.0		LDGNGIWSGDMGLERKR
VA3_SOLIN[e]	Venom allergen 3[a,b]	*S. invicta*	90/90	64			BMPDLLWDNELATLAZR
			96/96	88		88	KMPDLLWDNELATLAQR
W4WNK2_ATTCE[e]	Uncharacterized protein[c]	*Atta cephalotes*		3	0.0		SSIISLK
E2A992_CAMFO[e]	Venom phospholipase A1 2[b]	*Camponotus floridanus*	71/71	53		76	LYETDTNNLVVLHTSALGMLGK
PA2B_VIPRE[f]	Basic phospholipase A2 vurtoxin[b]	*Vipera renardi*	90/90	100		72	SLLEFGMMILEE
E2AMM2_CAMFO[f]	Hypothetical protein EAG_14340 (b)	*C. floridanus*	32/160	75		80	YAADVLYCRR
E2BA88_HARSA[f]	Hypothetical protein EAI_17579[b]	*Harpegnathos saltator*	41/129	100		76	TQALLGLLGGKGHK
E2BUY1_HARSA[f]	Coiled-coil domain-containing protein 39[d]	*H. saltator*	43/52	100			HKFGIMLAVICLIPLK

[a]Identification by sequencing "de novo," assisted by BioTools software, version 3.2.
[b]Identification by sequencing "de novo," assisted by Peaks software, version 7.0 and MS Blast.
[c]Identification assisted by Peaks software, version 7.0, database Formicidae.
[d]MS/MS ion search; database metazoa.
[e]Reverse-phase chromatography on HPLC and MALDI-TOF/TOF analysis.
[f]Shotgun strategy and ESI-Q/TOF.
[g]Result validation with false discovery rate.
[h]Average local confidence score.

TABLE 3 BLASTp of the "uncharacterized" or "putative uncharacterized" proteins identified from 2-D gels of *Pachycondyla striata* venom with MALDI-TOF/TOF analysis

Spot ID	Protein Access Uniprot	Protein Access NCBInr	Protein Name	Max Score	Query Cover (%)	e-Value	Ident. (%)
1	W4VV29	KYN10968	Myosin-XV	1,007	91	0.0	86
2	W4VV29	KYN10968	Myosin-XV	1,007	91	0.0	86
3	W4VV29	KYN10968	Myosin-XV	1,007	91	0.0	86
4	G3TM62	XP_003420296	PREDICTED: cationic trypsin-3-like	503	100	4×10^{-180}	100
5	W4VV29	KYN10968	Myosin-XV	1,007	91	0.0	86
6	E2B8I3	XP_011154548	PREDICTED: uncharacterized protein LOC105192254	346	100	5×10^{-121}	100
7	W4VV29	KYN10968	Myosin-XV	1,007	91	0.0	86
8	E9IRW0	EFZ16727	Cyclin G-associated kinase	2,086	100	0.0	90
9	F4 × 1A7	EGI59779	Hypothetical protein G5I 12066	421	100	4×10^{-149}	100
10	W4VV29	KYN10968	Myosin-XV	1,007	91	0.0	86
11	W4VV29	KYN10968	Myosin-XV	1,007	91	0.0	86
12	E9IPW8	XP_011161124	PREDICTED: serine protease easter	488	100	3×10^{-172}	100
13	V8P8L6	XP_015669244	PREDICTED: fibrocystin	460	86	3×10^{-136}	90
14	W4VT65	KYM77137	Splicing factor 1	1,241	95	0.0	99
15	W4WBM1	XP_012055865	PREDICTED: methylcytosine dioxygenase TET2	766	96	0.0	99
16	W4WBM1	XP_012055865	PREDICTED: methylcytosine dioxygenase TET2	766	96	0.0	99
17	W4WED9	XP_018045287	PREDICTED: protein APCDD1-like	1,110	99	0.0	99
18	W4WBM1	XP_012055865	PREDICTED: methylcytosine dioxygenase TET2	766	96	0.0	99
19	W4WBM1	XP_012055865	PREDICTED: methylcytosine dioxygenase TET2	766	96	0.0	99
20	F4WC18	EGI68258	Hypothetical protein G5I 03090	235	100	2×10^{-78}	100
21	E9J6C8	XP_011169821	PREDICTED: glucose dehydrogenase [FAD, quinone]-like	1,211	98	0.0	99
22	W4VW88	XP_012062854	PREDICTED: glutamate receptor ionotropic, kainate 2-like	1,038	90	0.0	99
23	E9J676	EFZ11677	Hypothetical protein SINV 10409	311	100	6×10^{-107}	100
28	W4WDX0	XP_012054709	PREDICTED: circadian clock-controlled protein-like	494	100	2×10^{-176}	97
41	W2TQ82	XP_013305483	Hypothetical protein NECAME 07492	178	100	6×10^{-57}	100

TABLE 4 BLASTp of the "uncharacterized" or "putative uncharacterized" proteins identified in *Pachycondyla striata* venom from reverse-phase chromatography on HPLC and MALDI-TOF/TOF analysis, and using shotgun strategy with ESI-Q/TOF

Protein Access Uniprot	Protein Access NCBInr	Protein Name	Max Score	Query Cover (%)	e-Value	Ident. (%)
E9J797	EFZ11306	Hypothetical protein SINV 09883	582	100	0.0	100
E9IVS5	EFZ15328	Hypothetical protein SINV 00659	396	100	7×10^{-140}	100
W4WNK2	KYM76259	Hypothetical protein ALC53 13286	451	97	3×10^{-159}	95

the venom of *P. striata*. Among the neurotoxic proteins present in the venom of *P. striata*, there are venom PLA1 2, basic PLA2 vurtoxin, conotoxin, and U10-ctenotoxin-Pn1a.

PLs are relatively common in ant venoms (Bouzid et al., 2014) and are among the most studied enzymes in the venom of Hymenoptera (Lima & Brochetto-Braga 2003). In animals, four main types of PLs are known: PLA1, PLA2, PLB, and PLC (Touchard et al., 2016a). In the venom of *P. striata*, two different PLs are present: basic PLA2 vurtoxin and venom PLA1 2. The PLA1 and PLA2 catalyze the specific hydrolysis of ester bonds of 1,2-diacyl-3-sn glycerophospholipids in the positions sn-1 and sn-2, respectively, converting these substrates into their corresponding lyso-associated compounds with the release of fatty acids (Santos et al., 2011)

PLA2 is among the most common PL found in ant venom (Touchard et al., 2016a), and has been described in the venom of different species (Pinto et al., 2012; Santos et al., 2011; Schmidt & Blum, 1978; Touchard et al., 2016a), whereas PLA1 has been described in several Hymenoptera venoms (Hoffman, 1994; King, Lu, Gonzalez, Qian, & Soldatova, 1996; Pinto et al., 2012). Venom PL from various animals have been described as a potent venom allergen (Lima & Brochetto-Braga, 2003) with neurotoxic, platelet activation, hemolysis, and tissue damage effects (Pinto et al., 2012; Touchard et al., 2016a).

Conotoxin is a family of proteins that target and block potently a wide range of ion channels, such as voltage-gated sodium, calcium, and potassium channels, as well as nicotinic acetylcholine and other membrane receptors (The Uniprot, 2017). Peptides that affect ion channels has been described frequently in some species of ants as: *H. saltator* and *C. floridanus* (Bonasio et al., 2010), *S. invicta* (Wurm et al., 2011), *A. cephalotes* (Suen et al., 2011), *Cerapachys biroi* (Oxley et al., 2014), *A. echinatior* (Nygaard et al., 2011), *L. niger* (The Uniprot, 2017), *P. clavata* (Piek et al., 1991), and *E. tuberculatum* (Pluzhnikov et al., 1999). Peptides of this group induce paralysis when injected into mice and fish (Lewis, Dutertre, Vetter, & Christie, 2012). *Pachycondyla striata* is a predator species, possibly this peptide is involved in prey paralysis process.

The U10-ctenitoxin-Pn1a (fragment) found in *P. striata* is a TX3 family peptide present in the venom of the spider *Phoneutria nigriventer* and has potent neurotoxic effects, acting on ion channels, and chemical receptors of neuromuscular system of insects and mammals (Cardoso et al., 2003). The U10-ctenitoxin Pn1a is also known as neurotoxin Pn3-5A, described only in *P. nigriventer* (Cardoso et al., 2003), and so far its natural occurrence was not reported in none other animal species (The Uniprot, 2017). Despite showing homology (58%, 48 amino acids) with TX3-5 toxin of *P. nigriventer*, this protein in *P. striata* has not conserved domain with another toxin or protein, suggesting that its physiological effects need further studies.

The presence of neurotoxins is not common in the venom of social insects due to the defensive nature of the venom in these insects (Pinto et al., 2012). However, *P. striata* is a predatory ant using their venom for both defense and predation. The presence of neurotoxins in the venom of *P. striata* may be related to its use in prey capture. The presence of compounds that paralyze or kill the prey quickly is important to the success of solitary foraging ants (Aili et al., 2014), as *P. striata*.

Proteins that promote venom diffusion or modulate prey defense mechanisms present in the venom of *P. striata* are hyaluronidase. This protein is a common in venom of ants, wasps, bees, and vertebrates (Pinto et al., 2012), and is a key

enzyme for the diffusion of toxic molecules from the venom into the inoculated area of the prey/victim. This enzyme hydrolyses hyaluronic acid in the extracellular matrix of connective tissue decreasing the milieu viscosity, allowing rapid diffusion of venom toxins, potentiating their effect and damaging the tissue, contributing to the inflammatory process in the inoculated region (Kemparaju & Girish, 2006). Hyaluronidases are in general among the more conserved hymenopteran allergens. In ants, enzymes with hyaluronidase activity are not described as a major component of the venom except for the Harvester ant *Pogonomyrmex* sp. (Bouzid et al., 2014) and they have low activity in comparison with those from social wasps (El-Safory, Fazary, & Lee, 2010).

Among the proteins causing tissue damage or inflammation present in the venom of *P. striata* are aminopeptidase N (APN), hyaluronidase, basic PL A2 vurtoxin, and venom PL A1 2, with the last three described above.

The APN is a multifunctional protease present in the venom of some snakes (Matteo et al., 2011) and in some organs, tissues, and cells, from bacteria to vertebrates (Chen, Lin, Peng, & Li, 2012). In mammals, the APN play a role in the degradation of neuropeptides, cytokines, angiotensins (Ogawa, Murayama, Fujita, & Yanoshita, 2007), and proteins (Matteo et al., 2011) and may be associated with the pain process (Chen et al., 2012). The presence of APN in *P. striata* venom may be involved in colony defense causing pain and injury in their enemies. In addition, as *P. striata* has carnivorous feeding habits, the presence of APN in its venom may be explained by the activity that this enzyme has on degradation of prey tissues, facilitating digestion. The aminopeptidases are among the poorly understood enzymes in the venom of snakes and other animals, and its real function remains unknown (Matteo et al., 2011).

The valacyclovir hydrolase and WNT-7a protein found in the venom of *P. striata* with the former described as enzyme and the second as rare in animal venom (The Uniprot, 2017). The WNT-7a protein occurs in some tissues of vertebrates and invertebrates binding to membrane receptors (Solis, Luchtenborg, & Katanaev, 2013). Thus, WNT induces the release of intracellular calcium (Huelsken & Birchmeier, 2001), resulting in cell necrosis. It is possible that valacyclovir hydrolase and WNT-7a protein play some role tissue damage, degrading proteins with damage to the prey.

Venom allergen 3 and allergen 5 were also found in the venom of *P. striata*, which stimulates antibody production and/or reactions. Venom allergen 3 (Sol i3) from the fire ant *S. invicta* is the major allergen of a series of Sol i peptides identified as the most frequent cause of hypersensitivity reactions following stings from this species (Padavattan, Schmidt, Hoffman, & MarkovicHousley, 2008). This allergen has homology (44% sequence identity) with Ves v 5 (allergen 5) from wasp venoms, which also occurs in *P. striata* here studied.

Venom allergens are among the most expressed proteins in the venom of *P. striata*. Additionally, the presence of venom PL A1 2, basic PL A2, hyaluronidase, U10-ctenitoxin, and proteins that also stimulate the production and/or antibodies reactions, suggest that *P. striata* has a venom with high allergenic potential.

Other proteins of *P. striata* venom identified are ribosomal protein S29, coiled-coil domain-containing protein 39, harmartin, pyruvate dehydrogenase E1 component subunit alpha, and retroelement polyprotein. The former is a mitochondrial protein, the second has conserved domain with cell cycle control protein, the third with membrane family proteins. The pyruvate dehydrogenase E1 component subunit alpha belongs to the thiamine pyrophosphate family and pyruvate dehydrogenase complex subfamily, which catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl-CoA in the bridging step between glycolysis and the citric acid cycle (https://www.ncbi.nlm.nih.gov/). The retroelement polyprotein interacts selectively and noncovalently with nucleic acids. All these proteins are probably soluble cellular proteins from the venom reservoir wall and/or convoluted gland present inside the reservoir (Ortiz & Camargo-Mathias, 2006; Schoeters & Billen, 1998), which may be ruptured during venom extraction contaminating the venom samples.

Twenty one (48%) of the proteins identified in this study are inferred proteins from the genome. In addition various sequences with high level of confidence obtained from de novo analyses show the limited information available for *P. striata* and other ants in databases.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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