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Peptidomic dissection of the skin secretion of *Phasmahyla jandaia* (Bokermann and Sazima, 1978) (Anura, Hylidae, Phyllomedusinae)

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ABSTRACT

The systematic investigation of the peptidic composition of the skin secretion of *Phasmahyla jandaia*, a phyllomedusine anuran endemic to the southern region of the *Espinhaço* range in Brazil, is herein reported. By means of *de novo* interpretation of *tandem* mass spectrometric data, Edman N-terminal sequencing and similarity searches, 57 peptides - including phylloseptins, dermaseptins *stricto sensu*, dermatoxins, hyposins, tryptophyllins, caerulein-related, bradykinin-related, bradykinin potentiating, tyrosine-rich, and opioid peptides - were sequenced. Moreover, five peptide families without significant similarity to other known molecules were verified. Differently from most Phyllomedusinae genera, the molecular diversity in the skin of representatives of *Phasmahyla* remained unprospected until now. Therefore, besides disclosing novel natural variants of number of bioactive peptides, the present study contributes to the understanding of the evolution of biochemical characters of the phyllomedusines.

1. Introduction

The anuran skin secretion is widely recognized by its diversity of biologically active peptides. These molecules have been subjected to intense investigation due to their potential applications in biophysical research, biochemical taxonomy, and as a guide for the development of new therapeutic agents (Chen et al., 2004; Conlon et al., 2004, 2009a, 2009b; Amiche et al., 2008; Nicolas and El Amri, 2009; Faivovich et al., 2010).

Evidencing such diversity, within the dermaseptin superfamily of antimicrobial peptides, which is present in the skin of Hylidae frogs, the inter- and intra- specific variety is such that only rare examples of peptides from one species being found with an identical primary structure in other organisms have been reported. Moreover, these differences in amino acid sequences often imply in differential biological activity (Nicolas and El Amri, 2009; Faivovich et al., 2010).

Notwithstanding, it is worth mentioning that despite extensive investigation of their properties *in vitro*, the precise biological roles of the skin peptides remain unclear (Conlon et al., 2009a). Moreover, the growing knowledge pertaining to amphibian species and to their molecular repertoire diversity is coincident with a massive and global decline in amphibian populations due to factors including, *inter alia*:

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habitat loss and fragmentation; global environmental changes; and emerging infectious diseases (Donnelly and Crump, 1998; Alford and Richards, 1999; Houlahan et al., 2000; Young et al., 2001; Blaustein and Kiesecker, 2002; Collins and Storfer, 2003; Licht, 2003; Stuart et al., 2004; Green, 2005; Halliday, 2005; Frost et al., 2006). Therefore, additional studies on the evolution and diversity of frog skin peptides may help reveal biochemical features which may lead to the elucidation of their *in vitro* activity and their physiological and ecological roles, besides securing the potential loss of invaluable biological information.

Amongst anuran groups, the Phyllomedusinae[1] subfamily (Hylidae) has been previously highlighted as displaying the greatest variety and abundance of active peptides described to date (Erspamer et al., 1986). Phyllomedusines occur in Latin America, from México to Argentina. Its 59 species (Frost, 2010) are distributed into 5 genera (Faivovich et al., 2010), including *Phasmahyla* Cruz, 1991.

Phasmahyla is endemic to the Brazilian Atlantic Forest Domain (Ab'Saber, 1977; Cruz, 1991; Carvalho-e-Silva et al., 2009). It currently includes the 7 following species (Frost, 2010): *P. cruzi* Carvalho-e-Silva, Silva, and Carvalho-e-Silva, 2009; *P. spectabilis* Cruz, Feio, and Nascimento, 2008; *P. timbo* Cruz, Napoli, and Fonseca, 2008; *P. cochranae* (Bokermann, 1966); *P. exilis* (Cruz, 1980); *P. guttata* (Lutz, 1924); and *Phasmahyla jandaia* (Bokermann and Sazima, 1978). *P. jandaia* is endemic to the *Espinhaço* range in the state of Minas Gerais, southeastern Brazil, where it is known from few localities (Leite et al., 2008a). It is worthy mentioning that the *Espinhaço* range has been deemed as of 'special biological importance'[2] for amphibian conservation in the state of Minas Gerais (Drummond et al., 2005) (Fig. 1). Moreover, differently from other Phyllomedusinae genera, *Phasmahyla* has not been subjected to investigations dedicated to the prospection and characterization of bioactive peptides in its skin secretion up until the date of conclusion of the present study, as evidenced by a search using the term "*Phasmahyla*" in the PubMed and ISI Web of Knowledge databases (last accessed in 7/06/2010) and further supported by Faivovich et al. (2010).

In light of the facts presented, the aim of the present study was the systematic sequencing/identification of peptides present in skin secretion of *P. jandaia*, by means of one- and two-dimensional high performance chromatography (HPLC), MALDI-TOF/TOF and ESI-Q-TOF mass spectrometry and *de novo* interpretation of peptide fragmentation spectra.

2. Materials and methods

2.1. Animals and extraction of skin secretion

Two *P. jandaia* specimens, one adult male and one juvenile whose sex was not determined, were collected in the municipality of Itabirito (WGS84, 20°09′59.87"S, 43°35′25.43"W, Elevation *ca.*1190 m), state of Minas Gerais, Brazil, within a region known as *Quadrilátero Ferrífero* in the southernmost portion of the *Espinhaço* range (Fig. 1). The specimens used in this study were deposited in the Herpetological Collection of the Biological Sciences Institute of the Federal University of Minas Gerais (*Coleção de Herpetologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais*) (Belo Horizonte, Minas Gerais, Brazil) under the following registration numbers: UFMG 1573 and UFMG 1574.

The skin secretion was obtained by means of mild electric stimulation applied on multiple sites on the frogs' dorsal region, as previously described (Tyler et al., 1992). The secretion was washed out from the animals' skin with chilled deionized water. The material was pooled and promptly frozen and lyophilized. The dried secretion was stored in freezer (−20 °C) until its use.

2.2. Fractionation of skin secretion

Fractionation of the skin secretion of *P. jandaia* was performed employing an ÄKTA Explorer 100 HPLC platform (GE Healthcare, Uppsala, Sweden), controlled by the UNICORN 4.11 software. Fractions were collected by an automated fraction collector Frac920 (GE Healthcare, Uppsala, Sweden). Elution was monitored by absorbance readings at 214 and 280 nm. Solutions used in the chromatographic separations were degassed (reversed-phase and cation-exchange chromatographies) and filtered (cation-exchange) using 0.45 μm nylon membranes (Sigma–Aldrich, Saint Louis, USA).

2.2.1. One-dimensional chromatography

One-dimensional chromatography consisted in the fractionation of the skin secretion by means of reversed-phase chromatography (RPC). A sample of the skin secretion of *P. jandaia* (dried weight, *ca.* 1.5 mg) was dissolved in 0.1% TFA in deionized water (2.0 mL), centrifuged (9500 RCF, during 10 min at 5 °C). The supernatant fraction was filtered in 0.22 μm nylon membranes (Sigma–Aldrich, Saint Louis, USA) and was loaded onto a monolithic RPC column (Chromolith Performance RP-18 100 mm × 4.6 mm - Merck - Darmstadt, Germany), which was previously equilibrated with 0.1% TFA in deionized water. Elution was achieved by means of a linear gradient (0–70%) of 0.1% TFA in acetonitrile, in 5 min. RPC analysis were carried out using a 4.0 mL/min flow.

2.2.2. Two-dimensional chromatography

Two-dimensional chromatography consisted in the fractionation of the skin secretion by means of cation-exchange chromatography (CIEX) followed by further separations of fractions obtained in the previous step by RPC.

A sample of the skin secretion of *P. jandaia* (dried weight, *ca.* 1.5 mg) was dissolved in 50 mM sodium-acetate buffer (pH = 5.0) (2.0 mL) and centrifuged (9500 RCF, during 10 min at 5 °C). The supernatant fraction was filtered in 0.22 μm nylon membranes (Millipore, Bedford, USA) and the CIEX step was carried out as previously

[1] Species names depicted in the present study are in accordance with Frost, 2010. Only currently valid names are used. Therefore, names may differ from synonyms adopted in the cited literature.

[2] "Areas of special biological importance: areas bearing species with distribution restricted to the region in question or bearing environments unique to the state of Minas Gerais" (Drummond et al., 2005). 47.

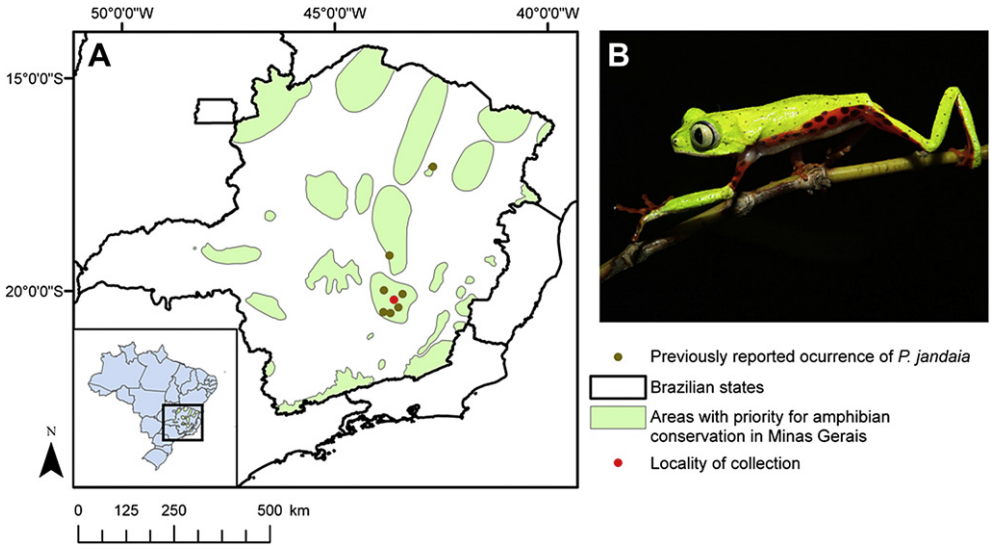

Fig. 1. Geographic range of *Phasmahyla jandaia*. (A) Geographic distribution of *P. jandaia*, which is endemic to the *Espinhaço* range in the state of Minas Gerais, southeastern Brazil. The distribution of *P. jandaia* is depicted as the seat of the municipalities where it is know to occur, namely: Santana do Riacho, Leme do Prado, Catas Altas, Congonhas, Nova Lima, Ouro Branco, and Ouro Preto (Leite et al., 2008a). Its distribution range is coincident with a number of regions deemed as having priority for amphibian conservation. Data sources: Brazilian states 'shapefile' downloaded from the *Centro de Sensoriamento Remoto* (Remote Sensing Center - CSR) of the *Instituto Brasileiro de Meio Ambiente* (Brazilian Environment Institute – IBAMA) (available at http://siscom.ibama.gov.br/shapes/, last accessed in 20/05/2010); Areas with priority with amphibian conservation in the state of Minas Gerais 'shapefile' downloaded from *Fundação Biodiversitas* (available at http://www.biodiversitas.org.br/atlas/repteis.asp, last accessed in 20/05/2010) and; Seat of municipalities of Minas Gerais 'shapefile' downloaded from the *Programa Integrado de Uso da Tecnologia de Geoprocessamento pelos Órgãos do Estado de Minas* Gerais (Integrated Program for the Use of Geoprocessing Technology by the Bodies of the State of Minas Gerais - GEOMINAS) (available at, http://www.geominas.mg.gov.br/, last accessed in 20/05/2010). Map datum: WGS84. Map generated in the software ArcMap 9.2. (B) Unvoucher adult specimen of *P. jandaia* (photo: Leite, F. S. F.).

described (Rates et al., 2007). The fractions obtained in the CIEX step were further separated by means RPC as described above (Item 2.2.1).

2.3. Mass spectrometry

RPC fractions obtained after one- and two-dimensional chromatography were submitted to mass spectrometric analyses, which were carried using an AutoFlex III or Ultraflex III MALDI-TOF/TOF mass spectrometers (Bruker Daltonics, Billerica, USA), both controlled by the FlexControl 3.0 software (Bruker Daltonics, Billerica, USA).

RPC fractions were mixed with α-cyano-4-hydroxycinnamic acid matrix solution (1:1, v/v) directly into a MTP AnchorChip 400/384 or 800/384 MALDI target plates (Bruker Daltonics, Billerica, USA) and dried at room temperature. For increasing signal, some fractions were submitted to concentration by means of vacuum centrifugation prior to mass spectrometric analysis. Protein average masses were obtained in linear mode with external calibration, using the Protein Calibration Standard (Bruker Daltonics, Billerica, USA). The peptide monoisotopic masses were obtained in reflector mode with external calibration, using the Peptide Calibration Standard (Bruker Daltonics, Billerica, USA). Peptide MS/MS spectra were obtained by means of LIFT fragmentation. The software FlexAnalysis 3.0 (Bruker Daltonics, Billerica, USA) and PepSeq (Waters, Manchester, UK) were used for mass spectrometric data analysis. Peptide primary structures were inferred by means of the manual *de novo* interpretation of fragmentation spectra.

Alternatively, high-resolution *tandem* mass spectrometric spectra of a selection of peptides were obtained by mean of ESI-Q-TOF, in a SYNAPT HDMS™ (Waters, Manchester, UK) instrument equipped with a nano Z-spray electrospray ionization (ESI) source. The electrospray capillary was operated at a voltage of 1.6 kV. MSMS data were acquired in the continuum and V-modes.

2.4. Peptide N-terminal sequencing

N-terminal sequencing of selected native peptides was performed by means of Edman degradation using an automated PPSQ-21A protein sequencer (Shimadzu, Tokyo, Japan) coupled to reversed-phase separation of PTH-amino acids on a WAKOSIL-PTH (4.6 × 250 mm) column (Wako, Osaka, Japan).

2.5. Sequence analysis

The search for similar sequences was performed with the FASTA3 program (Pearson, 1990), tool using the Swiss-Prot database (Bairoch and Apweiler, 2000). Alternatively, the BLASTP 2.2.23+ (Altschul et al., 1997) was employed, using the Swiss-Prot database with or without taxonomy filter for the Hylidae family (Taxon identity: 8418). Multiple sequence alignments were performed by means of the ClustalW 2.0.12 software (Thompson et al., 1994; Larkin et al., 2007).

Secondary structure prediction was performed by means of the SOPMA program (Geourjon and Deléage, 1995). Schiffer-Edmunson wheel projections (Schiffer and Edmunson, 1967) were plotted using the Pepwheel program.

3. Results and discussion

3.1. Fractionation and mass profiling of skin secretion

Direct fractionation of the skin secretion of *P. jandaia* by means of RPC (one-dimensional chromatography) yielded 18 fractions (Fig. 2). In order to increase the resolution, the skin secretion of *P. jandaia* was also fractionated by two-dimensional chromatography. In the former strategy, the CIEX step yielded 23 fractions, which were individually submitted to RPC (Fig. 3(A). After the RPC of the fractions obtained in the CIEX step, the skin secretion of *P. jandaia* was resolved in 88 fractions (B). Fractions obtained after RPC in both one- and two-dimensional chromatographies were submitted to mass spectrometric analyses.

A total of 203 molecular components were found: 71 signals (35%) exclusively detected after one-dimensional chromatography; 103 signals (51%) exclusively detected after two-dimensional chromatography; and 29 signals (14%) detected after both chromatographic strategies. This rather small intersection of the sets of molecules detected after the chromatographic strategies herein employed may be possibly attributable to MALDI signal suppression due to lower resolution in one-dimensional chromatography (more abundant components suppressing the signal of co-eluting lower abundant molecules) or to sample concentration effects (Kratzer et al., 1998; Pimenta et al., 2001; Annesley, 2003). It is noteworthy that the differences in the sets of molecules detected in the distinct chromatographic strategies are not attributable differences in the employed mass spectrometric instrumentation, since the results depicted in this section were obtained in an Autoflex III instrument whereas the employment of Ultraflex III and Synapt instrumentation in this study was restricted to the attainment of higher resolution MS/MS spectra of a selected number of peptides. Moreover, these differences are also not attributable to natural (seasonal or intra-specific) variations in the composition of the skin secretion, since the samples used in the different chromatographic strategies proceeded from the same pool, nonetheless *in vitro* sample degradation effects at some extent may not be discarded.

The masses of the detected molecular components ranged from 802.23 to 13,631.3 Da. Fig. 4 shows the histogram of the molecular masses of the components detected in the skin of *P. jandaia*. By analyzing this figure, one notices the preponderance of molecules within the 800–3500 Da range (87% of the total of molecular components detected). Ions within that scope and having sufficient signal intensities were systematically submitted to MS/MS analyses. Additionally, those peptides found to be in homogeneous state were also submitted to N-terminal sequencing by automated Edman's degradation.

3.2. Peptide sequencing and sequence analyses

In total, 57 peptides, or approximately 28% of the total number of detected ions, could be successfully sequenced/identified. The residues Leu/Ile and Gln/Lys that could not be distinguished by means of the MALDI-TOF-TOF *tandem* mass spectrometric data, whenever possible, were suggested based upon similarity with homologous Hylidae sequences or based upon similarity with related *P. jandaia* molecules whose N-terminal sequences could be undoubtedly determined by Edman degradation. Alternatively, Leu/Ile residues were assigned by means of high-resolution *tandem* mass spectrometric data acquired by means of Synapt ESI-Q-TOF instrumentation. The peptide

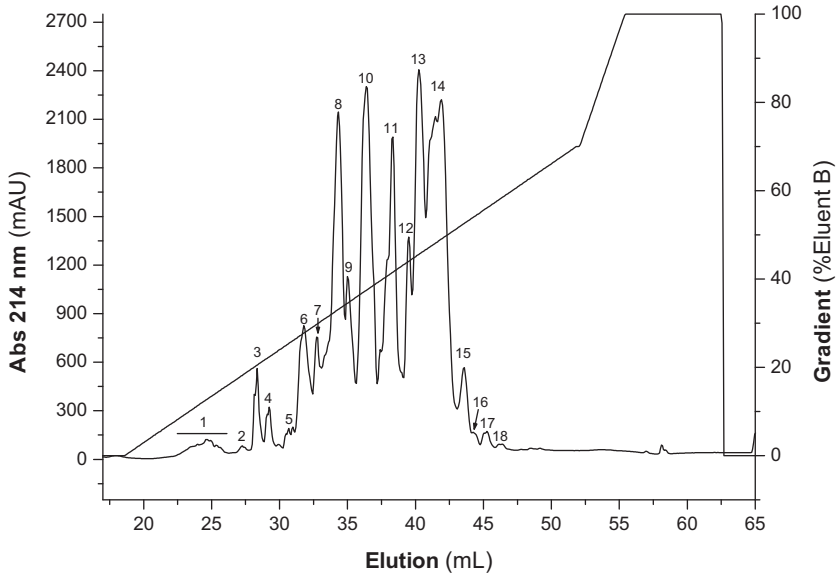

Fig. 2. One-dimensional chromatography of the skin secretion of *Phasmahyla jandaia*. Sample was loaded onto the RPC column (Chromolith Performance RP-18 100 mm × 4.6 mm) previously equilibrated with 0.1% TFA in deionized water (Eluent A) and elution was achieved by means of a linear gradient (0–70%) of 0.1% TFA in acetonitrile in 5 min (Eluent B). Flow rate: 4.0 mL/min. Readings at 214 nm (left vertical axis) and Eluent B gradient (right vertical axis) are shown.

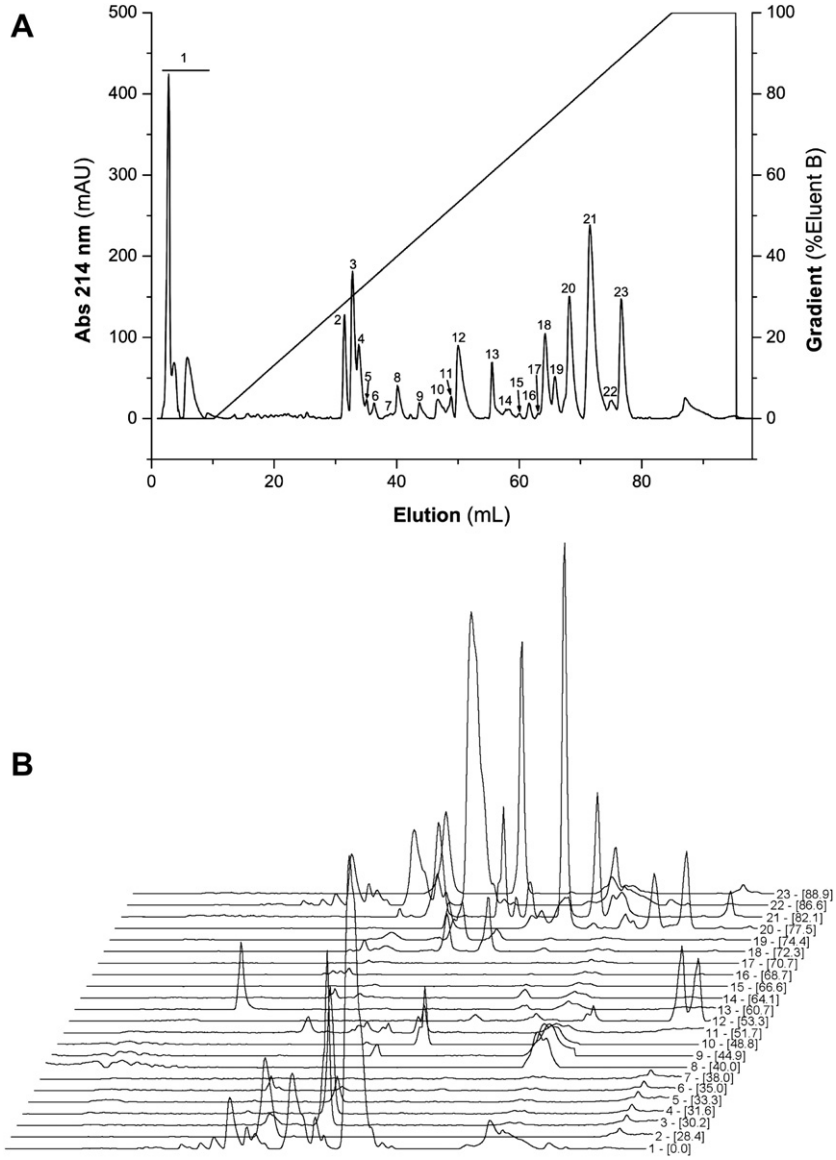

Fig. 3. Two-dimensional chromatography of the skin secretion of *Phasmahyla jandaia*. A) Cation-exchange chromatography (CIEX). Sample was loaded onto the CIEX column (TSK-Gels CM-SW, 15 cm × 4.6 mm) previously equilibrated with 50 mM sodium acetate buffer (pH = 5) (Eluent A). A linear salt gradient (0–1 M [NaCl] at a 10 mM/min rate) was completed by increasing the concentration of 50 mM sodium acetate buffer (pH = 5) with 1 M NaCl (Eluent B). Flow rate: 0.75 mL/min. Readings at 214 nm (left vertical axis) and Eluent B gradient (right vertical axis) are shown. B) Reversed-phase chromatography (RPC). CIEX fractions were loaded onto the RPC column (Chromolith Performance RP-18 100 mm × 4.6 mm) previously equilibrated with 0.1% TFA in deionized water (Eluent A) and elution was achieved by means of a linear gradient (0–70%) of 0.1% TFA in acetonitrile (Eluent B) in 5 min. Flow rate: 4.0 mL/min. The number of the CIEX fractions and their respective [NaCl] (mM) elution are depicted. Readings at 214 nm are shown.

sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession numbers depicted in Tables 1–7.

Phylloseptins, dermaseptins *stricto sensu*, dermatoxins, hyposins, tryptophyllin, caerulein-related, bradykinin-related, bradykinin potentiating, tyrosine-rich, and opioid peptides could be identified in the skin secretion of *P. jandaia*. Moreover, five peptide families without significant similarity to other known molecules could be verified. Novel phylloseptin, dermatoxin, dermaseptin, and hyposin peptides were named as per the most recent proposed nomenclature (Amiche et al., 2008). The novel tryptophyllin-related peptides and the tyrosine-rich peptide were named according to their structural class and species of origin, as previously suggested (Chen et al., 2004; Thompson et al., 2007a, 2007b). Peptides not displaying similarity with other known molecules were generically named as ***P. jandaia* P**eptide **x**–**y** (PjPx–y, for short), where 'x' denotes the Arabic numeral arbitrarily attributed to a given peptide family and 'y' denotes the Arabic numeral attributed to a given peptide within a family 'x' according to the increasing order of its molecular mass (Table 1).

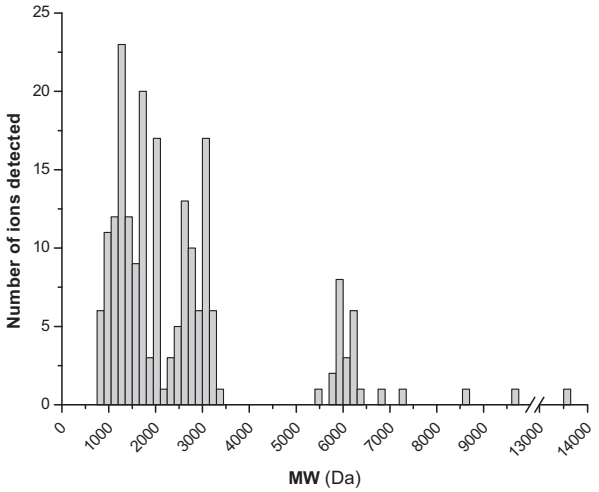

Fig. 4. Histogram of the molecular masses of components detected in the skin secretion of *Phasmahyla jandaia*. Components detected after one- and two-dimensional chromatographies are depicted without double-counting. Horizontal axis is broken between 10,000 and 13,000 Da for better visualization. Bin size: 150 Da.

3.2.1. Bradykinin (BK), bradykinin-related (BRP) and bradykinin potentiating (BPP) peptides

BK and seven analogs (BRPs) could be identified in the skin secretion of *P. jandaia* (Table 2). The presence of such peptides in the amphibian skin is widely reported. For instance, over 60 results are displayed after a search with the terms "bradykinin", "amphibian" and "skin" in the PubMed database (Accession date: 20/05/2010). It has been suggested that BRPs play a defensive role rather than possessing endogenous hormonal activity such as their counterparts in other vertebrate groups (Conlon, 1999). This hypothesis is supported by the apparent lack of a kallikrein–kinin system in amphibians (Conlon, 1999). In that sense, BK and its analogs might stimulate gastric and esophageal motility in predators, possibly stimulating emetic reflex resulting in the ejection of the prey (Conlon, 2006). Therefore, the current understanding is that the diversity of BRPs in the skin secretion of this and other anurans reflects the range of coexistent predators (Conlon, 1999). In line with that statement, the biological significance of the presence of BK variants (i.e. primary structure changes modulating specificity) may be illustrated by the fact that BK preferentially binds B_2 receptors, whereas the C-terminally truncated peptide des[Arg]^9-bradykinin has a high affinity to B_1 receptors (Regoli et al., 1993).

Amongst the BRPs herein reported figure phyllokinin and [Thr]^6-phyllokinin (Table 2). These molecules have been previously identified in the skin of other Phyllomedusinae members and display a [Tyr]^{11} sulfonation (Erspamer et al., 1985; Mignogna et al., 1997; Chen and Shaw, 2003; Brand et al., 2006a; Chen et al., 2006). The loss of 80 Da in MS/MS spectra of the 1415.7 and 1429.8 Da molecules is consistent with the previous reports of sulfonation in these peptides, which were also detected in de-sulfonated form.

The bradykinin potentiating peptide (BPP) Phypo Xa, featuring an N-terminal pyrogutamate modification, was identified amongst the molecules secreted in the sin of *P. jandaia* (Table 2). Phypo Xa, originally identified in the skin of *Phyllomedusa hypochondrialis*, potentiates bradykinin activities *in vivo* and *in vitro*, and is a competitive inhibitor of the Angiotensin Converting Enzyme (ACE) (Conceição et al., 2007). In light of these observations, synergic effects between BK/BRPs and Phypo Xa have been suggested (Conceição et al., 2007).

3.2.2. Caerulein-like peptide

One may also mention the presence of the novel phyllocaerulein variant, [Arg]^4-phyllocaerulein (Table 2). Phyllocaerulein, originally detected in the skin of *P. sauvagii*, displays an N-terminal pyroglutamic acid, [Tyr]^3 sulfonation and C-terminal amidation (Anastasi et al., 1969), whereas [Arg]^4-phyllocaerulein was only detected in de-sulfonated form (the mass corresponding to its sulfonated form −1293.5 Da – was not observed).

Tyr sulfation is catalyzed by tyrosylprotein sulfotransferase enzymes (TPSTs), which are located in the trans-Golgi network (Lee and Huttner, 1983; Baeuerle and Huttner, 1987). Such location enables the access of the TPSTs to secreted and membrane-bound proteins (Kehoe and Bertozzi, 2000). To date, there is not a defined sequence motif for Tyr sulfation, although the occurrence of acidic residues in the vicinity of the sulfation site is evidently preponderant (Kehoe and Bertozzi, 2000; Moore, 2003). Hence, this observation may be related to the fact that the sulfated form of [Arg]^4-phyllocaerulein was not detected.

Previous study has shown that the pharmacological effects elicited by phyllocaerulein and caerulein were identical in the models evaluated. Amongst the observed effects of phyllocaerulein one could mention prolonged blood pressure fall *in vivo* (Anastasi et al., 1969), making possible to speculate that [Arg]^4-phyllocaerulein, which may also possess such pharmacological activity, could act

Table 1
Amino acid sequence and molecular mass of peptides detected in the secretion of *P. jandaia* without detectable similarity to other known molecules.

Peptide name	Sequence	Experimental mass (Da)	Theoretical mass (Da)	Accession number
PjP1-1	kPEEDWGHk	1124.5	1124.5	P86602
PjP1-2	kPEEDWGHR	1152.6	1152.5	P86603
PjP1-3	kPEEDWGRES	1231.6	1.231.5	P86604
PjP1-4	kPEEDWDRTD	1289.6	1289.6	P86605
PjP2-1	GPPJJPPJP	899.4	899.5	P86606
PjP3-1	JFFkGEkkJ	1108.7	1108.7	P86607
PjP4-1	kPENENEEAJHE	1437.6	1437.6	P86608
PjP5-1	JLGMIPVAISAINJMkJa	1795.2	1795.1	P86609

Lower case 'a' indicates C-terminal amidation. Lower case 'k' indicates undifferentiated Lys/Gln residues. 'J' indicates undifferentiated Ile/Leu. Theoretical masses calculated assuming Lys in the dubious Lys/Gln residues.

Table 2
Amino acid sequence and molecular mass of known peptides identified in the skin secretion of *Phasmahyla jandaia* and of [Arg]^4-Phyllocaerulein.

Peptide name	Sequence	Experimental mass (Da)	Theoretical mass (Da)	Accession number
Bradykinin and related peptides				
Bradykinin	RPPGFSPFR	1059.5	1059.6	P86627
des[Arg]^9-bradykinin	RPPGFSPF	903.4	903.5	P86628
[Thr]^6-bradykinin	RPPGFTPFR	1073.6	1073.6	P86629
des[Arg]^9-[Thr]^6-bradykinin	RPPGFTPF	917.4	917.5	P86630
Phyllokinin de-sulfonated	RPPGFSPFRIY	1335.7	1335.7	P86631
Phyllokinin	RPPGFSPFRIY$_{SO3}$	1415.7	1415.7	
[Thr]^6-phyllokinin de-sulfonated	RPPGFTPFRIY	1349.8	1349.7	P86632
[Thr]^6-phyllokinin	RPPGFTPFRIY$_{SO3}$	1429.8	1429.7	
Bradykinin potentiating peptide				
Phypo Xa	qFRPSYQIPP	1214.9	1214.6	P86643
Caerulein-like peptide				
[Arg]^4-phyllocaerulein	qEYRGWMDFa	1212.4	1212.5	P86625
Opioid peptides				
Dermorphin	YAFGYPSa	802.2	802.4	P86633
Deltorphin (D-Met^2-delt)	YMFHLMDa	954.4	954.4	P86634
[Met(Ox)]^6-Deltorphin	YMFHLM$_{ox}$Da	970.4	970.4	

Y_{SO3} indicates sulfonated tyrosine residue. M_{ox} indicates an oxidated methionine residue. Lower case 'q' stands for pyroglutamic acid. Lower case 'a' indicates C-terminal amidation.

synergistically with BK, BRPs and Phypo Xa. Moreover, considering that caerulein is a potent analgesic (100–700 times more potent than morphine) (Bowie and Tyler, 2006), a synergic action of [Arg]^4-phyllocaerulein with the opioid peptides dermorphins and deltorphins (discussed below) may also be speculated. Nonetheless, it is noteworthy that the bioactivity of caerulein is significantly diminished if the sulfate group is hydrolyzed (Bowie and Tyler, 2006) as observed for phyllokinin (Anastasi et al., 1966).

3.2.3. Tryptophyllins

Another peptide family, the tryptophyllins, are herein represented the by the so-called *P. jandaia* T3 tryptophyllins (Pj-T3-1 to Pj-T3-3) (Table 3). Tryptophyllins constitute a numerous and structurally diverse group of molecules, which are divided into the sub-groups tryptophyllin-1 (T-1), tryptophyllin-2 (T-2) and tryptophyllin-3 (T-3) based upon their sequence similarities (Chen et al., 2004). The sub-group T-1 includes hepta- or octapeptides possessing an N-terminal basic amino acid residue and an internal PW or PPW motif. Most members of the sub-group T-2, which is constituted of peptides with variable lengths containing an internal PW-doublet, are C-terminally amidated. The sub-group T-3, the most highly conserved amongst the tryptophyllin sub-groups, consists of peptides with an internal PPPIY motif (Chen et al., 2004; Thompson et al., 2007a).

Despite the tridecapeptides Pj-T3-1, Pj-T3-2 and Pj-T3-3 being similar to the tryptophyllins belonging to the T-3 sub-group, as shown in Table 3, the sequenced *P. jandaia* tryptophyllins display the internal motif SPPIY or DPPIY, instead of the 'classical' PPPIY motif. *P. jandaia* tryptophyllins feature a N-terminal pyroglutamate as determined for their counterparts in *Phyllomedusa rohdei* (Montecucchi et al., 1985), *P. bicolor* (Erspamer et al., 1985), *P. sauvagii* (Erspamer et al., 1985), and *Phyllomedusa azurea* (Thompson et al., 2007a). By analyzing the sequence of Pj-T3 peptides along with the previously described peptides, one can notice the consensus KP-doublet prior to the doublet of aromatic residues in the T-3 tryptophyllins (Table 3).

The biological activity of T-3 tryptophyllins remains elusive (Erspamer et al., 1985, 1986; Montecucchi, 1985; Chen et al., 2004) although effects on liver protein synthesis and body weight have been reported for Tryptophyllin-13 from

Table 3
Amino acid sequence and molecular mass of the *Phasmahyla jandaia* T-3 tryptophyllin-1 (Pj-T3-1) and its alignment with previously sequenced T-3 tryptophyllins.

Peptide name	Species	Sequence	Exp. mass (Da)	Theor. mass (Da)	Accession number
Pj-T3-1	P. jandaia	qDKPFWSPPIYPV–	1555.9	1555.8	P86610
Pj-T3-2	P. jandaia	qDKPFWSPPIYPH–	1593.6	1593.8	P86611
Pj-T3-3	P. jandaia	qDKPFWDPPIYPV–	1583.7	1583.8	P86612
Un-named	P. sauvagii	qDKPFWPPPIYPV–	N.A.	1565.8	–
Pha-T3-1	P. azurea	qDKPFWPPPIYIM–	N.A.	1613.8	P84949
Pha-T3-2	P. azurea	qDKPFWPPPIYPM–	N.A.	1597.8	P84950
Tryptophyllin-13	P. rohdei	qEKPYWPPPIYPM–	N.A.	1627.8	P04096
Un-named	P. bicolor	qEKPFYPPPIYPV–	N.A.	1556.8	–
GM-14	B. variegata	-GKPFYPPPIYPEDM	N.A.	1649.8	P84215
Consensus	N/A	**:: ****	N.A.	N.A.	N.A.

Differentiation of Leu/Ile and Lys/Gln residues in Pj-T3-1 was based upon similarity to previously sequenced tryptophyllins. Multiple sequence alignment generated using ClustalW 2.0.12, where gaps (–) were introduced to maximize alignment. Lower case 'q' stands for pyroglutamic acid. 'N.A.' stands for non-applicable. Consensus symbols, namely '*', ':' and '.', means that the residues in that column are identical in all sequences in the alignment, that conserved substitutions have been observed, and that semi-conserved substitutions are observed, respectively.

Table 4
Amino acid sequence and molecular masses of Hyposin-J1 and its alignment with previously sequenced hyposins and related peptides.

Peptide name	Species	Sequence	Exp. mass (Da)	Theor. mass (Da)	Accession number
HPS-J1	P. jandaia	FRPAJIVRTKGK–a	1383.8	1383.9	P86613
HPS-H4	P. azurea	FRPAJIVRTKGTRJa	N.A.	1626.0	P84957
HPS-H3	P. azurea	LRPAVIVRTKGK–a	N.A.	1335.9	P84956
Sauvatide	P. sauvagii	LRPAILVRTK—a	N.A.	1164.8	CAX48601
AMP-1	P. hypochondrialis	LRPAVIVRTKAJ–	N.A.	1335.9	P84524
HPS-H1	P. azurea	LRPAVI-RPKGK–a	N.A.	1232.8	P84954
HPS-H2	P. azurea	LRPAFI-RPKGK–a	N.A.	1280.8	P84955
Consensus	N/A	:***.: *.*	N.A.	N.A.	N.A.

Differentiation of Leu/Ile or Lys/Gln residues in Hyposin-J1 was based upon similarity to Hyposin-H3 and Hyposin-H4. Lower case 'a' indicates C-terminal amidation. 'J' indicates undifferentiated Ile/Leu. Multiple sequence alignment generated using ClustalW 2.0.12, where gaps (–) were introduced to maximize alignment. Hyposin-H5 (P84958) was not included in this table due to an inconsistency between its experimental mass (1514.0 Da) and the mass calculated for its reported sequence (1588.0 Da). 'N.A.' stands for non-applicable. Consensus symbols, namely '*', ':' and '.', means that the residues in that column are identical in all sequences in the alignment, that conserved substitutions have been observed, and that semi-conserved substitutions are observed, respectively.

P. rohdei (Montecucchi, 1985), which is consistent with a more recent report of insulin-releasing activity for GM-14 from *Bombina variegata* (Marenah et al., 2004).

Moreover, the presence of the PP, PPP, and KP motifs in T-3 tryptophyllins allows the speculation that these peptides could act upon the vascular system as hypotensive agents. This assumption is based on the studies recently developed by our group on the so-called Hypotensins (TsHpt), a family of BPPs present in the venom of the Brazilian scorpion *Tityus serrulatus* (Verano-Braga et al., 2008, 2009; Verano-Braga, 2009). Structure minimization assessment of TsHpt-I, have demonstrated that the tripeptide KPP consistently reproduces all vascular effects of the 25 amino acid-long native TsHpt-I (i.e. BK-potentiation, transient BK-independent hypotension in rats, and NO-dependent vasodilatation in aortic rings preparations) (Verano-Braga, 2009). Moreover, the dipeptide KP reproduces the transient BK-independent hypotension, whereas BK-dependent hypotension elicited by TsHpt-I was reproduced by the dipeptide PP (Verano-Braga, 2009).

3.2.4. Opioid peptides

The opioid-peptides dermorphin and deltorphin (D-Met^2-Deltorphin) could also be identified in the skin secretion of *P. jandaia*. The pharmacological properties of these peptides and their analogs have been extensively reviewed elsewhere (Erspamer et al., 1989; Melchiorri and Negri, 1996; Amiche et al., 1998; Lazarus et al., 1999; Negri et al., 2000; Negri and Melchiorri, 2006). Briefly, dermorphin, a selective and potent μ-opioid receptor agonist (100 times more potent that morphine in guinea pig ileum preparation), produces antinociception, catalepsy, respiratory depression, constipation, tolerance and depression (Negri and Melchiorri, 2006). Differently, deltorphin has higher selectivity and affinity for δ-opioid receptor (Negri and Melchiorri, 2006). It is noteworthy that the intensity of the analgesia induced by δ-agonists depends on the co-activation of μ-opioid receptor by endogenous or exogenous molecules (Negri and Melchiorri, 2006). Injection of deltorphins in rats (0.06–3.8 nmol/animal *i.c.v*) increases locomotor activity and induces stereotyped behaviour (Negri and Melchiorri, 2006). Yet, deltorphin improves memory consolidation in a passive avoidance test in mice (Negri and Melchiorri, 2006).

Importantly, the post-translational modifications in these opiate peptides include C-terminal amidation and the presence of dextrorotatory residues (D-Ala or D-Met), both playing roles in the augmentation of the potency and stability of the molecules by which they are borne (Broccardo et al., 1981; Negri et al., 2000). Such modifications were assigned to the opioid-peptides herein identified based upon the information available from the molecules previously characterized in other phyllomedusines. This was due to the fact that an amidated Asp residue is *quasi* isobaric to a Asn residue (114.02 *versus* 114.04, respectively), and that dextrorotatory and levorotatory residues are not readily distinguishable by MALDI-TOF/TOF (Sachon et al., 2009).

Moreover, deltorphin was also identified in a form bearing an oxidation in [Met]^6. The oxidized methionine residue was distinguished from a *quasi* isobaric phenylalanine residue (147.04 *versus* 147.07 Da, respectively) by means of the verification of the loss of CH_3SOH (64 Da) from the parent (y_7), y_{3-6} and b_6 ions in the MS/MS spectrum of the so-called [$Met_{(ox)}$]^6–deltorphin (Fig. 5) (Jagannadham, 2009).

Table 5
Amino acid sequence and molecular masses of Tyrosine-Rich Peptide-J1 and its alignment with previously sequenced peptides.

Peptide name	Species	Sequence	Exp. mass (Da)	Theor. mass (Da)	Accession number
TRP-J1	P. jandaia	LMYYTLPRPVa	1250.7	1250.7	P86709
Un-named	P. sauvagii	LMYYTLPRPVa	N.A.	1250.7	–
TRP-HA1	P. azurea	VMYYSLPRPVa	1222.7	1222.7	P84960
Consensus	N/A	:***:*****	N.A.	N.A.	N.A.

Differentiation of Leu/Ile residues in TRP-J1 was based upon similarity to its un-named counterpart in *P. sauvagii* and TRP-HA1. Lower case 'a' indicates C-terminal amidation. Multiple sequence alignment generated using ClustalW 2.0.12. 'N.A.' stands for non-applicable. Consensus symbols, namely '*', ':' and '.', means that the residues in that column are identical in all sequences in the alignment, that conserved substitutions have been observed, and that semi-conserved substitutions are observed, respectively.

Table 6
Amino acid sequence alignment and molecular mass of the *Phasmahyla jandaia* Phylloseptins (PLS-J) and their identified truncated forms.

Peptide name	Sequence	Experimental mass (Da)	Theoretical mass (Da)	Accession number
Full length peptides				
PLS-J1	FLSLIPHAINAISAIANHFa	2047.2	2047.1	P86614
PLS-J2	FLSLIPHAINAISAIADHFa	2048.1	2048.1	P86615
PLS-J3	FLSLIPHAINAISAIANHLa	2013.2	2013.2	P86616
PLS-J4	FLSLIPHAINAISAIAHHLa	2036.1	2036.2	P86617
PLS-J5	*FLSLIPHAISAISAIAHHLa*	2009.3	2009.2	P86618
PLS-J6	FLSLIPHAISAISAIANHLa	1986.3	1986.1	P86619
PLS-J7	FLSLIPHAISAISAIADHLa	1987.1	1987.1	P86620
Consensus	********.******.*:	N.A.	N.A.	N.A.
Truncated forms				
PLS-J1[1–12]	FLSLIPHAINAI———	1307.8	1307.8	N.A.
PLS-J5[1–12]	FLSLIPHAISAI———	1280.7	1280.7	N.A.
PLS-J5[1–14]	FLSLIPHAISAISA——	1438.7	1438.8	N.A.
PLS-J3[3–19]	-SLIPHAINAISAIANHLa	1752.8	1753.0	N.A.
PLS-J4[3–19]	-SLIPHAINAISAIAHHLa	1776.1	1776.0	N.A.
PLS-J5[3–19]	-SLIPHAISAISAIAHHLa	1748.9	1749.0	N.A.
PLS-J6[3–19]	-SLIPHAISAISAIANHLa	1726.0	1726.0	N.A.
PLS-J1[4–19]	—LIPHAINAISAIANHFa	1699.8	1700.0	N.A.
PLS-J1[5–19]	——IPHAINAISAIANHFa	1586.9	1586.9	N.A.
PLS-J5[5–19]	——IPHAISAISAIAHHLa	1548.9	1548.9	N.A.

Underlining indicates sequence confirmation by Edman degradation. The identity of the C-terminal Leu residue in PLS-J4[3–19] and PLS-J6[3–19] was determined by the verification of its corresponding w_{a1} ion in high-resolution MSMS spectrum. The identity of the C-terminal Leu residue in PLS-J3 to PLS-J7, and in their truncated forms, was suggested based on its correspondence to PLS-J4[3–19] and PLS-J6[3–19]. Differentiation of the remaining Leu/Ile residues in PLS-J1 to PLS-J4, PLS-J6 and PLS-J7 was based upon similarity to PLS-J5. Lower case 'a' indicates C-terminal amidation. Multiple sequence alignment of full-length peptides generated using ClustalW 2.0.12. 'N.A.' stands for non-applicable. Consensus symbols, namely '*', ':' and '.', means that the residues in that column are identical in all sequences in the alignment, that conserved substitutions have been observed, and that semi-conserved substitutions are observed, respectively.

3.2.5. Hyposin

The sequenced molecules also include the so-called hyposin-J1 (HPS-1), which bears a C-terminal amidation Table 4. The hyposins, identified in the skin secretion of *P. azurea*, consist of a family of five peptides with lengths spanning from 11 to 15 residues (Thompson et al., 2007a). The sequences of hyposin-H1 to 5 (Table 4) were determined by Q-TOF MS/MS analysis, which did not distinguish Ile/Leu residues. However, the structure of hyposin-H1 (formerly, hyposin-HA1) was validated by cDNA sequencing, confirming the identity of [Ile]^6 and allowing the assumption that the corresponding position of hyposin-H2 to 5 also

Table 7
Amino acid sequence alignment and molecular mass of the *Phasmahyla jandaia* Dermaseptins (DRS-J) and Dermatoxins (DRT-J) and their identified truncated forms.

Peptide name	Sequence	Exp. mass (Da)	Theor. mass (Da)	Accession number
Dermaseptins				
DRS-J1	GLWKNMLSGIGKLAGQAALGAVKTLVa	2594.3	2594.5	P86635
DRS-J2	GLWKNMLSGIGKLAGEAALGAVKTLVa	2595.4	2595.5	P86636
DRS-J3	ALWKNMLSGIGKLAGQAALGAVKTLVa	2608.4	2608.5	P86637
DRS-J4	ALWKDMLSGIGKLAGQAALGAVKTLVa	2609.3	2609.5	P86638
DRS-J5	GLWSKIKEAGKAAVKAAGKAALGAVANSVa	2764.4	2764.6	P86683
DRS-J6	GLWSKIKEAGKAAVKAAGKAALGAVADSVa	2765.4	2765.6	P86684
DRS-J7	GLWSKIKAAGKEAAKAAAKAAGKAALNAVSEAVa	3149.9	3149.8	P86639
DRS-J8	GLWKSLLKNVGKAAGKAALNAVTDMVNQA	2967.5	2967.6	P86640
DRS-J9	GLWKSLLKNVGKAAGKAALNAVTDMVNQS	2983.5	2983.6	P86641
DRS-J10	ALWKSLLKGAGQLVGGVVQHFMGSQGQPES	3108.6	3108.6	P86642
Truncated forms				
DRS-J7[3–33]	-WSKIKAAGKEAAKAAAKAAGKAALNAVSEAVa	2980.0	2979.7	N.A.
Dermatoxins				
DRT-J1	SLGGFLKGVGKALAGVGKVVADQFGNLLQAGQa	3098.0	3097.7	P86621
DRT-J2	SLGGFLKGVGKALAGVGKMVADQFGNLLQAGQa	3129.6	3129.7	P86622
DRT-J3	SLGGFLKGVGKVLAGVGKVVADQFGNLLEAGQa	3126.9	3126.8	P86623
Consensus	**********.******:*********:***	N.A.	N.A.	N.A.

Underlining indicates sequence confirmation by Edman degradation. Differentiation of Leu/Ile and Lys/Gln residues in DRS-J1, DRS-J2, DRS-J3 and DRS-J4 was based upon similarity to P84926; in DRS-J5 and DRS-J6, based upon similarity to Q90ZK5 and AAB24271.1; in DRS-J7, based upon similarity to P83638; in DRS-J8 and DRS-J9, based upon similarity to CAI99866; in DRT-J1, DRT-J2 and DRT-J3, based upon similarity to Q9PT75 and O93221. Lower case 'a' indicates C-terminal amidation. Multiple sequence alignment of dermatoxin peptides generated using ClustalW 2.0.12. 'N.A.' stands for non-applicable. Consensus symbols, namely '*', ':' and '.', means that the residues in that column are identical in all sequences in the alignment, that conserved substitutions have been observed, and that semi-conserved substitutions are observed, respectively.

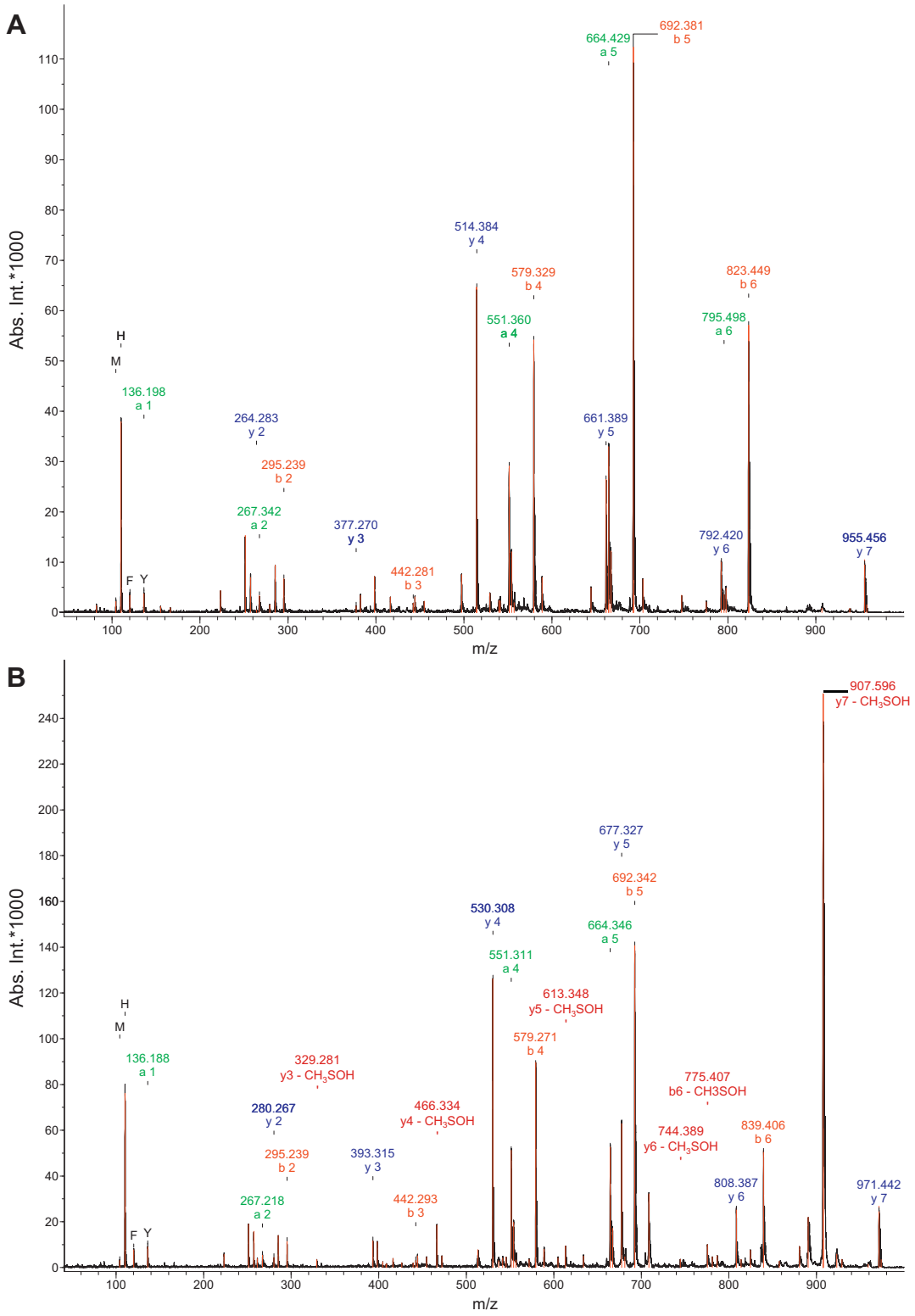

Fig. 5. MS/MS spectra interpretation for Deltorphin (A) and [$Met_{(ox)}$]^6-Deltorphin (B). Observed MW: 954.44 Da (Deltorphin) and 970.44 Da ([$Met_{(ox)}$]^6-Deltorphin). Observed ions are depicted in bold. Leu/Ile, and C-terminal Asn/Asp-NH_2 residues were differentiated based upon information available pertaining to molecules previously identified in other Phyllomedusinae species. The loss of CH_3SOH (64 Da) from the ions y_{3-6} and b_6, indicating the oxidation of [Met]^6 is depicted. Precursor charge state: 1. Tolerance: 0.3 Th.

contain an Ile residue. The Ile/Leu residues in position 5 of hyposin-H4 and 5 and in the C-terminal of hyposin-H4 remain undifferentiated (Thompson et al., 2007a).

The biological activities of the hyposins are yet undetermined (Thompson et al., 2007a). However, sauvatide, a peptide identified in the skin secretion of *P. sauvagii* similar to hyposin-H3 (Table 4), was found to be a potent contractile agent upon the rat urinary bladder ($EC_{50} = 2.2$ nM) (Wang et al., 2009). Although sauvatide failed to demonstrate activity against *Escherichia coli* and *Staphylococcus aureus* at concentrations up to 250 μM (Wang et al., 2009), a similar peptide from *P. hypochondrialis* (AMP-1) (Table 4) was reported to possess antimicrobial activity against the gram-positive bacterium *Micrococcus luteus*, even though further details on the potency and spectrum of this molecule were not disclosed (Thompson et al., unpublished results).

The sauvatide precursor displays the characteristic structural arrangement of the dermaseptin superfamily, with a signal peptide followed by an acidic spacer (Nicolas and El Amri, 2009; Wang et al., 2009). Contrastingly, previous attempts of cloning hyposins by means of the employment of primers targeting conserved dermaseptin signal peptide have failed (Thompson et al., 2007a). The incipient knowledge pertaining to the structural diversity and biological activity of the hyposins and related peptides poses these molecules as promising investigation subjects.

3.2.6. Tyrosine-rich peptide

The molecular repertoire of the skin secretion of *P. jandaia* includes the tyrosine-rich peptide TRP-J1, which was found to be identical to its un-named counterpart in the skin secretion of *P. sauvagii* (Erspamer et al., 1986) (Table 5). A similar peptide (TRP-HA1), which possesses conservative substitutions (V1L and S5T), has also been detected in *P. azurea* (Thompson et al., 2007b). The abovementioned molecules bear C-terminal amidation and lack information pertaining to their biological actions.

The MSMS spectrum of TRP-J1 was obtained in Synapt instrumentation, which rendered high-resolution mass-spectral data. Nonetheless, a search for *w* and *d* ions aiming the mass-spectral assignment of Ile/Leu residues was inconclusive. Hence, Ile/Leu assignments were performed as per the similarity with the previously sequenced molecules.

3.2.7. Phylloseptins, stricto sensu dermaseptins, and dermatoxins

The most numerous peptides sequenced in the skin secretion of *P. jandaia* were those belonging to the dermaseptin superfamily, which is defined as a group of host-defense peptides present in the skin of Hylidae and Ranidae frogs (Nicolas and El Amri, 2009). The peptides in this superfamily are genetically related and possess conserved signal sequences and intervening segments in their pre-proforms although having diverged into structurally and functionally distinct families (Nicolas and El Amri, 2009).

Grippingly, preprodermaseptins-encoding peptides also include neuropeptides such as the opioid peptides (*P. sauvagii* and *P. bicolor*), $[Thr]^6$-phyllokinin (*P. sauvagii*) and tryptophyllin-1 (*Agalychnis dacnicolor*) (Nicolas and El Amri, 2009). The conserved intervening segments or 'acidic propieces' carry negative charges that may interact with the cationic lytic peptides, thus possibly preventing the host cell from self-inflicted damage (Michaelson et al., 1992; Tennessen, 2005). Accordingly, a number of peptides detected in our analysis (2318.1, 2553.3, 2664.32, 2682.3 and 2767.5 Da) are likely to represent fragments of dermaseptin acidic propieces. However, due to the repetitive nature of their primary structure, which bear numerous Asp and, most preponderantly, Glu residues, and due to the general poor ion abundance of their MS/MS spectra, their amino acid sequences could not be undoubtedly resolved.

In the skin of *P. jandaia*, dermaseptin *stricto sensu* (DRS), dermatoxin (DRT) and phylloseptin (PLS) families represented the dermaseptin superfamily (Table 6 and Table 7). Table 6 lists the sequenced *P. jandaia* PLS peptides and their detected truncated forms. In total, 7 full-length peptides were sequenced, in addition to 3 C-terminally and 7 N-terminally truncated forms. Truncated forms of PLS peptides have been reported previously (Thompson et al., 2007a). The peptide PLS-J5 was submitted to Edman N-terminal sequencing, by means of which it was possible to confirm the identity of its residues $[Leu]^{2;4}$ and $[Ile]^{5;9;12;15}$. The identity of the Leu/Ile residues in the remaining PLS-J peptides, and in their truncated forms, was suggested based upon the identity of the residues assigned to PLS-J5 in their corresponding positions.

The MSMS spectrum of the truncated peptides PLS-J4 [3–19] and PLS-J6[3–19] was also obtained in Synapt instrumentation, by which it was possible to notice the presence of the 72.09 $m/z_{obs.}$ (w_{a1}) ion in both spectra (data not shown). This suggests that a Leu residue occupies the C-terminal position in both peptides. It should also be noticed that *w* (86.03 and 100.08 *m/z*) and *d* (1653.94 and 1667.90 *m/z*) ions corresponding to an Ile residue in the C-terminal were not detected in both peptides. In light of this, the identity of the C-terminal Leu residue was assigned to PLS-J3 to PLS-J7, and to their truncated forms. Moreover, in PLS-J4[3–19] the identity of the $[Ile]^{3;7;10}$ residues, previously suggested based on its correspondence with PLS-J5, was supported by means of the verification of the ions d_{a3} (258.10 $m/z_{obs.}$), d_{b10} (988.54 $m/z_{obs.}$), w_{a7} (815.44 $m/z_{obs.}$) and w_{a11} (1113.64 $m/z_{obs.}$) (data not shown).

An N-terminally extended form of PLS-J4 (observed mass: 2220.2 Da) was also verified (not shown). The poor abundance of low-range b ions and high-range y ions in the obtained MS/MS spectrum of this molecule resulted in the impossibility of the confident assignment of its N-terminal extension, which may be constituted by an AL- or VP-doublet.

PLSs were originally characterized in the skin of *P. hypochondrialis* and *Phyllomedusa oreades* (Leite et al., 2005), although their presence has also been registered in other members of the *Phyllomedusa* genus such as *P. burmeisteri*, *Phyllomedusa distincta*, *P. rohdei*, *P. tarsius* and *P. tomopterna* (Leite et al., 2005), *P. azurea* (Thompson et al., 2007b; Kuckelhaus et al., 2009) and *Agalychnis lemur* (Conlon et al., 2007; Abdel-Wahab et al., 2008). These 19–21 amino acid-long antibacterial and anti-protozoan peptides, feature a conserved N-terminal region and a C-terminal amidation (as observed for the *P. jandaia* PLSs - Table 6). CD and NMR investigations revealed that the *P. hypochondrialis* peptides PLS-H1, PLS-H2 and PLS-H3

(formerly PS-1, PS-2 and PS-3, respectively) exhibit random coil conformation in aqueous solution and continuous helix conformation in membrane-mimetic media (Resende et al., 2008). Yet, the helical structures of these peptides exhibit an amphipathic character (Resende et al., 2008), which allows the efficient partition into the membrane interface, resulting in their membrane-destabilizing and pore-forming activities (Shai, 1999; Zasloff, 2002; Bechinger, 2005). It has been also found that the electrostatic interactions between the cationic side chains of residues in position 16 (PLS-H1 and PLS-H2) and 17 (PLS-H1, PLS-H2 and PLS-H3) with the negative end of the helix dipole stabilize and promote the helical conformation at the C-terminal (Resende et al., 2008).

The prediction of secondary structure of *P. jandaia* PLSs indicated that the α-helix content of these peptides ranges from 74% to 100% (not shown). Moreover, when plotted in Schiffer-Edmunson wheel projections, the amphipathic nature of their cylindrical surfaces is evidenced (Fig. 6). However, it is worthy to notice the presence of the residue [Asp][16] in PLS-J2 and PLS-J7. An acidic residue in proximity to the negative end of the helix dipole could destabilize the helical conformation, with possible implications to the biological actions of the peptides. For instance, PLS-L2 (FLSLIPHVISALSSL-NH_2), present in the skin of *A. lemur*, does not feature basic residues next to the negative end of the helix dipole (Abdel-Wahab et al., 2008). Interestingly, PLS-L2 was found to possess *in vitro* and *in vivo* insulin

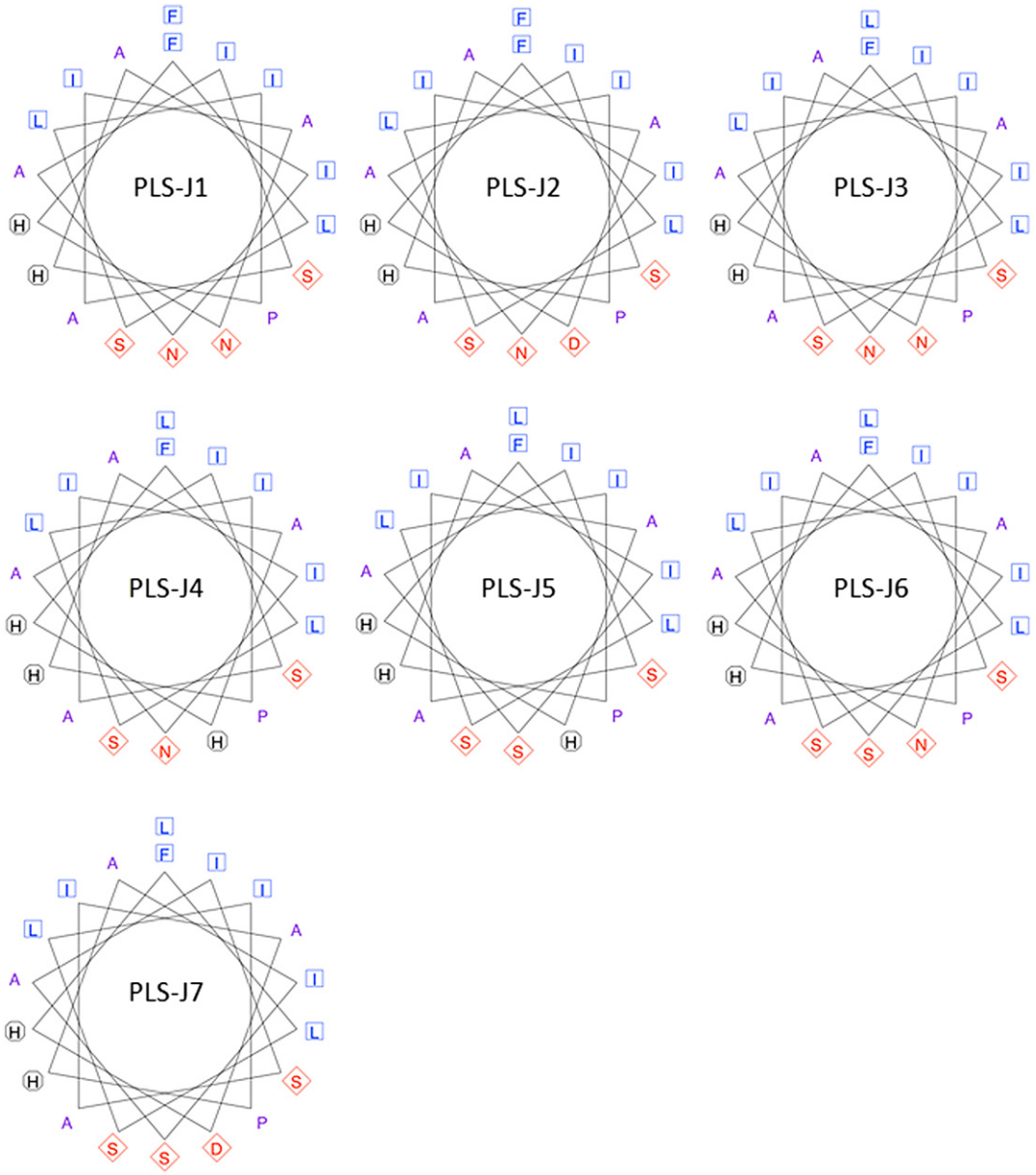

Fig. 6. α-helical (Schiffer-Edmunson) wheel plots of *Phasmahyla jandaia* phylloseptins. Hydrophobic residues marked by squares. Hydrophilic residues marked by diamonds. Positive residues marked by octagons. The amphiphilic structure prediction of the peptides showed. Plot created by the pepwheel software.

releasing activity, but was inactive against *E. coli* and weakly active towards *S. aureus* (MIC = 50 μM) (Abdel-Wahab et al., 2008). Contrastingly, PLS-L1 (LLGMIPLAISAISALSKL-NH_2), embodying a $[Lys]^{17}$, is devoid of insulin releasing activity and is highly active against *S. aureus* (MIC = 8 μM) (Conlon et al., 2007). Moreover, it has been demonstrated that there is an inverse relationship between cationicity and insulin-releasing activity in peptides belonging to the temporin family (Abdel-Wahab et al., 2007) and pseudin-2 (Abdel-Wahab et al., 2005). Therefore, the investigation of the structure and function of the variants PLS-J2 and PLS-J7 could lead to insightful information. Yet, one may mention the similarity of the peptide PjP5-1 with the phylloseptins as evidenced in Fig. 7. The projection of PjP5-1 into a Schiffer-Edmunson wheel (not shown) fails to evidence an amphipathic structure, which suggests some distinct function for it.

Eleven *stricto sensu* dermaseptins (including a truncated form) and three dermatoxins were sequenced (Table 7). The peptide DRS-J10 was submitted to Edman N-terminal sequencing, by means of which it was possible to confirm the identity of its residues $[Leu]^{2;6;7;13}$, $[Lys]^{4;8}$ and $[Gln]^{12;19;25;27}$. The identity of Leu/Ile and Lys/Gln residues in the remaining DRS-J and DRT-J peptides was suggested based upon the similarity with previously characterized molecules (refer to Table 7 and Fig. 8). In order to further confirm the C-terminal sequence the listed dermaseptins and dermatoxins, an attempt of digesting these peptides with carboxypeptidase-Y was made (not shown). However, peptides DRS-J1 till DRS-J7 and DRT-J1 till DRT-J3 were protected from the reaction, thus further evidencing their amidated C-terminal.

The amino acid sequence of DRS-J7 is identical to DRS-DI1 (former Dermadistictin K, or DD K) from *P. distincta* (Batista, 1999). One should notice that DRS-J7 is C-terminally amidated whereas DRS-DI1 was originally reported without an amidation (Batista, 1999). The determination of the primary structure of DRS-DI1 was performed by Edman degradation and the sequence data was validated by means of the verification the molecular mass of the native peptide (Batista, 1999). Nonetheless, the reported molecular masses consisted in average masses (Batista, 1999), instead of monoisotopic masses, which hindered the confident determination of whether the peptide possessed a carboxyamide terminal or not. Posterior studies pertaining to the characterization of the biological activity, three-dimensional structure, peptide–liposome interactions and liposome-fusion/lysis of DRS-DI1 interchangeably considered it in non-amidated (Brand et al., 2002) and amidated (Brand et al., 2002; Leite et al., 2008b; Silva et al., 2008; Verly et al., 2009) forms (similar situation is observed to DRS-DI2, former Dermadistictin L or DD L). Moreover, the truncated DRS-J7[3–33] was verified, although DRS-DI1[5–33] (2578.5 Da), previously reported in *P. distincta* (Batista, 1999), could not be detected in *P. jandaia*. As per its biological functions, DRS-DI1 possesses broad-range antibacterial (Batista, 1999), anti-*Trypanosoma cruzi* (Brand et al., 2002) and antifungal activities without toxicity to mammalian cells (Brand et al., 2002; Leite et al., 2008b). It has been observed that the liposome lysis caused by DRS-DI4 was preceded by a liposome-fusion phase (Silva et al., 2008). Yet,

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PLS-J5  FLSLIPHAISAISAIAHHJa
PJP5-1  JLGMIPVAISAINIMk--Ja
        :*.:** *****. :   *
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Fig. 7. Amino acid sequence alignment of PLS-J5 and PjP5-1. Underlining indicates sequence confirmation by Edman degradation. Differentiation of Leu/Ile residues in PjP5-1 was based upon similarity to PLS-J5. Lower case 'k' indicates undifferentiated Lys/Gln residues. 'J' indicates undifferentiated Ile/Leu. Lower case 'a' indicates C-terminal amidation. Multiple sequence alignment generated using ClustalW 2.0.12. Sequence consensus displayed below aligned sequences. Consensus symbols, namely '*', ':' and '.', means that the residues in that column are identical in all sequences in the alignment, that conserved substitutions have been observed, and that semi-conserved substitutions are observed, respectively.

multidimensional solution NMR spectroscopy evidenced its α-helical conformation spanning from position 7 till the carboxyamide terminal, when in membrane-mimetic environment (Verly et al., 2009). This peptide aligns itself in parallel to phospholipid membranes, and was suggested to partition into the bilayer, thus disrupting the membrane arrangement (Verly et al., 2009).

DRS-J1 till DRS-J4 are similar to DRS-TA6 (former Dermaseptin-6) from *P. tarsius* (Prates et al., unpublished results), to DRS-DI4 (former DD Q1) from *P. distincta* (Batista, 1999) and to DRS-B3 from *P. bicolor* (Charpentier et al., 1998) (Fig. 8A). DRS-TA6, DRS-DI4 and DRS-B3 are active against the Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *Pseudomonas aeruginosa*) bacteria (Prates et al., unpublished results; Batista, 1999; Charpentier et al., 1998). For DRS-DI4 the moderate activity against *Enterococcus faecalis* (MIC = 22.0 μM) has also been reported (Batista, 1999). Hemolytic effects of DRS-DI4 and DRS-TA6 are only observed at 36 and 612 μM, respectively (Prates et al., unpublished results; Batista, 1999).

DRS-J8 and DRS-J9 are identical to DRS-S7 from *P. sauvagii* (Chen et al., 2003) (Fig. 8B), except by the presence of an extra C-terminal Ala or Ser residue, respectively, and by the lack of C-terminal amidation. Those two facts are related since DRS-J8 and DRS-J9 lack the Gly residue necessary for the action of the α-amidating enzyme (Kim and Seong, 2001). Fig. 8B also reports other peptides similar to DRS-J8 and DRS-J9, including DRS-DI2 (former Dermadistinctin L, or DD L) from *P. distincta* (Batista, 1999), DRS-H1 (Chen et al., 2006) and DRS-H4 (former DShypo02) (Brand et al., 2006b) from *P. hypochondrialis*. As per the biological properties of the phyllomedusine counterparts of DRS-J8 and DRS-J9, it is worthy pointing that DRS-DI2 had limited activity against *P. aeruginosa* (MIC = 38 μM) and *E. faecalis* (MIC = 10 μM), but was highly active against *E. coli* (MIC = 2.5 μM) and *S. aureus* (MIC = 1.3 μM) (Batista, 1999). Similarly to DRS-DI1, DRS-DI2 possesses anti-*Trypanosoma cruzi* (Brand et al., 2002) and anti-fungal activities without toxicity to mammalian cells (Brand et al., 2002; Leite et al., 2008b). Also, it has been observed that the liposome lysis caused by DRS-DI2 was preceded by a liposome-fusion phase longer than that observed for DRS-DI1, (Silva et al., 2008).

DRS-J5 and DRS-J6 are similar to DRS-B8 (former DRS DRG2) from *P. bicolor* (Vouille et al., 1997) (Fig. 8C), whereas DRS-J10 is similar to DRS-S11 from *P. sauvagii* (Lequin et al., 2006) (Fig. 8D). No biological activity has so far been reported to both DRS-B8 and to DRS-S11. This fact renders

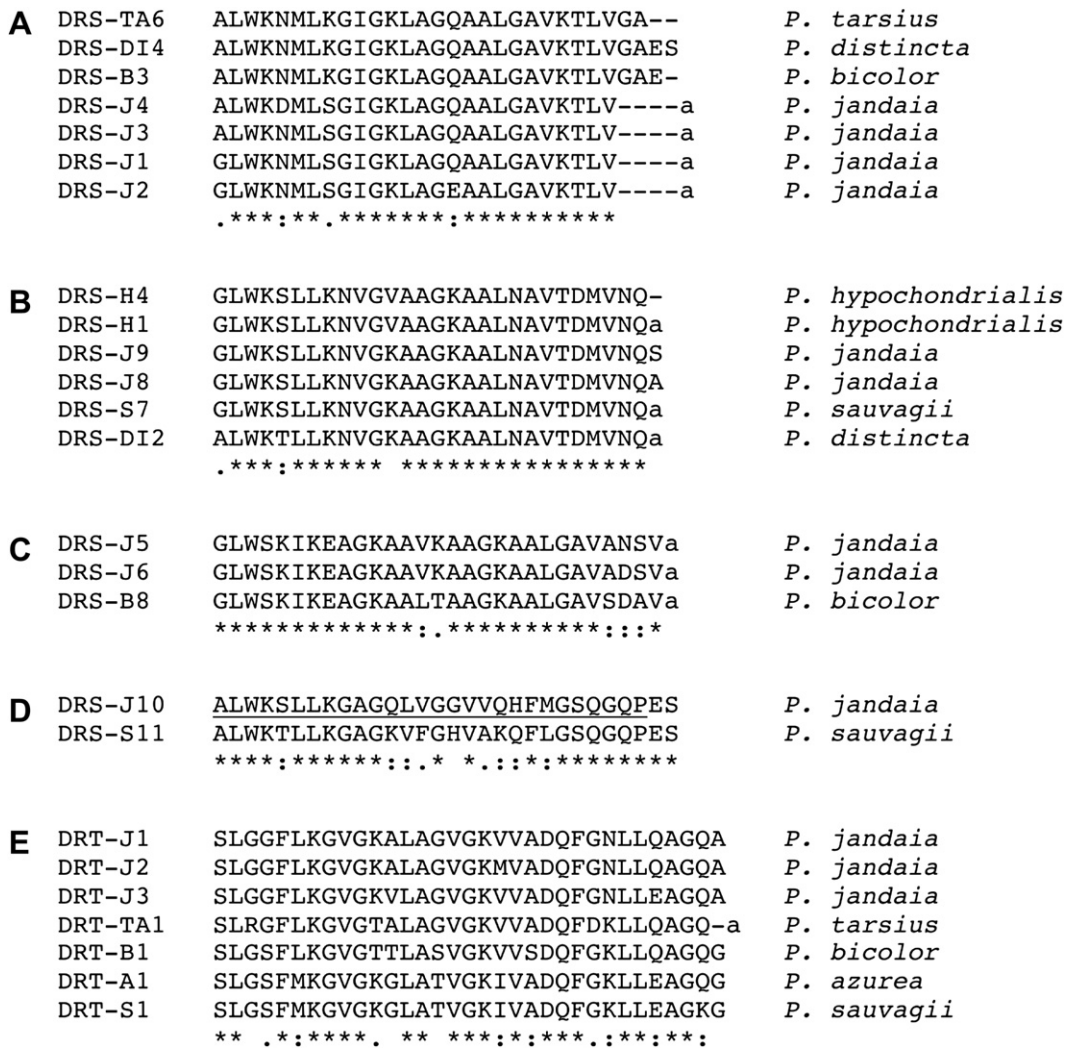

Fig. 8. Amino acid sequence alignment of dermaseptins and dermatoxins from *P. jandaia* with previously sequenced phyllomedusine peptides. (A) Alignment of DRS-J1 (P86635), DRS-J2 (P86636), DRS-J3 (P86637) and DRS-J4 (P86638) with DRS-TA6 (P84926 - original name: Dermaseptin-6), DRS-DI4 (P83641 - original name: DD Q1), DRS-B3 (P81485). (B) Alignment of DRS-J8 (P86640) and DRS-J9 (P86641) with DRS-H4 (P84597 – original name: DShypo02), DRS-H1 (CAJ76139), DRS-S7 (Q7T3K8) and DRS-DI2 (P83639 – original name: Dermadistinctin L). (C) Alignment of DRS-J5 (P86683) and DRS-J6 (P86684) with DRS-B8 (Q90ZK5 – original name: DRS DRG2). (D) Alignment of DRS-J10 (P86642) with DRS-S11 (CAI99866). (E) Alignment of DRT-J1 (P86621), DRT-J2 (P86622) and DRT-J3 (P86623) with DRT-TA1 (P84928 - original name: Dermaseptin-8), DRT-B1 (Q9PT75 - original name: Dermatoxin), DRT-A1 (O93221 - original name: DRP-AA-1-1), DRT-S1 (CAI26288 - original name: Dermatoxin S). Molecules with which *P. jandaia* peptides are aligned consist in the 'best hits' obtained by means of similarity search using BLASTP 2.2.23+ and the Swiss-Prot database. Underlining indicates sequence confirmation by Edman degradation. Differentiation of Leu/Ile residues in DRS-J1, DRS-J2, DRS-J3, DRS-J4, DRS-J5, DRS-J6, DRS-J10, DRT-J1, DRT-J2 and DRT-J3 was based upon similarity with previously known phyllomedusine sequences as depicted above. Lower case 'a' indicates C-terminal amidation. Multiple sequence alignment generated using ClustalW 2.0.12. Sequence consensus displayed below aligned sequences. Consensus symbols, namely '*', ':' and '.', means that the residues in that column are identical in all sequences in the alignment, that conserved substitutions have been observed, and that semi-conserved substitutions are observed, respectively. Peptide nomenclature as per Amiche et al., 2008.

DRS-J5, DRS-J6 and DRS-S11 and their phyllomedusine counterparts attractive investigation subjects.

As previously mentioned, three novel dermatoxin variants were sequenced in *P. jandaia* (Table 7 and Fig. 8E). Dermatoxins have been previously found in *P. bicolor* (Amiche et al., 2000), *P. sauvagii* (Chen et al., 2005), *P. tarsius* (Prates et al., unpublished results), *Agalychnis danicolor* and *Agalychnis annae* (Wechselberger, 1998). It has been reported that DRT-TA1 (originally named as Dermaseptin-8) from *P. tarsius*, is active against the Gram-positive, and Gram-negative bacteria besides displaying hemolytic activity (Prates et al., unpublished results). DRT-B1 (formerly known as dermatoxin) was found to be particularly active against mollicutes and Gram-positive bacteria, although having lower activity upon Gram-negative bacteria (Amiche et al., 2000). The bacterial plasma membrane is thought to be the primary target of DRT-B1 due to the membrane depolarization observed upon the application of this peptide, being its cell killing mechanisms likely involved with the alteration of membrane permeability rather than membrane solubilization (Amiche et al., 2000).

3.3. Evolution of biochemical characters in Phyllomedusinae

A recent study has proposed a well-supported phylogenetic hypothesis for phyllomedusines, based on sequence data from up to 10 mitochondrial and nuclear loci and three intervening transfer RNAs for 45 species of that group. *Phrynomedusa* was found to be sister to a group comprising all remaining genera, within which *Cruziohyla* is the sister to all remaining genera (*Phasmahyla*, *Phyllomedusa* and a clade containing the paraphyletic *Hylomantis* with *Pachymedusa* plus *Agalychnis*). The clade composed of *Hylomantis*, *Pachymedusa* and *Agalychnis* is sister to the clade containing *Phasmahyla* and *Phyllomedusa*, within which *Phasmahyla* is sister to *Phyllomedusa*. The monophyly of *Phasmahyla* and of Phyllomedusinae itself is supported (Faivovich et al., 2010).

Considering those results and the distribution of skin bioactive peptides families in Phyllomedusinae, isolated from 17 species within *Agalychnis* (5 species), *Phyllomedusa* (11 species) and *Cruziohyla calcarifer*, general predictions regarding their occurrence in yet unprospected species could be made (Faivovich et al., 2010). In that sense, the presence deltorphins, dermorphins, dermaseptins, dermatoxins, phyllokinins, PLSs, plasticins, tryptophyllins and the peptide sauvagine (hyposin-related - see Table 4) was expected across the clade containing *Agalychnis*, *Phyllomedusa* and *Phasmahyla* (Faivovich et al., 2010). Corroborating with that hypothesis, apart from plasticins, all peptide families cited above were detected in *P. jandaia*. Phylloxins, expected to be minimally present in the species of the clade contaning *P. camba*, *P. boliviana* and the *P. burmeisteri* and P. *tarsius* groups (Faivovich et al., 2010), were not detected in *P. jandaia*, further suggesting that this family may constitute a synapomorphy of that clade. The forecasted presence of caeruleins in *Phasmahyla* (Faivovich et al., 2010) was also confirmed upon the verification of $[Arg]^4$-phyllocaerulein. The presence of Phyllolitorins, whose distribution was deemed as 'ambiguous' (Faivovich et al., 2010) was not detected. Moreover, taking into account that Phypo Xa has been verified in *P. hypochondrialis* (Conceição et al., 2007) and *P. jandaia*, one can make the prediction that canonical BPPs (i.e. Pyr–Aaa_n–Gln–Ile–Pro–Pro) are to be minimally encountered across the clade containing *Phasmahyla* and *Phyllomedusa*. Corroborating with that supposition, according to Silva et al. (unpublished results), the ion 1215 $[M + H]^+$, corresponding to Phypo Xa, has been detected in *P. oreades*, *P. tomopterna*, *P. rohdei*, *P. tarsius*, *P. centralis*, *P. distincta*, *P. bicolor*, *P. burmeisteri*, and *P. ayeaye*. Similarly, considering that tyrosine-rich peptides have been detected in *P. azurea* (Thompson et al., 2007b), *P. sauvagii* (Erspamer et al., 1986), and *P. jandaia*, the occurrence of tyrosine-rich peptides is minimally expected across the clade containing *Phasmahyla* and *Phyllomedusa*.

It is currently understood that the genes encoding the peptides of the dermaseptin superfamily were originated from a common ancestral locus that has undergone several rounds of duplication and subsequent loci divergence events (Nicolas and El Amri, 2009). Moreover, most duplication events occurred before the radiations of South American hylids (Nicolas and El Amri, 2009). By analyzing Fig. 8, it is possible to notice that a number of sub-families within the *stricto sensu* dermaseptins occur, and to hypothesize that duplication events giving rise to such sub-families took place prior to, at least, the radiation of the clade including *Phasmahyla* and *Phyllomedusa*. Hence, efforts of systematic sequencing of *stricto sensu* dermaseptin peptides may assist the identification of their sub-families, the determination of the timing of the duplication events, and of which Phyllomedusinae clade a given sub-family of *stricto sensu* dermaseptin is a synapomorphy.

4. Conclusions

This study reports the sequencing/identification of 57 peptides from the skin secretion of P. *jandaia*, including phylloseptins, dermaseptins stricto sensu, dermatoxins, hyposins, tryptophyllins, caerulein-related, bradykinin-related, bradykinin potentiating, tyrosine-rich, and opioid peptides, in addition to five peptide families without significant similarity to other known molecules. The fact that 57 out of the 203 molecules detected were identified signalizes that other natural variants or even new molecular families still await discovery. Such natural peptidic repertoire may assist upcoming investigations pertaining to the relationships of molecular structure and function aiming at the development of therapeutical or other biotechnological application of phyllomedusine peptides. Besides, the present study contributes to the understanding of the evolution of biochemical characters of the phyllomedusines, since the molecular diversity in the skin of the genus Phasmahyla was hereby assessed for the first time.

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Conflict of interest Statement

None declared.

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