Gene expression analysis by ESTs sequencing of the Brazilian frog *Phyllomedusa nordestina* skin glands

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A R T I C L E   I N F O

A B S T R A C T

The subfamily *Phyllomedusinae* has attracted a great interest of many researchers mainly due to the high diversity of these frog species and plethora of pharmacological activities frequently observed for their skin secretions. Despite of this fact, mainly for new species, limited information is available regarding the molecular composition of these skin secretions and the cellular components involved in their production. *Phyllomedusa nordestina* is a recently described Brazilian frog species also popularly known as 'tree-frogs'. Aiming at contributing to the biological knowledge of this species, we show here the gene expression profile of this frog skin secretion using a global ESTs analysis of a cDNA library. The marked aspect of this analysis revealed a significant higher transcriptional level of the opioid peptide dermorphins in *P. nordestina* skin secretion than in *Phyllomedusa hypochondrialis*, which is its closest related species, belonging both to the same phylogenetic group. Precursors of bioactive peptides as dermaseptins, phylloseptins, tryptophyllins, and bradykinin-like peptideswere also found in this library. Transcripts encoding proteins related to ordinary cellular functions and pathways were also described. Some of them are chiefly involved in the production of the skin secretion. Taken together, the data reported here constitute a contribution to the characterization of the molecular diversity of gene-encoded polypeptides with potential possibility of pharmacological exploitation. The transcriptional composition of the skin secretion may also help to give the necessary support for the definition of *P. nordestina* as a new species, which actually relies basically on frog morphological characteristics and geographical distribution.

1. Introduction

Amphibian skin is characterized by the presence of mucous glands mainly associated to respiration and protection against desiccation, while granular (or poison) glands provide an arsenal of chemical compounds used for defense against opportunistic microorganisms and predators (Clark, 1997; Duellman and Trueb, 1986; Stebbins and Cohen, 1997; Toledo and Jared, 1993, 1995; Rollins-Smith et al., 2002, 2005). Under the control of a holocryne mechanism (Simmaco et al., 1998), poison glands secrete

a wide diversity of peptides, biogenic amines, steroids and alkaloids, all presenting a broad spectrum of biological activity (Auvymet et al., 2009; Bevins and Zasloff, 1990; Daly et al., 1987; Roseghini et al., 1989; Toledo and Jared, 1995; Van Zoggel et al., 2012). The family Hylidae (tree-frogs) is known to secrete polypeptide compounds, most of them with bioactive properties. Although the cutaneous secretions composition of the subfamily *Phyllomedusinae* is considered the most complex, it is well documented particularly for the genus *Phyllomedusa* (Conlon et al., 2004; Erspamer et al., 1986, 1993; Faivovich et al., 2010). In fact, several species were studied and numerous peptides have been isolated based on their antimicrobial and analgesic activities. Paramount interest has been arisen mainly due to the potential use of frog skin peptides as model molecules for the development of active compounds, alternative to the conventional drug therapies against microorganisms actually employed, which has been constantly tackled by the quickly evolving drug resistance (Zasloff, 1987).

Phyllomedusa genus comprises 30 species (Cruz, 1991; Faivovich et al., 2010), which are geographically distributed throughout Central and South America, as stated by American Museum of Natural History (AMNH), published online by Frost in 2011 (Frost, 2011). Recently, the frog species *Phyllomedusa nordestina* was described and included within the clade of *Phyllomedusa hypochondrialis*, according to its morphological characters (Caramaschi, 2006). This species is endemic to the Brazilian North-eastern, known as 'caatinga'. This is one of the main biomes in Brazil, characterized by a very dry and constant warm climate, with well-defined seasons and few rainfalls occurring only in the first months of each year. In contrast to the limited distribution of *P. nordestina*, *P. hypochon-drialis* is found spread along biogeographically different habitats, which also include the rich Amazon rainforest biome. Taking into account that amphibian skin secretions are highly related to the type of environment in which a given species of frog inhabit (Prates et al., 2011), it can be anticipated that the molecules secreted by *P. nordestina* should be different from that described for *P. hypochon-drialis* group.

Several studies describing the biochemical character-ization of the components from the skin secretion of *Phyllomedusa* genus have allowed the identification of biologically active peptides that are very similar to the mammalian hormones, neuropeptides, as well as the broad-spectrum cytolytic antimicrobial peptides (Conceição et al., 2006). To date these antimicrobial peptides are grouped in seven families namely derma-septins, phylloseptins, plasticins, dermatoxins, phylloxins, hyposins, and orphan peptides (Amiche et al., 2008). Some of these peptides were isolated and characterized from *P. hyponchondrialis* skin secretion, for instance derma-septins, phylloseptins, and hyposins, which were only described in this species (Conceição et al., 2006; Leite et al., 2005; Thompson et al., 2007b), and the bradykinin-related peptides (BRPs) (Brand et al., 2006a, 2006b; Conceição et al., 2007b). Activity against gram-positive and gram-negative bacteria, yeast and fungi were reported for dermaseptins (Mor et al., 1991, 1994),

while antibacterial activity and antiparasitic activity against *Trypanosoma cruzi* were demonstrated for phyl-loseptins (Leite et al., 2005). In addition to these reports, studies dedicated to characterize themain biological effects of crude *P. hypochondrialis* skin secretion showed that, at low doses, it is able to induce edema and inflam-mation in the cremaster mice (Conceição et al., 2007a). In addition, the same research team also observed pain, edema, and necrosis, 48 h after intraperitoneal injection in mice (*personal communication*).

Classical investigation of the main peptide constituents of frog skin using biochemical and protein chemistry techniques includes purification and characterization of the isolated components of these secretions by HPLC, followed by identification using mass spectrometry or protein fingerprint analysis. Despite of being fast and relatively inexpensive, these techniques present some problems such as inadequate fragmentation of molecules, besides the technical limitation in distinguishing amino acid residues with the same mass values, like Leu and Ile, making necessary the use of sophisticated equipment not always available (Kjeldsen et al., 2003; Tanaka et al., 2006). The generation of cDNAs libraries and their sequencing were shown to be a complementary technique that enables an accurate identification and characteriza-tion of gene-encoded proteins from diverse organisms (Adams et al., 1991; Chen et al., 2006; Junqueira-de-Azevedo and Ho, 2002; Okubo et al., 1992; Verdun et al., 1998). Recombinant DNA techniques, including cDNA cloning and sequencing, has also the advantage of providing information about cellular proteins involved in the processes of production and release of bioactive components into the glands of the studied venomous tissue. In addition, alternative splicing or post-translational modifications such as glycosylations, phos-phorylations, and disulfide bonds formation, that often limit the biochemical studies, can be predicted and circumvented.

P. nordestina was formerly comprised into the group of *P. hypochondrialis* and, only recently, they were recognized as different species (Caramaschi, 2006). Since a similar anal-ysis was also previously conducted for *P. hypochondrialis* skin gland tissue by others (Chen et al., 2006), here we report for the first time a survey of gene expression of the skin gland of *P. nordestina* species, based on the analysis of expressed sequence tags (ESTs), aiming to identify simi-larities and differences between these two species.

2. Materials and method

2.1. Collection of specimens

The Brazilian monkey tiger leg tree frog *P. nordestina* specimens ($n = 3$) were collected in Angicos in Rio Grande do Norte State and maintained at −80 °C, before tissue dissection and nucleic acid extraction. The tree frogs were collected according to the Brazilian Environmental Agency (IBAMA – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) under the License No. 02027.023238/03-91, and they were all treated according to the rules of animal care of local legislation.

2.2. Materials

Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs (Beverly, MA, USA). All chemical reagents were of analytical purity grade and were purchased from Sigma Aldrich Co (St Louis, MO, USA).

2.3. cDNA library construction

The skin was immediately dissected and pulverized under liquid nitrogen. The total RNA was extracted by using Trizol™ (Invitrogen, Eugene, OR, USA) (1 mL for 1 g of powdered tissue). Poly $(A)^+$ RNA was prepared by using pre-packed oligo-dT Sepharose columns (Invitrogen). The quality and quantity of obtained RNA were assessed by electrophoresis in denaturing agarose gel and spectro-photometric determination of the absorbance ratio at 260 and 280 nm. The cDNA was then synthesized, cloned, and packed using the ZAP-cDNA synthesis kit and the ZAP-cDNA Gigapack III Gold Packaging Extract (Stratagene, La Jolla, CA, USA) following the manufacture's instructions. After packaging, the obtained *P. nordestina* skin cDNA library was plated and the isolated phage clones were randomly collected in SM buffer (10 mM NaCl; 8 mM MgSO₄; 50 mM Tris-HCl pH 7.5; 0.01% gelatin) containing 0.3% chloroform, before the recovery of the phagemid containing the recombinant cDNA by *in vivo* excision. Alternatively, some of the clones were isolated after mass excision of the library. After *in vivo* excision the plasmid cDNA clones were then amplified and purified by alkaline lysis using Wizard Minipreps DNA Purification kit (Promega, Madison, WI, USA). The nucleotide sequence was determined by the dideoxy chain-termination method using the BigDye™ Terminator Cycle Sequence Kit and the ABI 310 automatic system (Applied Biosystems, Foster City, CA, USA).

2.4. Assembly and identification of ESTs

The analysis of sequences was conducted using a set of web based analysis programs. Sequence quality was first analyzed with the Phred and Crossmatch software packages to remove low quality ends (Green, 1996). After this preliminary analysis, only good quality sequences (phred > 20) with a length longer than 150 bp were considered for definitive annotation. The collection of good quality sequences was organized into clusters by using CAP3 software. We took into account overlaps of 50 bp that had at least 98% identity (Huang and Madan, 1999). The obtained sequences were compared to protein GenBank NR (http://www.ncbi.nih.gov) and Swissprot release 44 (ftp.ebi.ac.uk/pub/databases/swissprot/release/) databases using the BLASTx program (Altschul et al., 1990). Gene descriptions and EC numbers from Swissprot best hits and their associated product names were automatically assigned using 10^{-10} as the e-value cutoff. Thereafter, the ESTs were manually inspected by comparing the BLAST results with the automatically annotated EC numbers for functional classification. After this, an additional annotation allowing the alignment was conducted comparing the predicted protein sequences of clusters with Uniprot database and Swiss-Prot, SP-TrEMBL and stable Ensembl proteomes databases using the SMART software (Schultz et al., 1998).

3. Results

The average readable sequence length was of 390 bp, and only those considered having good quality were used to proceed with annotation. In this work, a total of 212ESTs or clusters were analyzed. The clusters were divided in three main groups based on their sequence similarity with the entry sequences found in GenBank, and thus, they were named as 'hit sequences' comprising (i) transcripts showing primary structure similarity to sequences deposited in databank (66%), (ii) transcripts with potential open reading frame (ORF) showing similarity to 'hypothetical proteins' with no biological function assigned (13.7%), and finally (iii) ESTs (20.3%) with no significant similarity to any sequences deposited in GenBank using the default parameters (i.e., Blosum62 matrix and expected threshold of 10) that were, so forth, defined as 'no hit' sequences (Table 1). From this point, only the group of 140 ESTs presenting sequence similarity to known sequences considered as valid for the functional annotation, also referred as "hit sequences", were included in the functional annotation describe hereupon. All obtained clusters were deposited in the EST database of GenBank (http://www.ncbi.nlm.nih.gov/dbEST) under accession numbers GenBank ID: JK811213–JK811339. Later the clusters analysis provides complete open reading frames (ORFs) comprising the assembled sequences GenBank ID: JK811213–JK811223 (dermorphins) and GenBank ID: JK811224–JK811227 (dermaseptins), which were deposited in GenBank under ID: JX127157, JX127158, and JX127159 respectively. Another complete open reading frames of clusters of protease inhibitors (P01, PI02, and P03), S100 like proteins (CP01 and CP560), and bradykinin-related peptides (BK01 and BK02), tryptophyllin (TP02) also was deposited in GenBank ID: JX879762, JX879763, JX879764, JX879760, JX879761, JX879758, JX879759, and JX879757, respectively.

3.1. Functional annotation

The functional annotation led tothe clustering of 140 ESTs in 8 contigs containing 42 ESTs, and the remaining 98 were singlets. The nucleotide sequences were then distributed in four main groups of proteins related to: (1) common cellular functions, corresponding to 64.3% of the ESTs from 'hit sequences' group, (2) antimicrobial and

Table 1
Annotation results of ESTs obtained from *P. nordestina* skin gland transcriptome. Total number of ESTs and relative percentages of hits in GenBank.

Name	ESTs	% of total mRNAs
Protein homologs	140	66.0
Hypothetical proteins	29	13.7
No hit	43	20.3
Total	212	100.0

opioid-like peptides (25.7%), (3) transcripts encoding other protein families (7.9%), and (4) ribosomal and mitochondrial proteins (1.4%).

3.2. Polypeptide components of P. nordestina skin secretion

We identified in this work, clusters of homologous sequences from seven different families of peptides, representing 25.7% of valid sequences, whose biological activities are related mainly to antimicrobial effects, as well as to a particular class of peptides with described actions in the nervous system (Table 3).

These peptides are relatively short, comprising molecules of 12–100 amino acid residues long, with diverse composition and mostly highly positively charged. They are expressed in frog skin both constitutively or by inducible mode, in which the expression is triggered by the presence of microorganisms or other pro-inflammatory stimuli (Cunliffe and Mahida, 2004).

The transcripts described here share a significant similarity to antimicrobial peptides (AMPs) namely dermaseptins, phylloseptins, and tryptophyllins. In addition to that we also observed transcripts encoding opioid peptides such as dermorphins, bradykinin-related peptides (BRPs), and kininogens, herein reported as 'neuropeptides' (Fig. 1).

Next, in the context of ESTs analysis, a brief discussion about the structural similarities and biological activities of each peptide family will be presented.

3.2.1. Dermorphins

Dermorphins have been isolated from *Phyllomedusinae* frogs and comprise heptapeptides that have high affinity and selectivity for opiate receptors (Broccardo et al., 1981; Erspamer et al., 1986; Mor et al., 1991; Kreil et al., 1989). First isolated from *Phyllomedusa sauvagii* by Montecucchi et al. (1981), dermorphins were shown to present analgesic effect eleven times more potent than morphine in mice (Broccardo et al., 1981). In this work we found that dermorphins precursors represent the most abundant peptides transcripts in the *P. nordestina* skin cDNA library (Fig. 1). Previous work also described a high content of dermorphins in the skin secretion of other members of *Phyllomedusinae* subfamily (Melchiori and Negri, 1996). The functional annotation performed here resulted in 12 ESTs sharing similarity to dermorphins, and they were grouped in three contigs. The contigs named DM01 and

Table 2
ESTs related to common cellular functions. The distribution of ESTs that were grouped according to the main cellular function and relative percentages inside group are shown, as well as the total matching sequences.

| Function	ESTs	% group	% of 'matching'
Structural	9	10	6.4
Metabolism	8	8.9	5.7
Transcription and translation	29	32.2	20.7
Degradation	1	1.1	0.7
Regulation	20	22.2	14.3
Retrotransposon	4	4.4	2.9
Other Functions	19	21.1	13.6
Total	90	100	64.3

Table 3
Peptides and other protein families ESTs. Distribution of groups of peptides and other protein families not related to common cellular functions are shown, and the relative percentage inside each group and the total matching sequences are indicated.

Name	ESTs	% group	% "matching"
Dermorphins	13	27.6	9.4
Dermaseptins	8	17.0	5.8
Phylloseptins	4	8.5	2.9
Tryptophyllins	7	14.9	5.0
BRPs	2	4.3	1.4
Kininogen	2	4.3	1.4
CRISPs	2	4.3	1.4
Protease inhibitors	9	19.1	6.5
Total	47	100	34.1

DM02 share similarities with demorphin-2, for both DNA and deduced protein sequence comparisons. The cDNA structures encoding the contigs sequenced here, DM01 and DM02, presented a signal peptide followed by five repeats of a propeptide and a mature peptide similarly, as observed for the dermorphin-2 from *P. sauvagii* (Richter et al., 1990). These contigs shared 80 and 84% of similarity with dermorphin-2 sequence found in databank (GenBank ID: M18031). The contig DM03 also showed similarities with *P. sauvagii* dermorphin-2, but differences in the number of copy of peptides repeats were observed, i.e. five in DM01 and DM02 sequences, and only three in DM03 (Fig. 2A).

Moreover, the consensual translation of contigs sequences showed that almost all peptides encoded by DM01 and DM02 clusters have the Tyr-Ala-Phe-Gly-Tyr-Pro-Ser sequence. One peptide encoded by DM01 showed the Tyr-Ala-Ser-Gly-Tyr-Pro-Ser sequence. There placement of a Phe by a Ser residue is not a conservative substitution, and it was not observed in other known dermorphins sequences. For this reason, an additional analysis using ProtParam webtool (http://web.expasy.org/protparam/) was conducted. In spite of the substitution of the non-polar amino acid residue (Phe) for a polar one (Ser), no change in the partial charge of the peptide could be detected (data not shown). Besides this amino acid substitution, all peptides encoded by both contigs have a C-terminal portion that is liable to amidation, which increases the peptide affinity for the receptor (Melchiori and Negri, 1996). But there is no clear indicative that the amino acid substitution observed here influence the biological activity of the peptide encoded by DM01 contig.

The analysis of the singlet DM03 also showed a similarity (identity) of about 95% to demorphin-2 (GenBank ID: M18030.1). The structure of the deduced transcriptional product showed five copies of propeptide and mature peptide, and the marked difference was the absence of a signal peptide. This may be an indicative of the existence of a precursor for putative intracellular peptides that, in on our view, is a novelty that deserves further investigation. The deduced amino acid sequences of all open reading frames of dermorphin contigs, as well as ESTs, and the respective sequences alignment are shown (see Supplementary material Figs. S1 and S2).
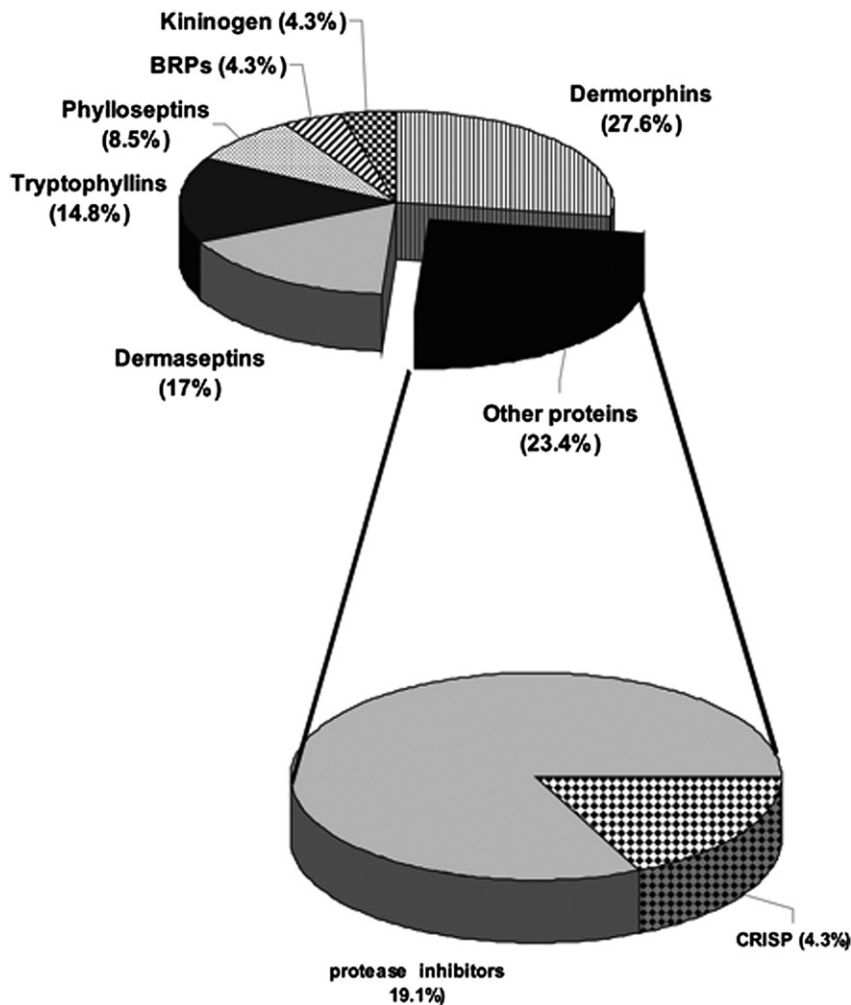
Fig. 1. Relative proportion of transcripts encoding peptide and other protein families non-related to cellular metabolism expressed in the *P. nordestina* skin gland. Homolog peptides were categorized by their structural type matching in GenBank. Percentages of ESTs in the category (in brackets) are presented. Proteins non-related to common cellular metabolism are also presented in a separated disk.

3.2.2. Dermaseptins and phylloseptins

Among several well-known families of antimicrobial peptides (AMPs), the superfamily of dermaseptins grouped several families, which include the phylloseptin family. Precursors mRNAs of dermaseptins have unique pattern with highly similar N-terminal preprosequences followed by a cleavage recognition site (KR) typical of prohormone processing signal and variable C-terminal domains encoding mature antimicrobial peptides (Amiche et al., 1999; Nicolas et al., 2003). Dermaseptins *strictu sensu* family comprises peptides typically of 27–34 amino acid residues, with 3–6 Lys residues and a highly conserved Trp residue in the third position (Zairi et al., 2009). They were the first vertebrate peptide to be described showing a potent antimicrobial activity against filamentous fungi, and that is implicated in severe opportunistic infections caused by immunodeficiency syndrome and immunosuppressive drug therapy (Amiche et al., 1994). Besides the antimicrobial activity another biological properties of dermaseptins was demonstrated, namely the chemotactic properties of

a peptide isolated from *Pacmedusa dacnicolor* DRS-DA4 upon leukocytes (Auvymet et al., 2009), and the anti-tumoral and angiostatic activities of dermaseptins B2 and B3 (Van Zoggel et al., 2012).

Dermaseptin family is represented in our dataset by 7 ESTs, all of them with similarity to dermaseptin-H3 from *Phyllomedusa azurea* (GenBank ID: AM269412.1) (Brand et al., 2006a,b). Four out of these 7 ESTs were grouped into a single contig named DS1. Three ESTs remained as a singlet named DS2 to DS4. Analysis of all these sequences also revealed high similarities with a dermaseptin isolated from *P. hyponchondrialis* skin secretion (Conceição et al., 2006), which contains 25 amino acid residues and shows antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus*, and *M. luteus*. Remarkably, they do not have hemolytic activity. With the exception for DS04, the ESTs analysis of *P. nordestina* dermaseptin-like precursors showed the conserved family structure consisting of a signal peptide that ends by a cleavage site (KR) typical of prohormone processing signal and a single copy of the mature peptide.
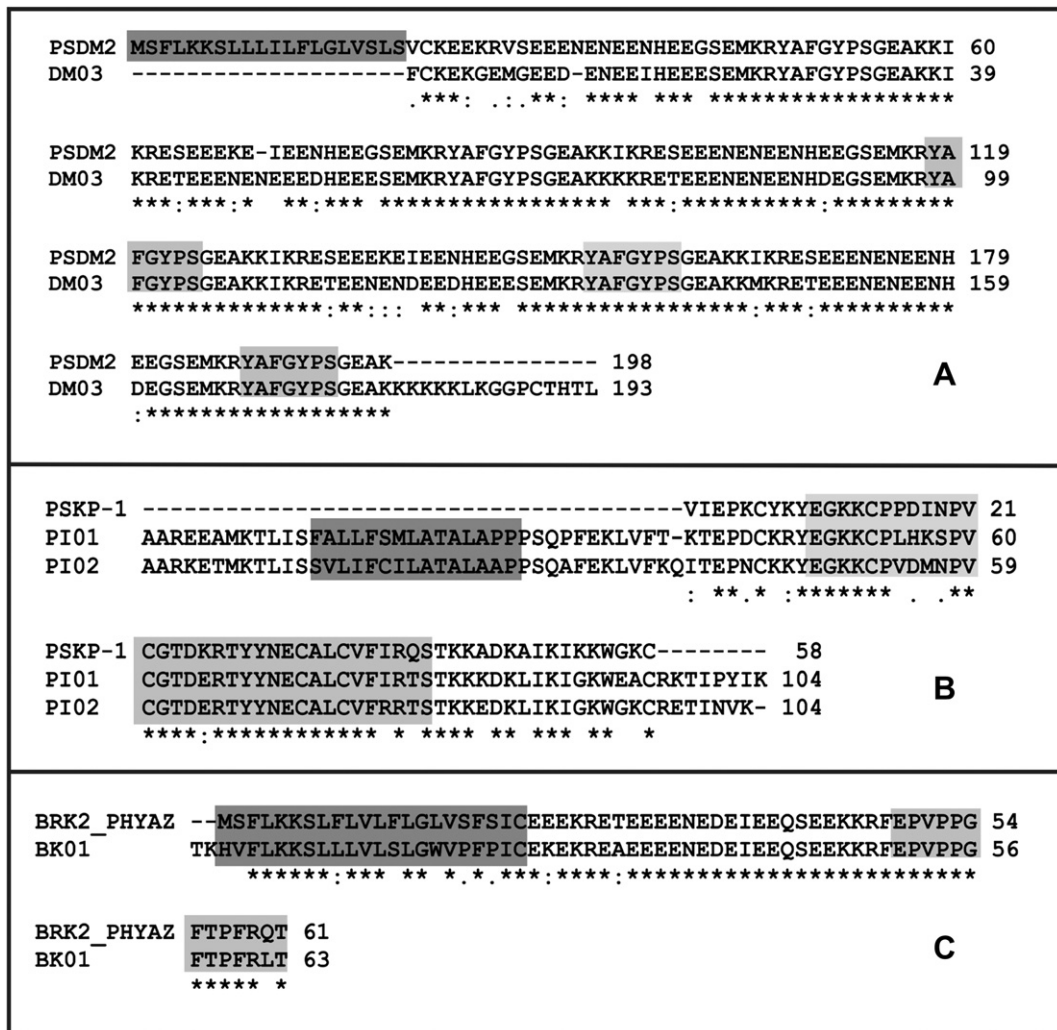
Fig. 2. Deduced amino acid sequences alignments of *P. nordestina* dermorphin (DM03), protease inhibitor (PI01 and PI02), and bradykinin-related peptide (BK01). Alignment of dermorphin singlet deduced primary sequence (DM03) and *P. sauvagii* dermorphin 2 (PSDM2) in shown in (A). Alignment of protease inhibitors clusters (PI01 and PI02) deduced sequence and *P. sauvagii* Kazal type 1 protease inhibitor (IKP1_PHYSA) is shown in (B). Alignment of bradykinin-related peptide singlet (BK01) and *P. azurea* bradykinin-related peptide 2 (BRK2_PHYAZ) is shown in (C). The signal peptide sequences are shaded in light grey and the mature peptide sequences are shaded in dark grey. The hyphens (-) indicate the gaps.

This latter one shares similarities with an isolated derma-septin from *P. azurea* DMS3_PHYAZ (GenBank ID: Q17UY8). The similarities of nucleotide sequences ranged from 77 to 90% (for DS04 and DS01, respectively). The search using BlastX, in which translated nucleotide sequences are used as query to search protein sequences, also resulted in a high score of similarities to dermaseptins (96% for contig DS02, and 94% for contig DS01, and singlet DS03). The analysis of singlet DS04 by BlastX resulted in 'no significant similarity' to known proteins using default parameters, but BlastN analysis showed 90% of similarity to *P. azurea* pre-prodermaseptin H3 (GenBank ID:AM269412.1). Multi-alignment of deduced amino acid sequences and homologous sequences retrieved from the databank showed that the signal peptide sequence and propeptide regions are both highly conserved. The nucleotide sequence stretch coding for the mature peptide showed a nucleotide insertion that introduced a stop codon in the ORF of DS04singlet (Fig. 3). This fact is an interesting difference. However, since this sequence is a product of one single pass sequencing, further investigations are still necessary to confirm if this transcript really encodes for a different active peptide or if it represents a truncated precursor of a non-functional peptide.

As mentioned, phylloseptins encompasses a family of related sequences included in the superfamily of derma-septins. The phylloseptins family comprises cationic peptides with 18–20 amino acid residuescharacterized by the conservation of several residues, including especially the sequence Phe-Leu-Ser-Leu-Ile/Leu-Pro at the N-terminus and a C-terminal amidation. These peptides were isolated from several species of *Phyllomedusinae*, and they have antibiotic activity against gram-negative and gram-positive bacteria, besides the activity against the *T. cruzi*
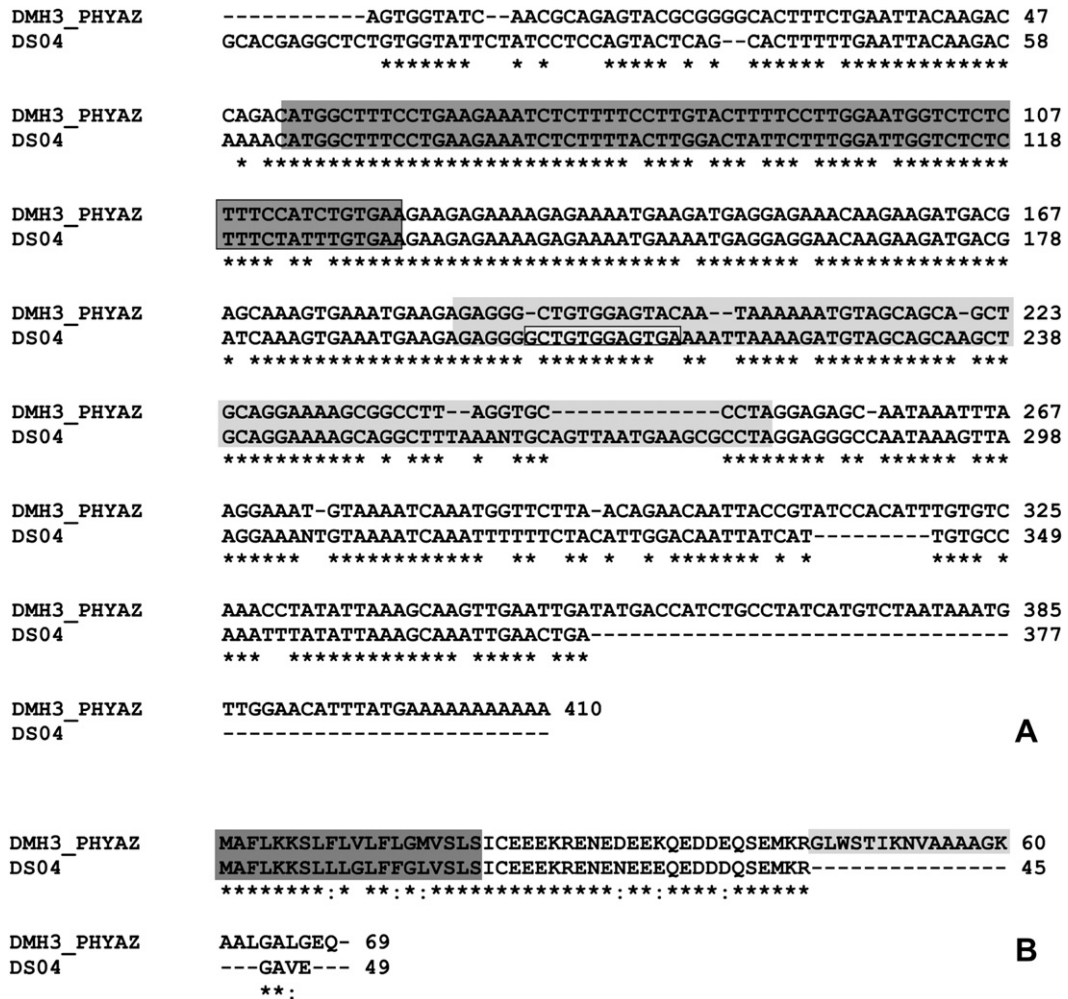
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DMH3_PHYAZ   -----------AGTGGTATC--AACGCAGAGTACGCGGGGCACTTTCTGAATTACAAGAC  47
DS04         GCACGAGGCTCTGTGGTATTCTATCCTCCAGTACTCAG--CACTTTTTGAATTACAAGAC  58
             ******  *  * ****  *  * ***** ************

DMH3_PHYAZ   CAGACATGGCTTTCCTGAAGAAATCTCTTTTCCTTGTACTTTTCCTTGGAATGGTCTCTC  107
DS04         AAAACATGGCTTTCCTGAAGAAATCTCTTTTACTTGGACTATTCTTTGGATTGGTCTCTC  118
             *  ************************** **** *** *** ***** ********

DMH3_PHYAZ   TTTCCATCTGTGAAGAAGAGAAAAGAGAAAATGAAGATGAGGAGAAACAAGAAGATGACG  167
DS04         TTTCTATTTGTGAAGAAGAGAAAAGAGAAAATGAAAATGAGGAGGAACAAGAAGATGACG  178
             **** ** ************************ ******* *************

DMH3_PHYAZ   AGCAAAGTGAAATGAAGAGAGGG-CTGTGGAGTACAA--TAAAAAATGTAGCAGCA-GCT  223
DS04         ATCAAAGTGAAATGAAGAGAGGGGCTGTGGAGTGAAAATTAAAAGATGTAGCAGCAAGCT  238
             * ********************* ********  **  ***** ********** ***

DMH3_PHYAZ   GCAGGAAAAGCGGCCTT--AGGTGC------------CCTAGGAGAGC-AATAAATTTA  267
DS04         GCAGGAAAAGCAGGCTTTAAANTGCAGTTAATGAAGCGCCTAGGAGGGCCAATAAAGTTA  298
             ********** * *** * ***       ******* ** ****** ***

DMH3_PHYAZ   AGGAAAT-GTAAAATCAAATGGTTCTTA-ACAGAACAATTACCGTATCCACATTTGTGTC  325
DS04         AGGAAANTGTAAAATCAAATTTTTTCTACATTGGACAATTATCAT--------TGTGCC  349
             ****** ************ ** ** * * ******* * *       **** *

DMH3_PHYAZ   AAACCTATATTAAAGCAAGTTGAATTGATATGACCATCTGCCTATCATGTCTAATAAATG  385
DS04         AAATTTATATTAAAGCAAATTGAACTGA-------------------------------  377
             ***  ************* ***** ****

DMH3_PHYAZ   TTGGAACATTTATGAAAAAAAAAA  410
DS04         ------------------------
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A

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DMH3_PHYAZ   MAFLKKSLFLVLFLGMVSLSICEEEKRENEDEEKQEDDEQSEMKRGLWSTIKNVAAAAGK  60
DS04         MAFLKKSLLLGLFFGLVSLSICEEEKRENENEEEQEDDDQSEMKR---------------  45
             ********:* **:*:***************:**:****:******
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DMH3_PHYAZ   AALGALGEQ-  69
DS04         ---GAVE---  49
                **:
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B

Fig. 3. Alignment of DNA and deduced amino acid sequences of dermaseptin contig DS04 and the *P. azurea* dermaseptin H3 mRNA (GenBank ID: AM269412.1). Panel (A) shows the nucleotide sequences and the panel (B) shows the respective deduced amino acid sequences of dermaseptin contig DS04 and the *P. azurea* dermaseptin H3 mRNA (GenBank ID: AM269412.1). The signal peptide sequences are shaded in light grey and the mature peptide sequences are shaded in dark grey. The nucleotide insertion that results in a stop codon is highlighted by the box in panel (A).

(Leite et al., 2005). In the *P. nordestina* skin cDNA library analyzed in this study, 4 ESTs forming one single cluster named PS01, showed similarity to phylloseptin-7 isolated from *P. azurea* (Thompson et al., 2007a), either for the DNA or also for the deduced protein sequences. The alignment of these transcripts showed a high identity (94%) for the signal peptide, propeptide and mature peptide. Sequence alignment of predicted protein also revealed a high structural conservation, showing a score of 96%. Besides this score, the propeptide sequence showed a deletion of a conserved Asp residue, which might reflect differences in the spectrum of biological activity (data not shown).

3.2.3. Tryptophyllins

The tryptophyllins were first isolated from *P. dacnicolor* (Meneses et al., 2011), and they belong to a large family of peptides with a conserved Trp residue at position P$_2$ of the active peptide, and a Pro residue at the N-terminal domain. Asynthetic replicate of tryptophyllin-1, a member of this family, the peptide PdT-1, was shown to be a potent myoactive agent, relaxing mammalian arterial smooth muscle and contracting small intestinal smooth muscle (Chen, 2004). We describe here two contigs and one singlet homologous to tryptophyllin. One of them TP01 showed similarity to sauvatide, which is a myotropic peptide from *P. sauvagii* (Wang et al., 2009), whereas TP02 was similar to *P. dacnicolor* tryptophyllin-1. The alignment of nucleotide sequences allowed observing 88% of similarity between TP01 and sauvatide, and 90% of similarity between TP02 and tryptophyllin-1. The singlet TP03 was 85% similar to aurein, a peptide with antimicrobial and antitumoral properties (Rozek et al., 2000). Sequence comparison performed using BlastX showed that only TP01 has significant structural conservation that is typical of secreted peptides, mainly characterized by a signal peptide followed by a propeptide and a mature peptide sequence. The open reading frames of the sequences corresponding to TP02 and the alignment of the deduced amino acid sequences are shown in Supplementary material Fig S3.

3.2.4. Bradykinin-related peptides (BRPs) and kininogen-like peptides

Bradykinin-related peptides (BRPs) are similar to the nonapeptide bradykinin originally described by Rocha e Silva et al. (1949) as a potent vasodilator. These peptides are expressed in many living organisms, including wasps (Picolo et al., 2010) and anurans including some species ofPhyllomedusa genus (Brand et al., 2006a,b; Chen and Shaw, 2003; Thompson et al., 2006). A high structural diversity of BRPs in the skin secretions of frogs and toads was described (Chen et al., 2011). The pharmacological effects induced by BRPs include antagonism of bradykinin effects on smooth muscle, vasodilatation, vasoconstriction, and hyperalgesia (Conceição et al., 2009; Picolo et al., 2010; Zhou et al., 2009). Two singlet sequences showing similarity to BRPs, coined as BK01 and BK02, were found in our database. BlastX did not show any significant similarity to known sequences for these singlets suggesting also the variability of DNA sequences in addition to the structural peptides variability. Thus the transcripts of P. nordestina may represent new transcripts encoding BRPs. On the other hand, BlastN analysis resulted in a hit to mRNA encoding for preprobradykinin-HA2, with a score of 93% identity. The P. nordestina cDNAs sequenced here encoded for a protein containing a signal peptide, a propeptide, and a single copy of mature peptide in each precursor, as already previously described for P. azurea, but differently from that observed for other frogs belonging to the genus Rana and Bombina, which seems to produce multiple copies of bradykinin-like peptides in a single precursor (Thompson et al., 2006). The consensual translation resulted in sequences with similarity of about 90% of identity. Besides this similarity, the consensual translation of BK01 showed similarity only for the frame +3 deduced sequence, but that resulted in a sequence without a Met residue as the start codon (Fig. 2C). Further investigations are necessary to determine if this cluster really encodes a non-secreted intracellular peptide or if it is just a non-functional protein.

Additionally, we found two ESTs, which were 94% similar to kininogen-1 for nucleotide sequence analysis (Chen et al., 2006), and that were grouped in contig KN01.

3.3. Protein clusters related to other functions

Besides the absolute majority of sequences encoding for peptides and common function cellular proteins, some ESTs studied here were shown to be similar to proteins related to non-common cellular functions (Fig. 1). These clusters belong basically to two classes: cysteine-rich secretory proteins (CRISPs) and protease inhibitors.

There are limited information on CRISPs and their biological activity, although their ability to inhibit smooth muscle contraction and to block the triggering of cyclic-nucleotide-gated ion channels was demonstrated (Osipov et al., 2005; Yamazaki and Morita, 2004). We found two ESTs, grouped in a single cluster, that share similarity to CRISPs expressed in the venom gland of snake Daboia russeli. However the similarity observed was below 50% identity (data not shown), making it difficult to infer any hypothesis about the probable function of this snake

counterpart molecule, we are identifying and describing for the first time in a frog skin.

The first molecule belonging to the class of protease inhibitors was isolated from the skin of Bombina bombina, and it was shown to be a trypsin inhibitor named bombinina. Thereafter, several other inhibitors from the skin of Rana and Phyllomedusa were described, indicating that these protease inhibitors may contribute to the broad spectrum of antimicrobial activity in frog skin secretion (Gebhard et al., 2004). From the present P. nordestina cDNA library, we identify nine sequences belonging to the class of protease inhibitors. Seven of these sequences were grouped in a contig named PI01, while other two sequences remained as singlets named PI02 and PI03. All clusters showed only a significant similarity by BlastX analysis, in which contig PI01 was shown to be 72% similar to protein PSKP1 isolated from P. sauvagii (GenBank ID:P83578.1). PSKP1 is a Kazal-type protein, but unlike Kazal, this protein is not an inhibitor of trypsin, chymotrypsin, S. aureus strain V8 protease or proteinase K, due to the presence of a Pro residue at position P_2 of the peptide. A Pro residue at this position seems to determine the loss of inhibitory activity (Gebhard et al., 2004). The comparison of the deduced amino acid sequence of contig PI01 and PSKP1 sequence showed that both proteins share this feature (Fig. 2B). The singlet PI02 also showed the same characteristic, thus the encoded proteins from both groups seem to lack the inhibitory activity. Additionally we could find a potential signal peptide in both DNA sequences of these clusters, using the software SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/).

On the other hand, the singlet PI03 showed low similarity ($\sim$43%) to Kazal type 2 (GenBank ID:XP_002940646), a serine proteinase inhibitor, in which all conserved residues common to this family members were present in the deduced amino acid sequence of the singlet PI03, with a single exception for the Pro residue at position P_1. This substitution is semi-conservative, but the effects on biological activity are not predictable since Pro residues impose important structural changes. Very limited information on structure-activity relationship was found in the literature for this type of Kazal type 2 serine proteases inhibitors (Gebhard et al., 2004), suggesting the importance of conducting further pharmacological studies to better understand the structural requirements for this biological activity.

3.4. Cellular functions related ESTs

The clusters encoding for sequences with similarity to cellular proteins represented 64.3% of total 'hit sequences', and they were categorized according to the demonstrated or presumed biochemical functions and pathways (Table 2).

Among these sequences, the most representative ESTs were those related to transcriptional and translational processes, covering about 20.7% of all analyzed contigs (Fig. 4). This group comprises ribosomal proteins, and transcription and elongation factor homologues. The expression of these precursors is indicative of the high biosynthesis process in response to the continuous production of skin secretion. Transcripts encoding for
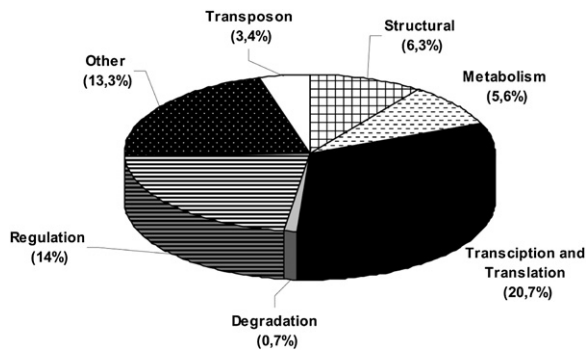
Fig. 4. Relative proportion of transcripts encoding common cellular metabolism ESTs expressed in the *P. nordestina* skin gland. Homolog proteins were categorized by their structural type matching in GenBank. Percentages of ESTs in the category (in brackets) are presented.

proteins related to regulatory processes represent the second most abundant group (14.2% of total). Amarked occurrence was observed for four transcripts encoding S100 calcium binding proteins. This large calcium binding protein family is composed by low molecular weight members with two different types of calcium binding domains. These proteins have an unusual calcium-binding EF-hand motif at the N-terminal portion, composed by a helix-loop-helix domains also referred as imperfect EF-hand motif with low affinity for calcium. The C-terminal portion has a conserved true EF-hand motif with high affinity to calcium. These proteins are associated with diverse cellular processes, both intracellular and extracellular, and the biological functions are suggested to be dependent of dimerization (Donato, 2001, 2003). Three transcripts were grouped in the contig CP01 with low similarity (39%) to S100 A11 protein from *Anoplopoma fimbria*(GenBank ID:ACQ58106.1),and the CP560 singlet is 86% similar to *Rana catesbeiana* S100 A10 protein (GenBank ID:ACO51990.1), as determined by BlastX analysis. Using SMART software, the sense deduced sequence of CP01 contig in frame 3 showed a protein sequence with an architecture composed by a calcium binding domain consistent with domains found in S100 and CaBP-9k calbidin protein, and a EF hand motif. The result of same analysis with singlet CP560 result in the identification of only one S100 calcium binding protein, and the EF-hand domain could not be identified (Fig. 5).

In mammals the protein S100 A10, also named p11, interacts with annexin II and is responsible for the regulation of the intracellular trafficking of membrane proteins (Harder and Gerke, 1993; Rescher and Gerk, 2008). This protein interacts with several other proteins such as cytosolic phospholipase A2 (Wu et al., 1997) and 5-HT1B receptor (Svenningsson et al., 2006). Besides these regulatory functions S100 A10 is related to inhibition of the extrinsic pathway of blood coagulation interacting with plasminogen (Fitzpatrick et al., 2000), so it would be interesting to further investigate and evaluate the participation of this polypeptide either in the cellular pathway of exportation in skin gland or even in the toxic effects of the secretion. Moreover, the S100 A11 is involved in regulation of annexin I, actomyosin ATPase inhibition (Donato, 2003)

and, possibly in cell growth regulation of human keratinocytes (Sakaguchi et al., 2003).

Our database reveals another cluster encoding for calmodulin, a calcium binding protein involved in protein processing, also expressed in the snake venom glands (Junqueira-de-Azevedo and Ho, 2002). Although amphibian dermal glands are anatomically and structurally different of animal venom glands, both tissues (i.e., venom glands and amphibian skin glands) have the convergent function of storage of pharmacological active molecules. Indeed, a handful of secreted proteins and peptides from both tissues have potentially similar biological purposes of defense and self-preservation.

4. Discussion

The study of secretions of amphibian epithelium is of great interest due the potential pharmacological properties of the various compounds, mainly peptides, contained in such biological material. The Hylidae family has hundred of species that have been extensively studied mainly due to the hallucinogenic properties of their skin secretion and constituents. In fact, several molecules have been described in frog skins (Broccardo et al., 1981; Conceição et al., 2006, 2007a, 2007b; Leite et al., 2005; Mor and Nicolas, 1994), most of them with analgesic and antimicrobial effects. Most of these studies are focused on the isolation of peptides and evaluation of their biological and pharmacological properties. Combined with these studies, those involving cloning and sequencing of peptides precursors are important to support information about molecular and gene structure of these compounds.

In the present work, we report a global pattern of gene expression in gland epithelium from the recently described new species of frog *P. nordestina* (Caramaschi, 2006). We observed several transcripts for bioactive peptides, and other protein precursors never isolated or described in *Phyllomedusa* family up to now. In addition, representative transcripts of protein families involved in basic cellular functions as metabolism, protein processing, and folding, were described representing new information about the regulation of metabolic processes, which may be related to production of skin secretion.

In the group of polypeptide categorized as having 'common cellular functions', we identified transcripts mostly encoding transcriptional factors and proteins involved in diverse regulatory process as exocytose, such as EF hand calcium binding proteins, which is a large family of proteins involved in diverse processes as folding and signaling. Despite of the fact that the skin gland cells release their content under a holocrine control mechanism, not involving exocytosis, precursors peptides of this biochemical route were not found, up to now – what still needs a careful investigation.

Several antimicrobial peptides like dermaseptins, phylloseptins, phyllokinins, tryptophyllins, and bradykinin-like peptide sequences were retrieved. A group of transcripts related to protease inhibitors, which seems to contribute to antimicrobial activity of the secretion, was also identified. They showed high degree of similarity to either DNA or protein sequence analysis, but several insertions, deletions,

```
A. fimbria S100A11  -MTDLENCMESLIKIFHCYADEDKDKTTLSKKELTKLIEKELPTFLKTQK  49
            CP01    MEIPIQDLMISMISAFNKYASGDGDNNTLTKKELVNMACKEFPSLEKHPQ  50
                     :::  * *:*.  *:  **.  * *:.**:****.::  **:*:: *   :

A. fimbria S100A11  NPKVVDEIKKDLDQNGDDKVDFQEFLSLVTGLSIACEKCFMLLEQKKGKK  99
            CP01    KDKFLNDIFKITDRDGDNKINFEEFMTLMTCMTIALRKSGTI--------  92
                     : *.:::* *   *::**:*::*:**::*:* ::** .*.  :

A. fimbria S100A11  CC 101
            CP01    --
```
A

```
R.catesbeiana S100A10  MGVPSQMEQAMENIMFTFHKYAGDKNYMSKEDLHHLMESQFPEFLKNQND  50
              CP560    M-VPSELEQAMETLMFTFHKYCGDKNYMSKDDLHKMMESEFPEFLKNQND  49
                        * ***:.*****.:*******.********:***.::***:*********
```
B

```
R.catesbeiana S100A10  PLAVDKIMKDLDQCRDGRVNFHSFSSLIAGLTIACNDYYCKHMKG-RN-  97
              CP560    PRAVDKIMKDLDQCRDGRVTFHSFFSLIAGLTIACNDYYCKNMKGGRKQ  98
                        * ****************** **** **************:*** *:
```

```
CP01   MEIPIQDLMISMISAFNKYASGDGDNNTLTKKELVNMACKEFPSLEKHPQKDK FLNDIFKITD RDGDNKINFEEFMTLMTCM TIALRKSGT

CP560  MVPSELEQAMETLMFTFHKYCGDKNYMSKDDLHKMMESEFPEFLKNQNDPRAVDKIMKDLDQCR DGRVTFHSFFSLIAGLTIACNDYYCKNMKGGRKQ
```
C

Fig. 5. Putative S100 proteins encoded by *Phyllomedusa nordestina* skin gland cDNAs. (A) Amino acid alignment of contig CP01 and *Anoplopoma fimbria* S100A11 protein. (B) Amino acid alignment of CP01 contig and *Rana catesbeiana* S100A10 protein. (C) Schematic representation of contigs S100 calcium binding domains (light grey) and CP01 EF-hand domain (dark grey).

and non-synonymous amino acid substitutions were also observed. Further investigations to utter the characterization of the biological activity of these modified peptides are undoubtedly deserved, but this transcriptomic and similarity analysis may greatly contribute to a rational design and for the planning of experimental biological and pharmacological characterizations, which are being planned by the group. For instance, the inflammatory response triggered by *P. nordestina* secretion was recently described by Conceição and colleagues (Conceição et al., 2007a). They also used specimens from the same provenience as ours, but that was previously believed to be a *P. hypochondrialis* member. Although we could not describe precursors related to proteases or phospholipases generally underlying such type of biological effects, we reported here the presence of bradykinin-like peptides precursors that might be involved in the pharmacological response described by this group. These bradykinin-like related peptides (BRPs) transcripts identified here may possibly contribute for the increased permeability and vasodilatation leading to edematogenic process. These findings were supported by a recent report of two novel BRPs isolated from *P. nordestina* skin secretion, which were able to induce vasodilatation (Conceição et al., 2009).

The main difference between *P. nordestina* and *P. hypochondrialis* transcriptome was the significant higher presence of dermorphin transcripts in *P. nordestina* skin secretion compared to *P. hypochondrialis*, whose main transcripts were encoding for dermaseptins and no transcript encoding dermorphin was described (Chen et al., 2006). Only one single dermorphin sequence from *P. hypochondrialis* was found deposited in NCBI databank, and description of experimental characterization of the biological effects of this peptide could not be found, although the anti-nociception action of this frog secretion has been justified by and associated to the presence of this peptide. This fact deserves further investigations to clarify if the major expression of a specific group of opioid molecules in

the *P. nordestina* skin peptidome is not due to an artifact from sample handling procedure. Once confirmed, this difference could be potentially used as a biochemical marker to differentiate these two so similar species.

5. Conclusion

We present here a survey of expression profile of skin gland from the Brazilian leaf frog *P. nordestina*, which is the first global study for this species. The data show an overall high similarity to transcripts from frog skin belonging to other closest genus and families. Despite of some similarity in the global expression pattern between *P. nordestina* and *P. hypochondrialis* skin glands, the few differences described here may potentially support a classification of a given frog group based on molecular data and composition, especially to differentiate closely related species like *P. nordestina* and *P. hypochondrialis*. Moreover, besides this high similarity, remarkable differences in the skin secretion composition were observed, with a special attention to the high number of transcripts for dermorphin in *P. nordestina*, which was rarely found in *P. hypochondrialis*.

In our view, these data also reinforce the importance of recombinant DNA techniques and high throughput analyses of frog skin as a way of obtain new molecular information on novel species. In addition, in our view, the isolation and characterization of these several cDNAs bring new tools and perspectives on the functional studies of transcript products from *P. nordestina* skin gland. This knowledge will pave the way for making more solid the potential future use of frog skin active peptides for biotechnological applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2012.10.016.

Conflict of interest

Non declared.

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