Insect Biochemistry and Molecular Biology 76 (2016) 49–61

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Identification of major *Toxoneuron nigriceps* venom proteins using an integrated transcriptomic/proteomic approach

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ARTICLE INFO

Article history:
Received 3 May 2016
Received in revised form
28 June 2016
Accepted 3 July 2016
Available online 5 July 2016

Keywords:
Venom
Hymenoptera
Venom glands
Endoparasitoids
Gene Ontology

ABSTRACT

Endoparasitoids in the order Hymenoptera are natural enemies of several herbivorous insect pest species. During oviposition they inject a mixture of factors, which include venom, into the host, ensuring the successful parasitism and the development of their progeny. Although these parasitoid factors are known to be responsible for host manipulation, such as immune system suppression, little is known about both identity and function of the majority of their venom components. To identify the major proteins of *Toxoneuron nigriceps* (Hymenoptera: Braconidae) venom, we used an integrated transcriptomic and proteomic approach. The tandem-mass spectrometric (LC-MS/MS) data combined with *T. nigriceps* venom gland transcriptome used as a reference database resulted in the identification of a total of thirty one different proteins. While some of the identified proteins have been described in venom from several parasitoids, others were identified for the first time. Among the identified proteins, hydrolases constituted the most abundant family followed by transferases, oxidoreductases, ligases, lyases and isomerases. The hydrolases identified in the *T. nigriceps* venom glands included proteases, peptidases and glycosidases, reported as common components of venom from several parasitoid species. Taken together, the identified proteins included factors that could potentially inhibit the host immune system, manipulate host physiological processes and host development, as well as provide nutrients to the parasitoid progeny, degrading host tissues by specific hydrolytic enzymes.

The venom decoding provides us with information about the identity of candidate venom factors which could contribute to the success of parasitism, together with other maternal and embryonic factors.

1. Introduction

Parasitoid wasps in the order Hymenoptera are important natural enemies of several insect pests having a potential use as biological control agents. They constitute one of the largest groups of venomous animals. Unique regulatory compounds, which functionally aid in subduing insect hosts, characterize Hymenoptera parasitoid venoms. Venom is the secretion of venom glands, conserved organs in Hymenoptera, located in the female reproductive system (Dorémus et al., 2013). Venom is stored in a sac-like reservoir and is injected into the host body during parasitization, in order to create a suitable environment for the development of progeny (Moreau and Asgari, 2015; Beckage and Gelman, 2004).

Abbreviations: HPLC-MS/MS, high performance liquid chromatography tandem mass spectrometry; 2DE, two-dimensional gel electrophoresis; PDV, polydnavirus; *Tn*BV, *Toxoneuron nigriceps Bracovirus*; PBS, Phosphate buffered saline solution; GO, Gene Ontology; IEF, first dimension isoelectric focusing; PO, polyphenol oxidase; HS, heparan sulfate; ECM, extracellular matrix.

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http://dx.doi.org/10.1016/j.ibmb.2016.07.001
0965-1748/

Wasp venom fluid is a complex mixture containing proteinaceous and non-proteinaceous compounds (Leluk et al., 1989; Moreau and Asgari, 2015). The effects of parasitoid wasp venom are related to the host colonization strategy. While ectoparasitoid wasp venom is mainly used to paralyze or rapidly kill the host (Quistad et al., 1994; Nakamatsu and Tanaka, 2003; Wharton, 1993), the venom of endoparasitoids devoid of polydnavirus (PDV) produces either a lethal (Parkinson and Weaver, 1999) or a transiently paralysing effect on their hosts (Moreau et al., 2002). In contrast, little or no paralysis effects, as well as no lethal effect, are observed in response to injection of venom by wasps associated with PDVs (Webb and Strand, 2005; Asgari, 2006). In these latter cases venoms are involved in host regulation (Vinson and Iwantsch, 1980). In endoparasitoid wasps, venom is co-injected with other maternal factors during oviposition to ensure the success of parasitism (Asgari and Rivers, 2011). Despite progress on the identification of venom fluid components, the functional variety and evolution of most venom proteins are unknown.

In this study we aimed to identify *Toxoneuron nigriceps* venom proteins using an integrated transcriptomic and proteomic approach. *Toxoneuron nigriceps* (Viereck) (Hymenoptera: Braconidae) is a larval endoparasitoid of the tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). The mature parasitoid larvae emerge from the parasitized host, which does not reach the pupal stage due to developmental arrest upon parasitization (Pennacchio et al., 1993). During parasitization *T. nigriceps* females inject the egg along with maternal fluids into the host haemocel (Malva et al., 2004). These fluids are a combination of secretions from both venom glands (venom) and ovarian calyx (ovarian calyx fluid) (Fig. 1). The latter is composed of ovarian proteins and Polydnavirus (PDV) particles (Whitfield, 1990; Moreau and Asgari, 2015; Lawrence and Lanzrein, 1993; Kaeslin et al., 2005; Vinson et al., 2001).

The combined action of parasitoid factors, both of maternal and embryonic origin (teratocytes) (Pennacchio et al., 2001; Consoli and Vinson, 2004; Consoli et al., 2004; Rossi et al., 2012; Valzania et al., 2014), is responsible for alteration of host physiology. Among the maternal factors, the polydnavirus associated with *T. nigriceps* (*Tn*BV) infects different larval tissues, but the main infection targets are hemocytes and prothoracic glands (Stoltz and Vinson, 1979; Wyder et al., 2003), with subsequent alteration of the host immune and endocrine systems (Valzania et al., 2014; Falabella et al., 2006). The individual roles of several *Tn*BV genes have been clarified (Lapointe et al., 2005; Falabella et al., 2007a, 2003, 2006; Provost et al., 2004).

Despite some data on *Tn*BV and *T. nigriceps* teratocytes, and a number of studies showing that injection of individual components is not sufficient to defeat the host (Formesyn et al., 2012; Moreau and Asgari, 2015), only little information is available on both composition and function of *T. nigriceps* venom. We used a combination of next-generation transcriptome sequencing and bottom-up proteomics to identify the major protein components of *T. nigriceps* venom, enabling the identification of biological processes also in non-model organisms (Safavi-Hemami et al., 2014; Tang et al., 2010; Escoubas et al., 2006; Labella et al., 2015). Combining transcriptomic and proteomic data, we tested the correspondence between RNA sequences and the actually expressed proteins by defining the effective translated regions. Moreover, using this approach, we identified a large number of *T. nigriceps* venom gland transcripts and venom protein components. Among these, besides proteins similar to known venom components (Asgari and Rivers, 2011; Dorémus et al., 2013; Moreau and Asgari, 2015), we provided the first outline of novel proteins (i.e., with no similarity in databases) identified in *T. nigriceps* venom glands. This study provides new opportunities for the investigation of the role of the complex venom fluid proteins to better clarify *T. nigriceps* parasitization success.

2. Materials and methods

2.1. Insect rearing

The parasitoid *T. nigriceps* was reared in the laboratory according to the protocol adopted by Vinson et al. (1973) in an environmental chamber under controlled conditions: cocoons and parasitized host were kept a 29 ± 1 °C and adults at 25 ± 1 °C, a photoperiod of 16:8 h [L:D] was adopted and the relative humidity was 70 ± 5%. *H. virescens* larvae were reared on artificial diet (Vanderzant et al., 1962), at 29 ± 1 °C, at 16 h light photoperiodic and relative humidity of 70 ± 5% (Ferrarese et al., 2005).

2.2. Venom glands collection and RNA isolation

Toxoneuron nigriceps females were anaesthetized on ice for several minutes and subsequently placed in a phosphate buffered saline solution (PBS) in a Petri dish. The whole reproductive apparatus of adult females was pulled out with a pair of forceps and placed in 20 μl of PBS solution. Subsequently, the venom glands and ovarian calyx were dissected and placed in a centrifuge tube (Eppendorf, Hamburg, DE) containing TRI Reagent (Sigma, St. Louis, Missouri, USA). Teratocytes were obtained from "*in vitro*" reared *T. nigriceps* embryo as previously described by Pennacchio et al. (1992) and then were transferred into a 1.5 ml tube (Eppendorf, Hamburg, DE) containing TRI Reagent (Sigma, St. Louis, Missouri, USA). Each tissue samples were pooled per tube and stored at -80 °C until RNA extraction (Parkinson and Weaver, 1999).

Total RNA was extracted using TRI Reagent following the manufacturer's instructions (Sigma, St. Louis, Missouri, USA). A DNase (Turbo DNase, Ambion Austin, Texas, USA) treatment was carried out to eliminate any contaminating DNA. The DNase enzyme was

Fig. 1. *Toxoneuron nigriceps* and its reproductive apparatus. A) *T. nigriceps* adult female. Scale bars 2 mm; B) Reproductive apparatus. Scale bars 250 μm; C) a) venom gland, b) reservoir. Scale bars 100 μm.

then removed and the RNA was further purified using the RNeasy MinElute Clean up Kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol and eluted in 20 μl of RNA Storage Solution (Ambion Austin, Texas, USA).

RNA integrity was verified on an Agilent 2100 Bioanalyzer using the RNA Nano chips (Agilent Technologies, Palo Alto, CA) while RNA quantity was determined by a Nanodrop ND1000 spectrophotometer.

2.3. RNASeq data generation and de novo transcriptome assembly

Tissue-specific transcriptome sequencing of the RNA samples was performed with poly(A)+ enriched mRNA fragmented to an average of 150 nucleotides. Sequencing was carried out by the Max Planck Genome Center (http://mpgc.mpipz.mpg.de/home/) using standard TruSeq procedures on an Illumina HiSeq2500 sequencer, generating appr. 40 Mio paired-end (2 × 100 bp) reads for each of the tissue samples. Quality control measures, including the filtering of high-quality reads based on the score given in fastq files, removal of reads containing primer/adaptor sequences and trimming of read length, were carried out using CLC Genomics Workbench v7.1 (http://www.clcbio.com). The *de novo* transcriptome assembly was carried out with the same software and selecting the presumed optimal consensus transcriptome as described in Vogel et al., 2014.

All obtained sequences (contigs) were used as query for a blastx search (Altschul et al., 1997) in the 'National Center for Biotechnology Information' (NCBI) non-redundant (nr) database, considering all hits with an e-value<1E-5. The transcriptome was annotated using BLAST, Gene Ontology and InterProScan searches using BLAST2GO PRO v2.6.1 (www.blast2go.de) (Götz et al., 2008). To optimize annotation of the obtained data, we used GO slim, a subset of GO terms that provides a higher level of annotations and allows a more global view of the result. The assembled and annotated venom gland transcriptome was used to generate a custom-made protein database. The six reading frames of the 17,472 nucleotide sequences were translated in their corresponding amino acid sequences by SEQtools software (http://www.seqtools.dk/), thus obtaining 104,832 predicted amino acid sequences ("***Tnigriceps protein database***").

2.4. Digital gene expression analysis

Digital gene expression analysis was carried out by using CLC Genomics workbench v7.1 (http://www.clcbio.com) to generate BAM (mapping) files and QSeq Software (DNAStar Inc.) to remap the Illumina reads onto the reference transcriptome and then counting the sequences to estimate expression levels, using previously described parameters for read mapping and normalization (Vogel et al., 2014).

In particular, the expression abundance of each contig was calculated based on the reads per kilobase per million mapped reads (RPKM) method (Mortazavi et al., 2008), using the formula: $RPKM(A) = (10{,}00{,}000 \times C \times 1000)/(N \times L)$, where RPKM (A) is the abundance of gene A, C is the number of reads that uniquely aligned to gene A, N is the total number of reads that uniquely aligned to all genes, and L is the number of bases in gene A. The RPKM method is able to eliminate the influence of different gene lengths and sequencing discrepancy in the calculation of expression abundance.

2.5. Collection of venom and two-dimensional gel electrophoresis

Wasps previously anaesthetized on ice were submerged in PBS solution and their venom apparatus (venom glands and reservoir) was isolated. Each reservoir was gently opened with dissecting needles in a 20 μL drop of water (ratio 1 μL of water: 1 reservoir). The resulting crude extract was centrifuged at 5000 g for 5 min at 4 °C, and the supernatant was used for electrophoretic analysis. For the proteome analysis, the venom from 120 *T. nigriceps* females was collected for a total of 267 μg of protein. Protein quantity was measured using the Bradford method, with bovine serum albumin as the quantitative standard (Bradford, 1976).

To remove any impurities like excess salts, charged detergents, lipids, phenolic and nucleic acids that could interfere both with first dimension IEF separation and visualization of the 2nd dimension (2-D) result, a 2-D Clean-Up Kit (GE Healthcare) was employed. The 1-D separation (Isoelectric focusing) was performed with a total of 200 μg of protein on 7 cm non-linear pH gradient (3–11) IPG Dry Strips (Amersham Biosciences, Buckinghamshire, UK), using an IPGphor system and a Multiphor II system (Amersham Biosciences, Buckinghamshire, UK).

The sample was mixed with IPG strip rehydration buffer (8 M urea, 2% CHAPS, 40 mM DTT, IPG buffer, 0.002% bromophenol blue, 2.5 ml) (Amersham Biosciences, Buckinghamshire, UK). The isoelectric focusing (IEF) was performed with 50 μA per strip in gradient mode at 300 V for 4 h, at 1000 V for 3 h, at 5000 V for 5 h and at 500 V for 10 h. The temperature was set at 20 °C. After IEF, IPG strip equilibration was carried out for 15 min in 1% DTT containing equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris–HCl, pH 8.8) and then for 15 min in the same buffer solution containing 4.5% (w/v) iodoacetamide.

Proteins were separated in the 2-D in a 10% (w/v) polyacrylamide running gel, by enclosing the IPG strip with 2 ml of 1% agarose solution in electrode buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS, pH 8.3 and a trace of bromophenol blue) preheated. The run was carried out on a Bio Rad Electrophoresis Cell Mini Protean II (Life science, Hercules, California, U.S.A.) first at 80 V for 30 min and then at 100 V for 1 ½ h.

The protein gel was stained with colloidal Coomassie Blue G-250 for 1 h and the excess dye removed by washing in deionized water for at least 12 h. The stained two-dimensional gels were scanned on an ImageMaster Gel Scanner (Amersham Biosciences, Buckinghamshire, UK). The image analysis was performed using the ImageMaster 2D Elite software version 3.1 (Amersham Biosciences, Buckinghamshire, UK).

2.6. Protein spot in situ digestion

A total of 111 spots were excised from the 2DE gel, destained by repetitive washes with 50 mM ammonium bicarbonate buffer (pH 7.8) and acetonitrile, and digested with trypsin (10 ng/μL) in the same ammonium bicarbonate buffer. Spots were incubated at 4 °C for 1 h and then for 16 h at 37 °C. A minimum reaction volume was used to obtain the complete rehydration of the gel. Peptides were extracted by washing the gel particles with 0.2% trifluoroacetic acid in ammonium bicarbonate buffer and then in acetonitrile at room temperature. The resulting peptide mixtures were lyophilized and then resuspended in 10 μL 0.2% formic acid.

2.7. LC-MS/MS and protein identification

Digested spots were analyzed by an LC-MSD Trap XCT Ultra (Agilent Technologies, Santa Clara, California, USA) equipped with an 1100 HPLC system and a chip cube (Agilent Technologies, Santa Clara, California, USA). After loading, the peptide mixture (8 μl in 0.2% formic acid) was first concentrated and then desalted at 2 μl/min on a column of reverse enrichment (chip, Agilent Technologies, Santa Clara, California, USA), with 0.1% formic acid as eluent. The sample was then fractionated on a C18 reverse phase capillary column (75 μm, 43 mm) at a flow rate of 400 nL/min, with a linear gradient from solvent B (0.2% formic acid, 4.8% water in

acetonitrile) to solvent A (2% acetonitrile, 0.2% formic acid in water) from 5% to 60% in 50 min.

The mass spectrometer was set in the MS/MS mode, with a collision energy in the range 30 and 60 eV according to the mass and charge of the precursor ion. The MS/MS spectra obtained were analyzed by Analysis List program. The peak list generated was uploaded in MASCOT software (http:\\www.matrixscience.com), using the Mascot option Ion Search, and a research against the "*Tnigriceps protein database*" was performed.

These parameters were fixed: "trypsin" as an enzyme allowing up to 3 missed cleavages, carbamidomethyl on as fixed modification, oxidation of M, pyroGlu N-term Q, as variable modifications, 0.6 Da MS/MS tolerance, 600 ppt peptide tolerance and +2, +3 peptide charge. The score used to evaluate quality of matches for MS/MS data were higher than 46. The output contains information about the proteins identified.

3. Results

3.1. Transcriptome assembly

To enable unambiguous identification of the candidate protein(s) in the venom glands and subsequently analyze tissue-specific gene expression levels, we performed Next-Generation sequencing (RNAseq) of RNA isolated from the ovarian calyx, teratocytes and venom glands of *T. nigriceps*. We combined the tissue-specific datasets to build the *de novo* transcriptome assembly (TA) that we also used for protein identification. The resulting final *de novo* reference transcriptome assembly of *T. nigriceps* venom glands contained 17,472 contigs with a N50 contig size of 840 bp and a maximum contig length of 11,840 bp.

3.2. Functional analysis by Gene Ontology

To identify similarities with known proteins, venom gland contig sequences were translated and searched by BLASTX algorithm (Altschul et al., 1997) against non-redundant (nr) NCBI protein database with an E-value cut-off of 10^{-5} identifying 12,457 contigs (71%) matching entries. Overall, the species distribution of the top BLAST hit against the nr database for the *T. nigriceps* venom gland transcriptome showed that the majority of top hits matched against *Microplitis demolitor*, reflecting the close phylogenetic relationship between these species (Fig. 2). For functional annotations, all sequences were subjected to Gene Ontology (GO) analysis in Blast2GO revealing that of the total number of contigs (17,472), 61% (10,758) shared significant similarity to proteins with assigned molecular functions in the GO database (Altschul et al., 1997). Some of these contigs could be assigned to one or more ontology terms and we thus assigned each contig to a set of non-redundant GO terms using GO slim. We found a wide diversity of functional categories represented on all levels of the Gene Ontology database. The annotated contigs were classified into the three main GO categories: biological process, cellular component, and molecular function. The most prominent GO Biological Process categories (Level 2) were cellular process and metabolic process (Fig. 3). This result was expected due to the very large number of general GO terms, which comprise basic processes needed to maintain a living organism. The most prominent GO Molecular Function categories (Level 3) were binding protein and proteins with catalytic activity like hydrolase and transferase. The most abundant groups were cell and organelle in the Cellular Component (Level 3). Contigs not matching to any known sequences in the nr database accounted for 29% of the total transcripts indicating a large number of species-specific or noncoding transcript. In general, *de novo* transcriptome assemblies obtained from RNA-Seq data tend to be rather fragmented, frequently resulting in contigs which cover only part of the transcript, i.e. fully or mostly consist of UTR regions and fragmented transcripts corresponding to weakly-expressed genes.

In *T. nigriceps* venom, the enzyme code distribution shows that the most abundant families of enzymes are hydrolases and

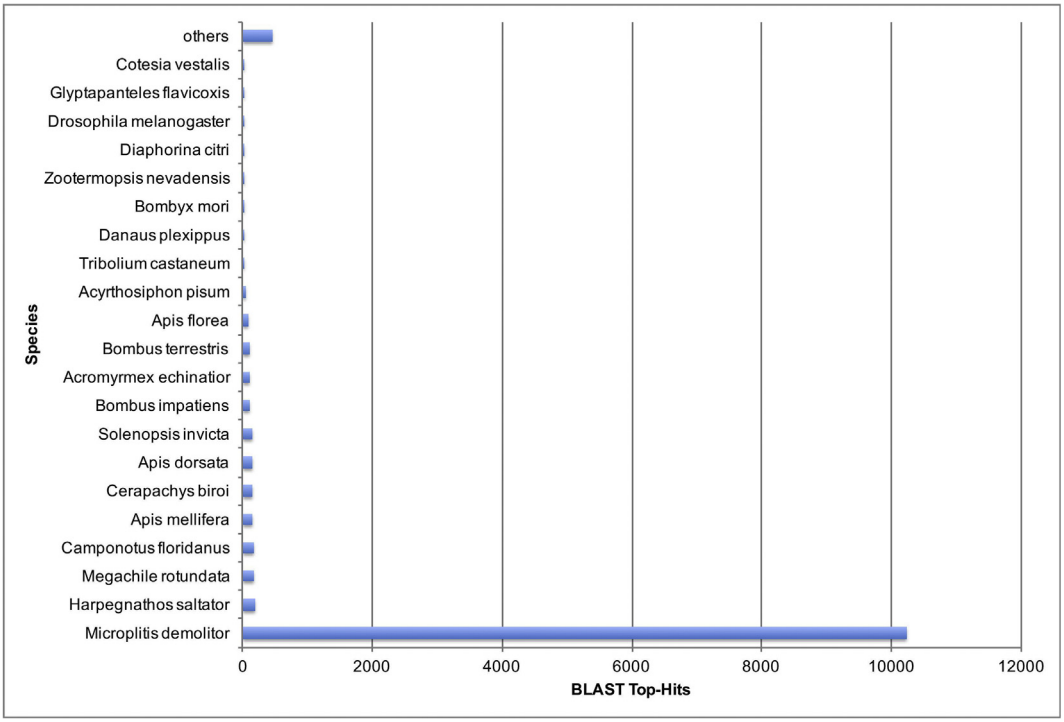

Fig. 2. Top BLAST hit species distribution of the *T. nigriceps* transcriptome assembly. Top BLAST hit species distribution obtained by BLASTx against the NCBI non-redundant (nr) protein database. The number of top BLAST hits per species is shown on the x-axis. The complete number of top hits of all related annotated organisms is shown. The most matches are to *Microplitis demolitor*.

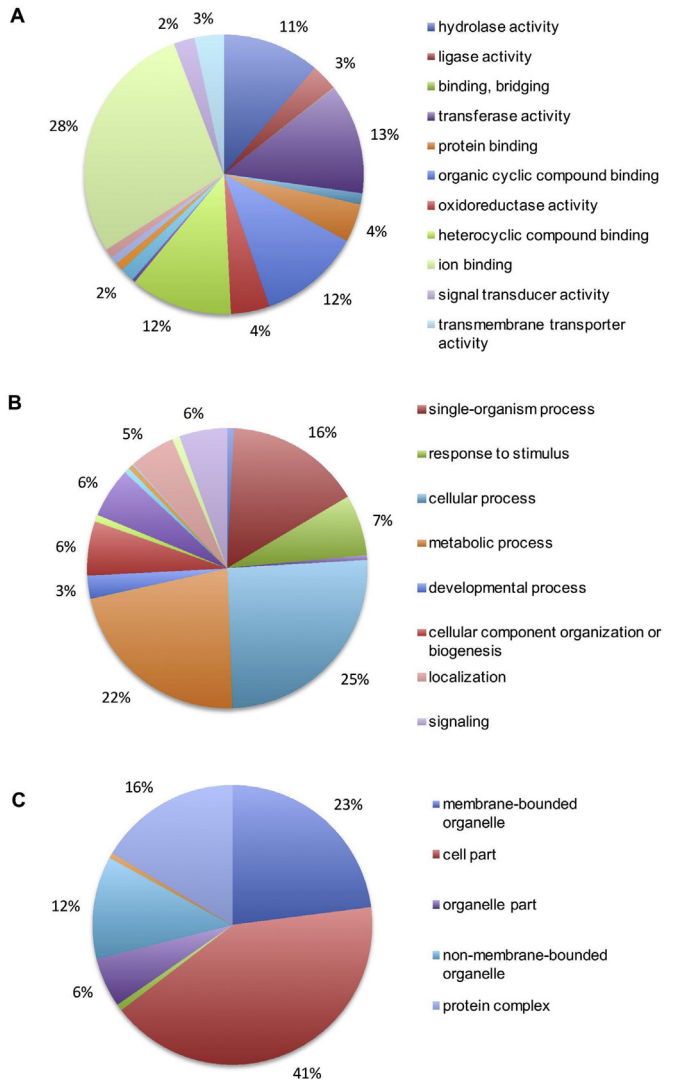

Fig. 3. Gene Ontology sequence annotation. Functional classification of all nr-matched transcripts from the *T. nigriceps* venom gland. A. Molecular function, B. Biological process, C. Cellular component. Data are presented as level 3 GO category for Molecular function and Cellular component and level 2 GO category for Biological process. Classified gene objects are displayed as percentages of the total number of gene objects with GO assignments; percentages below 2% are not shown.

transferases (Fig. 4). The identified proteins from *T. nigriceps* venom appeared to fall into different broad functional categories in agreement with literature data (see discussion section for a more detailed description). On the basis of proteomic identification (see below), a quantitative RNA-Seq analysis of the transcripts effectively encoding for putative venom proteins was carried out showing large differences in the expression levels, as visualized in the heat map with the normalized mapped read (RPKM) values (Fig. 5). The most strongly expressed transcripts in the venom gland included membrane metallo-endopeptidase (RPKM 8.74537), N-acetyllactosaminide β-1,3-N-acetylglucosaminyltransferase (RPKM 10.16535), glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1-like isoform x2 (RPKM 13.72048), heparanase (RPKM 11.53052), dihydrofolate reductase (RPKM 14.49132), galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase p (RPKM 12.15855), venom protein ci-48a (RPKM 10.40475), and venom allergen 5-like protein (RPKM 13.21409). As shown in Fig. 5 all of these transcripts were expressed at lower levels in other tissue such as ovarian calyx and teratocytes.

3.3. T. nigriceps venom components identified by two-dimensional gel electrophoresis and mass spectrometry (2DE-LC-MS/MS)

The crude extract from venom glands and reservoir was fractionated by 2DE using a 3–11 pH gradient. Following protein staining with Coomassie Brilliant Blue, the 2DE showed a protein profile ranging between MW 20–200 kDa (Fig. 6). A total of 111 spots were selected on the basis of their relative intensity (circled in red in Fig. 6), excised from the gel, destained and *in situ* digested with trypsin. The resulting peptide mixture was directly analyzed by LC-MS/MS and mass spectral data used to search the protein database obtained by translating the *T. Nigriceps* transcriptome.

A total of 31 proteins occurring in the "*Tnigriceps protein database*" matched with the peptide sequences derived from MS/MS spectra of individual venom components found in the 2DE analysis. The positive matches between proteins identified by the proteomic approach and the transcript sequence allowed us to confirm proteomics data and to obtain predicted full-length protein sequences.

Table 1 shows the list of proteins identified in the "*Tnigriceps protein database*". Protein regions included within the peptides sequence effectively identified by mass spectrometry were used in the BLAST program to find homologous proteins in Arthropoda.

The most representative proteins identified in *T. nigriceps* venom were: membrane metallo-endopeptidase (spots 1, 2, 3, 4, 5), heparanase (spots 6, 7, 12, 13, 14, 23, 24, 25, 26, 27, 28, 29, 30, 31), N-acetyllactosaminide β-1,3-N-acetylglucosaminyltransferase (spots 15, 16, 17, 18, 20, 21, 94), venom protein ci-48a (spots 35, 36, 37, 41, 42, 44), glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase-like (spots 51, 67, 87, 88, 89, 91, 92, 98) and galactosylgalactosylxylosyl 3-beta-glucuronosyltransferase P (spots 77, 79, 80, 81, 83). At lower levels other proteins could also be detected: retinoid-inducible serine carboxypeptidase-like (spot 43), calreticulin (spot 38), maternal protein tudor (50, 65, 70), venom metalloproteinase 3-like (spot 68), heat shock 70 kDa protein (spot 22), cathepsin (spot 59, 60), venom allergen 5-like (spot 82), elongation factor 1α (spot 69), phospholipase A2 (spots 78, 85), translationally controlled tumor protein (spot 108), peptidyl-prolyl cis-trans isomerase b (spot 111), 60S ribosomal protein l38 (spot 90), multidrug resistance protein (spot 93), serine protease homolog 90 (spot 9, 10), myrosinase 1-like (spot 8), phosphoglycerate kinase (spot 62), v-type proton ATPase subunit S1-like (spot 109), glucose dehydrogenase (spot 19), disintegrin and metalloproteinase domain-containing protein 10 (spot 72, 73, 100, 101, 102, 103, 104, 105, 106), enolase (spot 56), ovalbumin-related protein x-like (spot 56, 58), actin-4 (spot 54), spermine oxidase-like (spot 33, 34), retinal dehydrogenase 1 (spot 45) and dihydrofolate reductase (spot 107).

4. Discussion

The study of endoparasitoid venom would allow for detailed knowledge on the molecular biology and evolution of virulence components (Goecks et al., 2013) revealing the key role that this secretion plays in the host–parasitoid interactions. The activity of endoparasitoid venom is related to the inactivation of host immune system suppression and to the host developmental alterations, either independently or in association with mutualistic viruses and/or embryonic factors. Endoparasitoid progeny have to survive in the host hemolymph, and as a consequence will be exposed to the host immune responses. In order to avoid/suppress the host immunity, both at the cellular and non-cellular (humoral) level, parasitoids evolved a variety of strategies. The combinations of these strategies, grouped into passive and active mechanisms, are employed by individual parasitoids to ensure the development of their progeny. The major insect cellular immune response is the

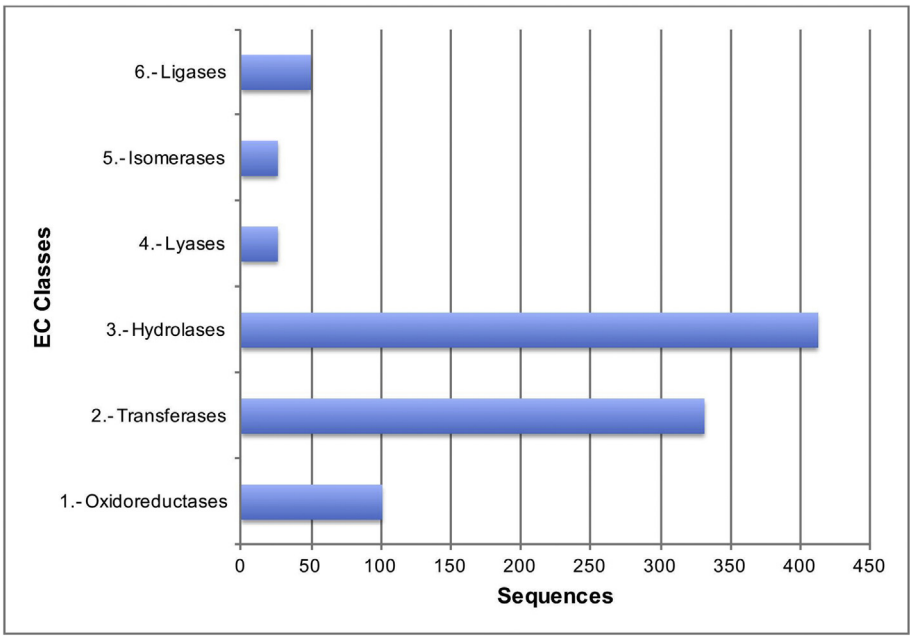

Fig. 4. Enzyme Code (EC) Classes of the *T. nigriceps* contigs encoding enzymes. Displayed are the most abundant families of enzymes found in the *T. nigriceps* venom gland transcriptome.

encapsulation response carried out by immunocytes response accompanied generally by melanization. Venom from several parasitoids has been shown to inhibit melanization, interfering with the phenoloxidase cascade. Since in most host–endoparasitoid systems studied the parasitoid-injected factors inhibit melanization, it was suggested that suppression of melanization is advantageous for successful parasitism (Asgari and Rivers, 2011; Moreau and Asgari, 2015).

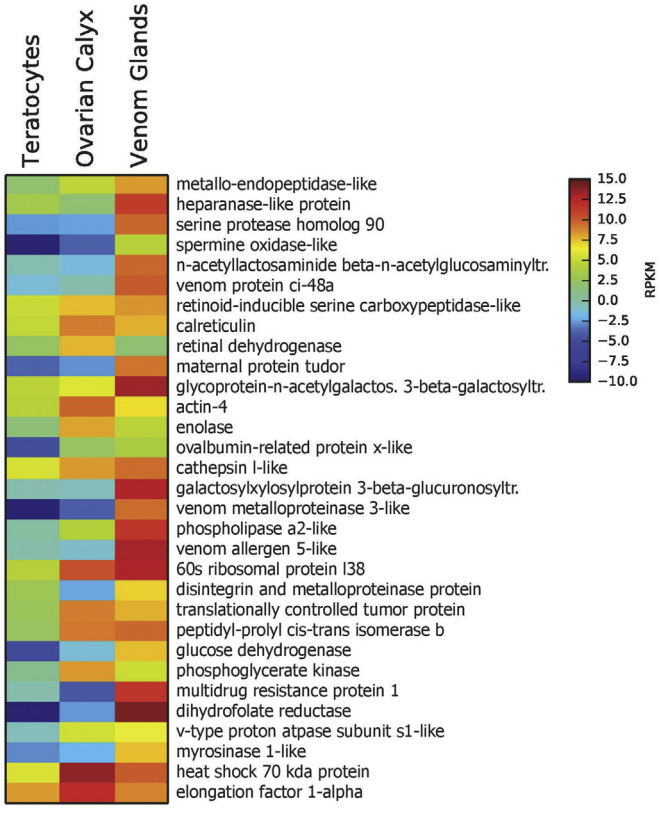

Fig. 5. Tissue-specific gene expression profiles of a selected set of venom gland candidate genes. Many of the candidate genes displayed tissue-specific expression patterns. The most strongly expressed transcripts in the venom gland were frequently expressed at lower levels in other tissues such as ovarian calyx and teratocytes. The annotations are reported on the right. Shown are log2-transformed RPKM values (blue resembles lower-expressed genes, while red represents highly expressed genes).

In endoparasitoids associated with PDVs, venoms contribute to the developmental alterations. Tanaka and Vinson (1991) demonstrated that venom in combination with *Cardiochiles nigriceps* calyx fluid prolongs larval development, inhibiting the pupation (calyx fluid alone is not able to induce the same effects in the host).

Although physiological effects of Hymenoptera parasitoid venoms are documented, relatively little is known on their protein composition, mostly due to the complex nature of venom components.

In the past, venomics (the analysis of venom compounds) was greatly limited because of technological restrictions and lack of genome sequences. Over the years, the advent of omics technologies, the accessibility of cDNA libraries from venom glands of several parasitoid wasps (Parkinson et al., 2001; Asgari et al., 2003; Falabella et al., 2007b; Crawford et al., 2008; Price et al., 2009; Baek and Lee, 2010; Vincent et al., 2010) and the availability of the *Nasonia vitripennis* genome sequence (http://hymenopteragenome.org/nasonia/) facilitated the identification and characterization of numerous venom proteins.

Venoms from Hymenoptera wasps are rich sources of biomolecules containing small peptides, including neurotoxins, amines and mid-to high-molecular-weight enzymes (Asgari and Rivers, 2011). Here we report the first identification of the major protein components of the *T. nigriceps* venom integrating transcriptomic and proteomic approaches. The venom gland transcriptome was obtained by extracting total RNA from *T. nigriceps* venom glands that was then used for RNA-seq analysis. Particular care had to be used to remove venom glands from other tissue since they are very small in size and very fragile. The *T. nigriceps* venom gland RNAseq data were assembled into 17,472 contigs and translated into the corresponding amino acid sequences.

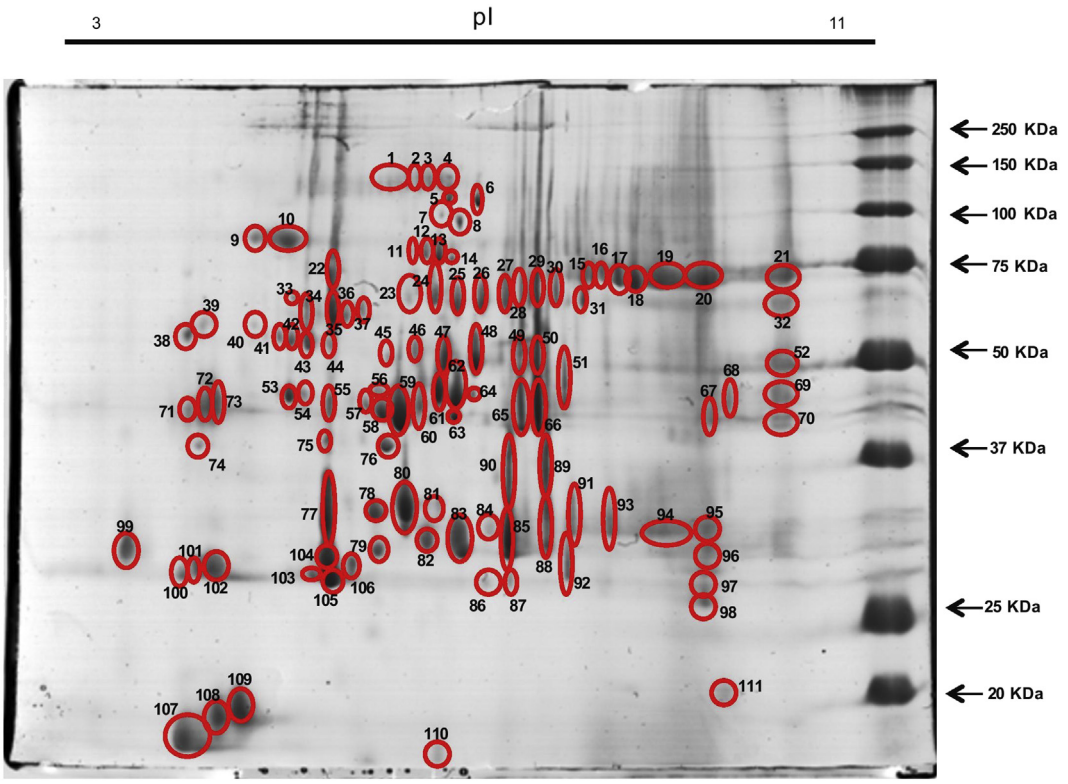

Fig. 6. 2DE analysis of *Toxoneuron nigriceps* venom. The first dimension comprised a 7 cm non-linear pH 3–11 immobilized pH gradient (IPG) subjected to isoelectric focusing. The second dimension was a 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The protein spots were stained with Coomassie Brilliant Blue. The sizes of protein standards are indicated on the right. Spots circled with a red line were then excised and submitted to in-gel digestion (with trypsin). The resulting peptides were analyzed by LC-MS/MS. The 31 identified proteins were listed in Table 1.

Peptide sequences identified in *T. nigriceps* venom by LC-MS/MS were matched against the predicted protein database, resulting in the identification of a total of thirty one different proteins. While homologs of some of the identified *T. nigriceps* venom proteins had already been described in venom from several parasitoids, others were identified for the first time in a venom.

Among the identified proteins, hydrolases constituted the most abundant family followed by transferases, oxidoreductases, ligases, lyases and isomerases. These findings seem to be in agreement with data reported for the venom of other parasitoids (Asgari and Rivers, 2011).

Hydrolases comprise a large group of different enzymes including proteases, peptidases and glycosidases that have already been reported as common component of venoms from several species of parasitoids (Moreau and Guillot, 2005). Among the hydrolases in the *T. nigriceps* venom glands, we identified metalloproteinases, serine protease homologue, heparanase, enolase and peptidase.

A number of ***metalloproteases*** have been identified in the venom of other hymenopteran parasitoid species (Consoli et al., 2004; Price et al., 2009; Danneels et al., 2010). In the venom analyzed we identified a venom metalloproteinase 3-like (spot 68) that contains a domain structure, with a C-terminal domain similar to ADAM/reprolysin zinc metalloproteinases. We also found a membrane metallo-endopeptidase-like protein (spots 1, 2, 3, 4, 5). According to the MEROPS database (http://merops.sanger.ac.uk) these *T. nigriceps* venom metalloproteases belong to the family M12 (M12B subfamily) and M13 respectively. Zinc metalloproteases of the M13 family include the animal peptidases neprilysin and endothelin-converting enzyme, which are involved in processing a number of neuronal and hormonal peptides (Rawlings and Barrett, 1995). Members of the M12 family are either secreted extracellular enzymes or membrane-bound enzymes such as adamalysins, capable of shedding a multitude of proteins from cell surfaces. M12 metalloproteases were shown to be involved in a multitude of biological and disease-related processes, such as digestion, intracellular signaling, matrix degradation and inflammation (Van Goor et al., 2009). In addition, a member of the M12B subfamily was also identified as a major component in snake venom. In *Eulophus pennicornis* (Price et al., 2009) it has been demonstrated that EpMP3 (*E. pennicornis metalloproteinase*) is a functional component of the venom, which is able to manipulate host development, reducing larval growth thus prolonging developmental time to pupation. For the metalloprotease identified in *T. nigriceps* venom we propose a similar role, where the delay in host pupation could promote parasitoid development, which would continue to grow in the host.

Although generally belonging to different subfamilies, microbial metalloproteases can be major virulence factors, allowing the attacking opportunistic pathogens to successfully invade host tissues The thermolysin-like microbial metalloproteases act as general toxic factors to the host, have proteolytic activity toward many kinds of host proteins, can cause necrotic or hemorrhagic tissue damage and allow systemic bacterial dissemination (Cabral et al., 2004; Miyoshi and Shinoda, 2000; Santi et al., 2010; St Leger et al., 1994). Although some insects have evolved counter adaptations to inhibit microbial metalloproteinases and to circumvent the negative effects (Griesch et al., 2000), in insect hosts the activity of pathogenic metalloproteases include the utilization of host proteins for nutrition, suppression of host cellular defense, and degradation of host defense molecules (Griesch and Vilcinskas, 1998; Liehl et al., 2006). Thus, it is tempting to speculate that the

Table 1
Proteins identified in the database of *Tnigriceps protein database* venom. Protein regions included within the peptides sequence effectively identified by mass spectrometry were used in the BLAST program to find homologous proteins in the Arthropoda taxonomy.

Spot	Contig	Mascot matches	Max ID score from Mascot	Corresponding description indicated in annotations obtained by *T. nigriceps* custom-made database	Corresponding Acc. N. NCBI protein indicated in annotations obtained by *T. nigriceps* custom-made database	Corresponding protein name indicated in annotations obtained by *T. nigriceps* custom-made database	Corresponding Acc. N. protein obtained by BlastP search	Corresponding protein name from BlastP search in Swiss prot
1,2,3,4,5	4489	13	479	membrane metallo-endopeptidase-like 1-like	gi|340723203|ref|XP_003399984.1|	PREDICTED: membrane metallo-endopeptidase-like 1-like [*Bombus terrestris*]	E2AFA5	Membrane metallo-endopeptidase -like 1 OS = *Camponotus floridanus*
6,7,8,12,13,14, 23,24,25,26, 27,28,29,30,31	18626	9	331	heparanase-like protein	gi|665784415|ref|XP_008559984.1|	PREDICTED: heparanase-like [*Microplitis demolitor*]	F4W9X3	Heparanase OS = *Acromyrmex echinatior*
9,10	18408	2	155	serine protease homolog 90 isoform x1	gi|315131321|emb|CBM69269.1|	venom protein Ci-40c [*Chelonus inanitus*]	A0A034V0K7	Serine protease easter OS = *Bactrocera dorsalis*
15,16,17,18, 20,21,94	18364	12	531	n-acetyllactosaminide beta--n-acetylglucosaminyltransferase	gi|665788683|ref|XP_008559945.1|	PREDICTED: N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase [*Microplitis demolitor*]	E2AMU3	N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase OS = *Camponotus floridanus*
22, 35, 36, 37, 41, 42, 44	18596	12	524	venom protein ci-48a	gi|665819695|ref|XP_008558721.1|	PREDICTED: uncharacterized protein LOC103579170 [*Microplitis demolitor*]	E6ZCK2	Venom protein Ci-48a OS = *Chelonus inanitus*
33, 34	14112	8	348	spermine oxidase-like	gi|665801233|ref|XP_008548621.1|	PREDICTED: spermine oxidase-like [*Microplitis demolitor*]	V9IIS9	Peroxisomal N(1)-acetyl -spermine/spermidine oxidase OS = *Apis cerana*
43	6885	3	138	retinoid-inducible serine carboxypeptidase-like	gi|665785882|ref|XP_008549603.1|	PREDICTED: retinoid-inducible serine carboxypeptidase-like isoform X1 [*Microplitis demolitor*]	E2BXB0	Retinoid-inducible serine carboxypeptidase OS = *Harpegnathos saltator*
38	5341	7	332	calreticulin	gi|665788653|ref|XP_008559929.1|	PREDICTED: calreticulin [*Microplitis demolitor*]	Q8IS63	Calreticulin OS = *Cotesia rubecula*
45	5964	7	282	retinal dehydrogenase 1	gi|665814527|ref|XP_008555876.1|	PREDICTED: aldehyde dehydrogenase X, mitochondrial-like [*Microplitis demolitor*]	E2AHA9	Retinal dehydrogenase 1 OS = *Camponotus floridanus*
50, 65, 70	19179	3	172	—NA—	—NA—	—NA—	F4WZD5	Maternal protein tudor OS = *Acromyrmex echinatior*
50,51,65,67,87, 88,89,91,92,98	6822	8	425	glycoprotein-n-acetylgalactosamine 3-beta-galactosyltransferase 1-like isoform x2	gi|665808476|ref|XP_008552562.1|	PREDICTED: glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1-like isoform X2 [*Microplitis demolitor*]	A0A067QWL0	Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 OS = *Zootermopsis nevadensis*
54	13583	5	229	actin-4	gi|17530805|ref|NP_511052.1|	actin 5C, isoform B [*Drosophila melanogaster*]	W5JXB3	Actin beta/gamma 1 OS = *Anopheles darlingi*
56	4620	6	256	enolase	gi|665799952|ref|XP_008547916.1|	PREDICTED: enolase isoform X1 [*Microplitis demolitor*]	E2A4J2	Enolase OS = *Camponotus floridanus*
59,60	6829	7	353	cathepsin l-like	gi|665792610|ref|XP_008543917.1|	PREDICTED: cathepsin L [*Microplitis demolitor*]	V9IF08	Cathepsin L OS = *Apis cerana*
77,79,80,81,83	14458	3	167	galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase p	gi|572307460|ref|XP_006619592.1|	PREDICTED: galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase P-like isoform X3 [*Apis dorsata*]	E0VA90	Glucuronyltransferase-S, putative OS = *Pediculus humanus*

68	7042	5	221	venom metalloproteinase 3-like	gi|665805846|ref|XP_008551135.1|	PREDICTED: phospholipase A2-like isoform X1 [*Microplitis demolitor*]	F4X7T0	A disintegrin and metalloproteinase with thrombospondin motifs 1 OS = *Acromyrmex echinatior*
78,85	16077	10	570	phospholipase a2-like	gi|665805846|ref|XP_008551135.1	PREDICTED: phospholipase A2-like isoform X1 [*Microplitis demolitor*]	C0LTQ4	Phospholipase A2D OS = *Tribolium castaneum*
82	17054	2	108	venom allergen 5-like	gi|665796216|ref|XP_008545886.1|	PREDICTED: venom allergen 5-like [*Microplitis demolitor*]	E2AD07	Tryptophan 5-hydroxylase 1 OS = Camponotus floridanus
90	19122	2	113	60s ribosomal protein l38	gi|665820171|ref|XP_008558979.1|	PREDICTED: 60S ribosomal protein L38 [*Microplitis demolitor*]	Q9VTG7	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase P OS = *Drosophila melanogaster*
72, 73, 100, 101, 102, 103, 104, 105, 106	6424	12	618	—NA—	—NA—	—NA—	W8AEN6	Disintegrin and metalloproteinase domain-containing protein 10 (Fragment) OS = *Ceratitis capitata*
108	4538	6	294	translationally controlled tumor protein	translationally controlled tumor protein	translationally controlled tumor protein [*Spodoptera frugiperda*]	V5NCU4	Translationally controlled tumor protein OS = Spodoptera frugiperda
111	7131	5	403	peptidyl-prolyl cis-trans isomerase b	peptidyl-prolyl cis-trans isomerase b	PREDICTED: peptidyl-prolyl cis-trans isomerase 5 [*Microplitis demolitor*]	E2B9N3	Peptidyl-prolyl cis-trans isomerase OS = *Harpegnathos saltator*
19	4451	7	239	glucose dehydrogenase	gi|665800394|ref|XP_008548159.1	PREDICTED: glucose dehydrogenase [FAD, quinone]-like [*Microplitis demolitor*]	A0A022T3N9	Glucose dehydrogenase acceptor-like protein-14 OS = *Microplitis demolitor*
62	6482	9	516	phosphoglycerate kinase	gi|665799330|ref|XP_008547575.1	PREDICTED: phosphoglycerate kinase [*Microplitis demolitor*]	K7IM64	Phosphoglycerate kinase OS = *Nasonia vitripennis*
93	17485	5	228	multidrug resistance protein 1	gi|405970698|gb|EKC35579.1|	Multidrug resistance protein 1 [*Crassostrea gigas*]	A0A067QWL0	Glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 OS = *Zootermopsis nevadensis*
107	15050	2	130	—NA—	—NA—	—NA—	S4PKE6	Dihydrofolate reductase OS = *Pararge aegeria*
57,58	4452	3	124	ovalbumin-related protein x-like			Q8IS84	Serine protease inhibitor serpin 1c OS = *Mamestra configurata*
69	4154	2	122	elongation factor 1-alpha	gi|665799004|ref|XP_008547400.1|	PREDICTED: elongation factor 1-alpha [*Microplitis demolitor*]	K7IVS1	Elongation factor 1-alpha OS = *Nasonia vitripennis*
109	18366	4	198	v-type proton atpase subunit s1-like	gi|665784417|ref|XP_008559996.1|	PREDICTED: V-type proton ATPase subunit S1-like [*Microplitis demolitor*]	D6WKR8	Guanylate cyclase OS = *Tribolium castaneum*

metalloproteases identified in *T. nigriceps* venom might have similar functions, namely in host immune suppression and protein and tissue degradation for nutritional purposes.

Serine protease homologues are similar to active serine proteases but lack one of the three amino acids in the catalytic domain (Hedstrom, 2002) and do not have proteolytic activity. Insect serine proteases are involved in numerous biological processes, including the activation of the Toll signalling cascade and the regulation of the polyphenol oxidase (PO) activation. Several serine proteases homologues were found in different parasitoid venoms (Hedstrom, 2002; De Graaf et al., 2010), where they can play a crucial role in host regulation, inhibiting melanization in host hemolymph by blocking the phenoloxidase cascade (Zhang et al., 2004). In *T. nigriceps* venom we identified a serine protease homologue (spots 9, 10), which could have a similar role.

We identified an ***Enolase*** (spot 56) and a ***Heparanase-like protein*** among our parasitoid venom components. Enolases catalyze the reversible dehydration of D-2-phosphoglycerate (PGA) to phosphoenolpyruvate (PEP) in both glycolysis and gluconeogenesis but are also known to be implicated in other intra- or extra-cellular functions in different organisms (Pancholi, 2001). Therefore Enolase is also noted to be a multifunctional protein (Pancholi, 2001). Falabella et al. (2009) and Grossi et al. (2016) have shown that *Aphidius ervi* teratocytes synthesize and release in the host haemocoel an extracellular Enolase (*Ae*-ENO) which mediates host tissue degradation for nutrition of the parasitoid larva. It could be speculated that enolase in *T. nigriceps* venom could act in a similar way. A similar mode of action could be used by venom heparanase, an enzyme involved in many biological processes, acting both at the cell-surface and the extracellular matrix to degrade polymeric heparan sulfate (HS) (Vlodavsky et al., 1999). In other endoparasitoid venoms, heparanase-like proteins have been identified, but no information is available regarding their function. The overexpression of heparanase has been observed in human tumour cells conferring them an invasive phenotype in experimental animals (Schubert et al., 2004). Heparanase activity is correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of HS cleavage and remodeling of the extracelluar matrix (ECM) barrier (Parish et al., 2001). A similar mechanism might be used by *T. nigriceps* venom heparanase to ensure both tissue degradation and availability of nutrients for the development of young parasitoid larvae that lack an elaborated mandibular apparatus. Particularly interesting was the observed good correlation between transcript abundance and the number of peptides identified by mass spectrometry. In addition to the heparanase-like protein, which was identified in a larger number of spots with high intensity in the 2D protein gel, we also found a high expression level in our venom gland transcriptome (RPKM value of 13.72048). The good correlation between the abundance of transcripts and the number of matches with mass spectrometry data was particularly interesting also for enzymes belonging to the transferase family. We found a glycoprotein-N-acetylgalactosamine 3-beta galactosyltransferase 1-like identified in spots 50, 51, 65, 67, 87, 88, 89, 91, 92, 98 with RPKM value 12.15855, a N-acetyllactosaminidebeta-n acetylglucosaminyltransferase identified in spots 15, 16, 17, 18, 20, 21, 94 with RPKM value 10.16535 and a galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase P was identified in spots 77, 79, 80, 81, 83 with RPKM value 11.53052. The role of these enzymes, all of which belong to the glycosyltransferases family, is still unknown.

Based on the literature and existing data on parasitoid venoms, below we discuss putative functions of other proteins identified in the *T. nigriceps* venom.

Calreticulin is a molecular chaperone that acts as Ca^{2+}-binding and lectin binding protein, found in several parasitoid tissues including venom glands. In insects, its role in encapsulation and phagocytosis has been reported (Choi et al., 2002; Asgari and Schmidt, 2003). Calreticulin was previously identified in the venom of other parasitoids, e.g. *N. vitripennis* (De Graaf et al., 2010), *Pteromalus puparum* (Zhu et al., 2010) and *Cotesia rubecula* (Asgari et al., 2003). Zhang et al. (2006) have demonstrated that calreticulin in the venom of *C. rubecula* inhibits *Pieris rapae* hemocyte diffusion, debilitating them from the encapsulation response. Calreticulin identified in *T. nigriceps* venom could alter the intracellular calcium balance, thus modifying pathways in which Ca^{2+} is involved, such as apoptosis, inflammation, and activation of hydrolytic enzymes.

Glucose dehydrogenase (spot 19) is an oxidoreductase transferring electrons to various natural and artificial electron acceptors, specifically those acting on the CH–OH group of donor with NAD^+ or $NADP^+$ as acceptor. Although a glucose dehydrogenase was found in *N. vitripennis* venom (Rawlings and Barrett, 1995), its role is still unknown.

***Venom protein Ci-48 like* protein** was found in Hymenoptera and Diptera and it was recently speculated to have specific functions in the early phases of parasitism in *M. demolitor* venom glands (Burke and Strand, 2014). The protein identified in spots 22, 35, 36, 37, 41, 42 and 44 displays 26% sequence identity with *Venom protein Ci-48* previously identified in *Chelonus inanitus* (Vincent et al., 2010). Thus the protein found in the *T. nigriceps* venom could have similar functions in the early phases upon parasitization.

Phospholipase A2 (*PLA2*) is one of the main Hymenopteran venom enzymes which is also the most studied in bee (Monteiro et al., 2009) and snake venoms (Kini, 2003). The Hymenopteran enzyme shares similarity both at the structural and catalytic level with mammalian enzymes, but in contrast to those is often toxic and induces a wide spectrum of pharmacological effects. PLA2 are enzymes that release fatty acids and lysophospholipids from the second carbon group of glycerol (Mingarro et al., 1995). The neurotoxic, myotoxic, anticoagulant and inflammatory effect of Phospholipase A2 is well described (Dotimas and Hider, 1987; Hoffman, 1996). In line with this toxic function, these enzymes have been found in the venoms of a wide range of organisms including insects, reptiles, amphibians, arachnids and coelenterates (Nicolas et al., 1997).

Cathepsin L is a lysosomal endopeptidase expressed in several eukaryotic cells in the papain-like family of cysteine proteinases. Cysteine proteases play key roles in extra- and intra-cellular protein degradation in a large range of organisms, from bacteria to mammals (Berti and Storer, 1995). Numerous studies have shown that in various insect orders, such as Coleoptera, Diptera and Hemiptera, cysteine proteases are important digestive enzymes and have been considered as targets for pest control (Cristofoletti et al., 2003). Our study describes the first Cathepsin L identified in Hymenopteran venom.

Venom allergen 5-like, also called antigen 5, is generally found in venoms of social Hymenoptera of the superfamily Vespoidea (Vincent et al., 2010). To date only two antigen 5-like venom proteins have been discovered in *N. vitripennis* venom but their biological function is still unknown (Bull et al., 2002; Danneels et al., 2010). The venom allergen 5-protein contains a sperm-coating protein (SCP)-like extracellular protein domain, and belongs to the SCP superfamily. It has been proposed that SCP domains may function as endopeptidases. We speculate that the venom allergen 5-like protein found in the *T. nigriceps* venom might be involved in peptide proteolysis, and could thus be one of a number of factors involved in tissue degradation, a key function of hymenopteran venom.

Elongation factor 1-alpha was previously also identified in the venom of another parasitoid, *Leptopilina heterotoma* (Colinet et al., 2013). EF1-alpha is essential for regulating polypeptide elongation

during translation. However, although it was also found as a secreted candidate virulence factor in *Leishmania protozoan* parasites, where it seems to be involved in the induction of host macrophage deactivation (Nandan et al., 2002), to date the putative function of EF1-alpha in host-parasitoid interaction is unknown.

Spermine oxidase-like (SMO) is a FAD-dependent enzyme that specifically oxidizes spermine (Spm) producing the reactive oxygen species H_2O_2 and playing a key role in numerous cell functions, such as DNA synthesis, cellular proliferation, alteration of ion channels function, nitric oxide synthesis and inhibition of immune responses (Cervelli et al., 2012). Although its biological role in venom fluids still has to be elucidated, it is tempting to speculate that a similar mechanism may occur in the insect to either directly facilitate parasitoid development or manipulate host physiological processes to the advantage of the parasitoid.

Ovalbumin-related protein x-like is a member of Ovalbumin family that consists of three proteins: ovalbumin, ovalbumin-related protein Y (OVAY), and ovalbumin-related protein X (OVAX). OVAX, similar to ovalbumin and OVAY, belongs to the ovalbumin serine protease inhibitor family (ov-serpin). The serpin role in insects is well known and its involvement in inhibiting the activation of the PO cascade controlling the melanization production (Kanost and Gorman, 2008) suggests a key role in parasitoid venom.

Maternal Protein Tudor occurs in *Drosophila melanogaster* where it is required during oogenesis for the formation of primordial germ cells and for normal abdominal segmentation. The molecular mechanism by which Tudor contributes to germ cell formation is unknown, however without proper Tudor function germ cell formation does not occur. No information is at present available for this protein in parasitoids and its possible role as venom component is still unknown. Here we identified for the first time a Maternal Protein Tudor in a venom.

The *T. nigriceps* venom analysis through an integrated transcriptomic and proteomic approach allowed us to obtain a global profile of its major components, starting from the nucleotide sequence to protein sequence validation. Furthermore, the functional annotation allowed us to speculate about the putative function of some of the identified venom proteins. It is known that one of the possible functions carried out by endoparasitoid venoms is a primarily and immediate inhibition of the host immune system activity, as previously reported also in case of *T. nigriceps* venom by Tanaka and Vinson (1991). Among the *T. nigriceps* venom components *Serpin*, *Serine protease homologue and Calreticulin* could be reasonably involved in this function. The second possible activity of venom could be the involvement in providing nutrients to the parasitoid progeny, degrading host tissues by specific enzymes such as hydrolases, as already observed in other host/parasitoid systems. In particular, the analysis of the *T. nigriceps* venom components, carried out in this work, supports these considerations by the presence of *Heparanase* and *Enolase*, proteins probably involved in this mechanism. The venom decoding provides us with information about the identity of venom factors, demonstrating that although this single parasitic factor alone is not enough, the venom contributes substantially and synergistically to other maternal and embryonic factors to ensure the success of parasitism.

Funding

This work was supported by University of Basilicata, the Italian Proteomics Association (ItPA) and Tab consulting S.r.l.

Conflict of interest statement

The authors have declared no conflict of interest.

Acknowledgements

We would like to thank Dr. Genoveffa Ciancio and Indira Kuriachan for their help in insect rearing.

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