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Analysis of the Differential Gene Expression, by the DD-RTPCR Technique, Of Workers *Atta sexdens rubropilosa* Mandibular Glands (Hymenoptera: Formicidae)

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Abstract: The DD-RTPCR technique is becoming more efficient nowadays to isolate and characterize genes differentially expressed among cells, tissues or individuals, being used here, for the first time in the study of mandibular glands of minina and media workers and soldiers of the ant species *Atta sexdens rubropilosa*. The expression profile of bands (transcripts), obtained in the present study, permitted the grouping of gene fragments into three distinctive classes. Class I fragments corresponded with those called constitutive genes, which could be associated with the metabolic processes of the mandibular glands. Class II could be the ones that would be codifying more important products in more specific stages than in others, related to the tasks performed in the colony by individuals of different castes. Class III gene fragments (transcripts 1, 2 and 3) must be caste-specific and they were found in the mandibular glands of soldiers (T1), media workers (T2) and minima workers (T3), respectively. Therefore the present study, besides having highlighted the differential gene expression in the mandibular glands of individuals of three castes of *A. s. rubropilosa*, demonstrated that the DD-RTPCR technique can be one more tool to be used in gene expression analysis, including establishing intra and inter-caste relations in insects.

Key words: Differential display, transcripts, caste, tasks

INTRODUCTION

Technical fundaments of *Differential Display Reverse Transcriptase Polymerase Chain Reaction* (DD-RTPCR) in the study of gene expression: The superior organisms contain around 100.000 different genes from which only 10.000-15.000 are expressed in the cell. The choice of the genes that will be expressed is what determines the biological processes such as: development, homeostasis, stimuli response, cell regulation, etc[1]. This way, comparisons of the gene expression in the different cell or tissue types, provide useful information in the understanding of such biological processes.

Due to the differentiated activity of gene expression, in several cells or tissues, the identification and isolation of the gene has been difficult, considering that their activities are reflected in the types and quantities of their mRNA species and proteins[2].

Many methods used to distinguish the mRNA are associated with subtractive hybridization techniques[3]. However, these techniques are not very sensitive and are difficult to perform, in addition to recovering genes in incomplete form and not identifying rare mRNA.

The DDRT-PCR technique has been widely used in studies that involve comparison between gene expression patterns. It is a powerful tool to detect genes that express differentiation between the cells, tissues, individuals or different development stages, or even genes expressed differentially in embryogenesis, cancer, diabetes, cerebral development and others[4].

The DDRT-PCR methodology was described by[5] to identify genes differentially expressed among the 15.000 mRNA species, in two populations of breast cells. Later, the technique was named DDRT-PCR in accordance with the terminology of polymerase chain reaction-PCR[6].

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DD-RTPCR is a method based in PCR that allows systematic comparisons of expressed mRNA in the cells. This method consists of reverse transcription (RT-PCR) of a mRNA population of samples studied using anchored primers that surround the mRNA poly-A tail and in the subsequent PCR amplification of cDNA, with an anchored primer and an arbitrary one. After amplification, the fragments are usually separated by electrophoresis, then the bands that will be differentially expressed are taken from the gel, reamplified, cloned, sequenced and analysed with the help of *GenBank*. The general scheme of the process is represented in Fig. 1.

The DD-RTPCR technique compared to subtractive hybridization is fast and more sensitive[7].

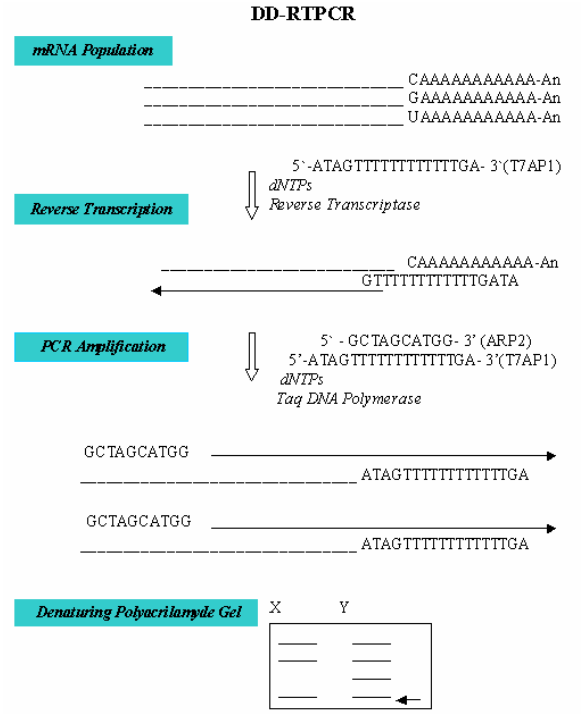

Fig. 1: Schematic diagram of the DDRT-PCR technique. The first step of the process is the total RNA extraction followed by the mRNA reverse transcription to cDNA. The mRNA are converted into cDNA by the reverse transcriptase enzyme and by an oligo-dT primer that can be anchored C, G or A. The next step is the amplification of a subgroup of cDNAs inside the pool with the same oligo-dT primers used for the reverse transcription and a 10-base arbitrary primer as a secondary primer. The products are separated by size, in denaturing polyacrilamyde gel. The band pattern is analyzed and the candidate bands to be differentially expressed are excised from the gel, purified, cloned and sequenced. T7AP1: anchored primer, ARP2: arbitrary primer, gel arrow: candidate gene to be differentially expressed

Study of gene regulation by the DD-RTPCR technique in social insects: Among the social insects, the study of gene expression has been performed with bees, aiming at gender and caste determination, metabolic ways related to the ontogenesis or even mechanisms of the division of labour.

Studies of caste determination, in *Apis mellifera* larvae, were conducted by[8]. The initial results showed 8 gene sequences differentially expressed in the queens and workers of the fourth larval instar. Two genes appeared in larger quantities in the workers, showing great similarity to the crystalline-lambda protein and one group of proteins present in the fat body of other holometabolous insects. Only three genes that were present in the queens were similar to the oxiacyl-reductase enzyme and with proteic factos of transcription.

In the development of queens and workers of *A. mellifera*, analysis demonstrated the presence of numerous genes being differentially expressed in both castes[8]. Two of them are potentially promising in the regulation of caste differentiation. The result clearly denoted that the process of caste determination in *Apis* involves the activation of specific genes in accordance with each caste.

Analysis of the caste-specific gene expression were also performed in termites by[9], using the mandibular glands of soldiers of *Hodotermopsis japonica*. The results showed the presence of one gene functioning especially in the mandibular glands of soldiers and the expressed protein could function in the regulation of the differentiation of caste or even as an alarm pheromone.

The division of labour between the castes of *A. s. rubropilosa* and the role of the mandibular glands in the workers: The ants divide their tasks inside the colony. The ant nest population is composed of individuals that are morphologically different (polymorphism), in accordance to the labour or role that they perform in the colony (polyethism).

In the literarure 29 tasks are described (task = determined sequence of acts with a specific purpose) that are performed by the castes of this species[10] and they are classified as follows:

* Gardeners or “sitters”, smaller individuals, with a head measuring between 0,8 to 1,0 mm. They take care of the offspring inside the nest, besides “piggy-backing” with the larger foraging workers over the vegetal material that is being transported.
* Generalists, workers of intermediary size, with a head measuring around 1,4 mm. They work inside the nest degrading the vegetation before its incorporation in the fungus garden. They transport other workers, help the offspring during ecdysis or

moulting, take care of the queen, discard the waste and also rebuild the fungus sponge.

* Foragers and excavators, bigger than the generalists, with a head measuring between 2,0 to 2,2 mm. They collect and cut the new vegetation, besides excavating the nest.
* Soldiers or defenders, bigger individuals, with a head measuring around 3,0 mm. They act in defending the nest.
* The adult workers change their activities as they age, from being sitters to foraging and many of these changes are followed by alterations to their exocrine glands[11].

The present study analysed the differential gene expression of mandibular glands of minima and media workers and soldiers of *A. s. rubropilosa*, by the DD-RTPCR technique already performed in other Hymenoptera, in trying to detect possible differences in the gene expression of the mandibular glands of the three castes and also trying to establish relationships with the different tasks that these individuals perform in the colony.

MATERIALS AND METHODS

Total RNA dosage

Material collection for extraction of total RNA: The mandibular glands of minima and media workers and soldiers of ants *A. s. rubropilosa* were removed and placed in a physiological solution for insects (NaCl, KH_2P_4, $NaHPO_4$, pH 7.2) containing DEPC-treated water, making sure that the gloves and the tools were also DEPC-treated to avoid any contamination with RNAses in the environment. The glands were placed in sterile eppendorfs (RNAse free), containing 500µL of Trizol (Trizol®, Life Technology, Gaithersburg, MD USA) and kept in ice during the whole process. After that, around 50 mandibular glands of each caste were placed in eppendorfs that were stored in a freezer at-80 ºC, until the RNA extraction.

Extraction of total RNA with trizol: The eppendorfs containing the homogenized material in trizol were placed in ice for a slow thaw. Using sterile tools, DEPC-treated water, the material was homogenized and the glandular tissue was dissolved.

After that, 100µL of DEPC-treated water were added to the eppendorfs and the solution was quickly homogenized in the vortex. These eppendorfs were then incubated for 5 min at room temperature, for a complete dissolution of the nucleoproteins of the material and 100µL of chloroform were added and the eppendorfs were shaken for 15 sec and then incubated for 2-3 min at room temperature. The samples were centrifuged at 4ºC (1200xg) for 15 min. The supernadant (aqueous), where the RNA was found, was transferred to a new tube. The lower portion or organic phase contained the RNA and proteins.

After the phase separation, the next step was the RNA precipitation, with 500µL of isopropilic alcohol added to the aqueous phase. They were incubated for 10 min at room temperature and then centrifuged at 1200xg for 10 min at 4ºC. The precipitated RNA, frequently invisible before, formed a pellet similar to a gel at the bottom of the tube.

The supernadant of each sample was carefully discarded and the RNA pellet was washed with 500µL of 75% ethanol using a micropipette, until it detached from the tube. The sample was treated in the vortex at 7500xg for 5 min (4ºC). The supernadant was then discarded and the pellet dried at room temperature for 5-10 min. Before the complete drying of the pellet, 20µL of 0.01% of DEPC-treated water were added to the sample which was incubated for 15 min in room temperature, for a complete dissolution of the pellet.

Total RNA dosage extracted from the samples: The quantification of the total RNA of the three castes of ants *A. s. rubropilosa*, was performed in a spectrophotometer using a quartz cuvette, with a wavelength of 260nm. Ten µL of the total RNA solution were added in 990µL DEPC-treated water (dilution factor 100). The following formula was utilized to determine the absorbancy value:

[RNA µg/mL] = Absorbancy (260nm) x 40 x Dilution Factor

Total RNA electrophoresis: For the preparation of the 1% agarose denaturing gel with formaldehyde, all the material was previously sterilized and DEPC-treated. Initially, 0,6g of agarose was dissolved in 22,8mL of 0.01% DEPC-treated water (in a microwave oven), adding, 12mL of 5X TBE buffer plus 10,8mL of 2,2M formaldehyde afterwards. This solution was placed in an electrophoresis horizontal tank, previously DEPC-treated and the polymerized gel was covered with a running buffer and subjected to a 5 min pre-run at 60V before the sample was applied.

Meanwhile, samples of the glands to be applied in the gel were prepared as follows: 9µL of the sample + 3µL of the sample buffer (50% glycerol, EDTA 1 mM, 0.25% bromophenol-blue and 0.25% xylene-cyanol). These samples were heated for 15 min at 65ºC, with the purpose of linearizing the RNA ribbons and immediately placed in ice to keep the ribbons linearized.

The samples were then placed in the wells and the running took place at 60V, for 45 min. Later, the gel

was stained in a tray containing ethide bromate (12µL at [10mg mL^{-1}], diluted in 1X TBE (200mL) and analyzed using an UV transilluminator for visualization and registry of rRNA bands. These bands were present in larger quantities and displayed low diversity (rRNA 28S and 18S), producing visible bands.

RT-PCT (Reverse transcriptase polymerase chain reaction): For this reaction, the anchored primer T7AP1 which contains a group of 12dTs that allow the curling of the poly tail (A) of the mRNA during the cDNA ribbon generation was used. A combination of two bases (GA) was added to the 3' portion of the anchor primer T7AP1 ('AATACGACTCACTATAGTTTTTTTTTTTGA'), to subdivide the mRNA which is transcript-reverse in the cDNA reaction.

After that, samples containing 9µL total RNA (mandibular gland of soldiers, media and minima workers) and 1µL of anchor primer T7AP1 (5µM) were prepared and heated at 70º C, for 5 min, in a thermocycler and then stored at 4ºC for 5 min, 20µL of the Mix (4µL 5X Buffer, 2µL of 0.1% DTT, 2µL dNTP (205µM) and 1µL of RNAse out) were added to the samples. After 5 min at 4ºC, 1µL of the Superscript III enzyme (reverse transcriptase) was added and left for 5 min at 42ºC, followed by 50 min at 50ºC and then after 15 min at 70ºC, the mRNA were converted into cDNAs (simple ribbons).

Differential display reverse transcriptase polymerase chain reaction (DD-RTPCR): The anchor primer T7AP1 TAMRA was used combined with 3 arbitrary primers ARP2, ARP3, ARP5, totaling 3 possible combinations (C1, C2, C3), as shown in Table 1. The anchor primer was marked with fluorescent pigment (TAMRA fluorophoge), which allowed the visualization of the amplified fragments without the use of radio-isotopes.

The anchor and arbitrary primers were drawn and given by the researchers of the Molecular Morpho-Physiology and Development Laboratory of the Zootechnia and Food Engineering Faculty of USP (São Paulo University)-Pirassununga-SP, as follows:

T7AP1 TAMRA: AATACGACTCACTATAG
ARP2: GCTAGCATGG
ARP3: GACCATTGCA
ARP5: ATGGTAGTCT

The DD-RTPCR technique was performed, as described in Table 2, in triplicate reactions in the cDNAs samples of mandibular glands of workers minima, media and soldiers of ants *A. s. rubropilosa*. All the obtained amplifications were verified in 1.5% agarose gel before following the 6% DDPCR denaturing polyacrilamyde gel protocol.

Table 1: Anchor primer T7AP1 and arbitrary primer ARPs combinations (C1, C2, C3)

Primers	ARP2	ARP3	ARP5
T7AP1	C1	C2	C3

Table 2: DDPCR technique protocol (Home Run)

Home Run 1		
1º MIX		10 cycles
Water	2,71 µL	95ºC 2 min
TP 10X	1,00 µL	
$MgCl_2$ (1,0mM)	0,20 µL	92ºC 15 sec 42ºC 30 sec
DNTP (250µM)	2,00 µL	72ºC 2 min
Arbitrary primer (5µM)	1,33 µL	
T7 marked primer (5µM)	1,33 µL	72ºC 10 min 4ºC
Taq	0,10 µL	
cDNA	1,33 µL	
Total	10 µL	
Home Run 2		
2º MIX		25 cycles
Water	2,44 µL	95ºC 2 min
TP 10X	1,00 µL	
$MgCl_2$ (3,5mM)	1,80 µL	92ºC 15 sec 42ºC 30 sec
DNTP (250µM)	2,00 µL	72ºC 2 min
Arbitrary primer (5µM)	1,33 µL	
T7 marked primer (5µM)	1,33 µL	72ºC 10 min 4ºC 24 hrs
Taq	0,10 µL	
Total	10 µL	
Final Volume	20 µL	

Table 3: Polyacrilamyde stock solution preparation

	Final Volume			
Reagents	30 mL	40 mL	120 mL	240 mL
40% Polyacrilamyde	4,5 mL	6 mL	18 mL	36 mL
Mili Q Water	13,1 mL	17,4 mL	52,2 mL	104,4 mL
Urea	12,6 g	16,8 g	50,4 g	100,8 g
10 X TBE	3 mL	4 mL	12 mL	24 mL
Ammonium Persulfate	200 µL	266 µL	800 µL	1,6 mL
TEMED	20 µL	26 µL	80 µL	160 µL

Denaturing Polyacrilamyde Gel preparation: To perform the vertical electrophoresis in 6% polyacrilamyde gel, the glass walls of the tank were washed with neutral soap, dried with dH_20 and mili-Q water and dried in a stove.

To the polyacrilamyde stock solution (T3) were added the ammonium persulfate and TEMED exactly when the gel was used. The wells were filled up with this solution with the help of a 20mL syringe to avoid bubbles.

The polyacrilamyde gel was polymerized approximately 1h before being used, when a pre-run of 30 min at 1500V took place.

Polyacrilamyde gel sample preparation: The volume 1, 5 µL of loading buffer (according to Table 4) and

Table 4: Loading buffer preparation

Reagents	Quantities
Formamide	1 mL
1M EDTA	21 µL
Mili Q Water	32 µL
Xylene cyanol	Enough to stain the solution

8 µL of PCR were added to the samples, which are the products' reaction of the triplicated amplification of the mandibular glands of the three castes of *A. s. rubropilosa*). Later, the samples were denatured at 95ºC for 2 min and placed in ice for application in the polyacrilamyde gel.

Drying and visualization of the 6% DDPCR polyacrilamyde gel: After running for approximately 3h and 30 min, the gel was dried in a stove at 60ºC. After 1 hour it was washed with Mili-Q Water for a complete urea removal. After it was dried, the gel was visualized with fluorescence scanner FLA3000G (Fuji Film, Tokyo-Japão).

Amplified gene fragments analysis: Differentially amplified fragments were selected, isolated from the DD-RTPCR gels and reamplified with the use of a combination of specific primers. Later, the fragments were cloned using pGEM-T Kit (Promega) and sequenced in the Synchroton Light National Laboratory-Structural Molecular Biology Center, Campinas-SP, Brazil.

The obtained sequences were compared to other in the GenBank using BLAST software[12].

RESULTS AND DISCUSSION

The quantitative evaluation of the total RNA, of mandibular glands of individuals of the three castes of *Atta sexdens rubropilosa*, showed variations among the castes since the soldiers presented 400µg mL^{-1}, the media workers 348µg mL^{-1} and the minima workers 380µg mL^{-1}. As well, it was verified that in gel the RNA was kept intact in the glands of the individuals of the three castes, where the two bands of the rRNA were highlighted (28S e 18S) (Fig. 2).

To obtain a subdivision of the mRNA population, a combination of 2 nitrogenated bases (GA) were added to the 3' portion of the anchor primer T7AP1 in the reverse transcription reaction which produces the cDNAs of the mandibular glands of the 3 castes of *A. s. rubropilosa* (Fig. 3).

The DDPCR gel band profiles showed the three combinations (C1, C2 e C3) proposed herein through the presence of gene fragments (Fig. 4).

For comparison purposes the gene fragments obtained were classified according to the intensity of

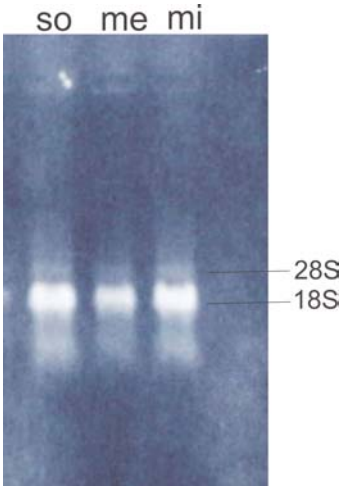

Fig. 2: Result of total RNA extraction of mandibular glands of soldiers (so), media (me) and minima workers (mi) of *A. s. rubropilosa* in 1% agarose denaturant gel

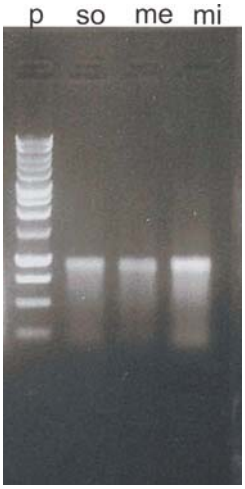

Fig. 3: Result of the RT-PCR reaction of mandibular glands of soldiers (so), media (me) and minima workers (mi) of *A. s. rubropilosa*, where p = DNA pattern, in 1% agarose gel

the gel markings and then divided into three classes: class I, transcripts that express themselves with the same intensity pattern in all combinations; class II or transcripts that express themselves with different intensities and class III or transcripts that are caste-specific (Fig. 4).

Some class III transcripts presented a peculiar expression pattern and among these, were highlighting T1, T2 and T3 present in the glands of soldiers, media and minima workers *A. s. rubr*opilosa, respectively. These gene fragments were also analyzed by cloning and sequencing and their descriptions and similarities were verified with the help of the *GenBank* as shown on Table 5.

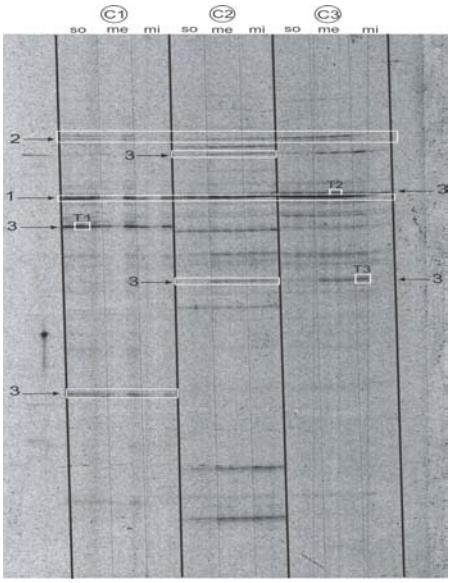

Fig. 4: DD-RTPCR result applied to the mandibular glands of the soldiers (so), media (me) and minima workers (mi) of *A. s. rubropilosa,* using 6% polyacrilamyde gel, where C1 = combination 1; C2 = combination 2; C3 = combination 3; arrow 1 = class I gene fragments; arrow 2 = class II gene fragments; arrow 3 = class III gene fragments (T1 = transcript 1; T2=transcript 2; T3 = transcript 3)

Table 5: Results of similarities of the transcripts T1, T2 e T3, found in the mandibular glands of soldiers, media and minima workers of *A. s. rubropilosa*, respectively, obtained according to the information from the *GenBank*

T1	Hialuronidase Precursor Protein of *Polistes annularis* (gi/5815251/gb/AAD52616.1/)
T2	Phosphate Transporter Protein of *Apis mellifera* (gi/48099304/ref/XP_392582.1/)
T3	Mitochondrial Ribosomic Protein of *Apis mellifera* (gi/66546420/ref/XP_396501.2/)

During the development of an organism, genetics programs are executed followed by changes in the pattern of genes that will be expressed in the proteins of the cells, tissues and organs determining all the vital processes[3].

Changes in the gene expression can be monitored using the Differential Display Reverse Transcriptase Polymerase Chain Reaction technique (DD-RTPCR), which was used for the first time in the mandibular glands of minima, media and soldiers of ants *Atta sexdens rubropilosa*. This technique is being used more each day to isolate and characterize differentially expressed genes in different parts of the same individual, mainly due to the high sensitivity and the requirement for a small RNA quantity for the analysis.

The original DDRT-PCR protocol develops by[3] was modified, including alterations in the primers[6,13], in the radioactive nucleotides[14,15], in the protocol of the electrophoresis gel processing[6,16] and in the purification and cloning of the amplified products (transcripts)[17].

These modifications were adopted for the present study and produced useful results in the analysis of the differential gene expression of the mandibular glands of the three castes of the species studied.

The use of the oligo-dT T7AP1 primer anchored to the "GA" bases permitted the subdivision of the mRNA population found in the mandibular glands of *A. s. rubropilosa* and reduced the number of necessary combinations for each sample.

The three arbitrary primers (ARP2, ARP3, ARP5) used in the study detected the gene expression and demonstrated the presence of different band patterns (transcripts) in the mandibular glands of *A. s. rubropilosa*. This finding, corroborated by[18] also demonstrated that the DD-RTPCR technique can be performed using a small number of primer combinations. However, a larger number of primers can be used to augment the message pool to be evaluated in the gene expression.

In this study with *A. s. rubropilosa*, the band expression profile presented in the DDPCR gel permitted the grouping of products of amplification into three distinctive classes, where the class I gene fragments corresponded to transcripts that were uniformely expressed in all of the combinations proposed here. These genes could probably be the constitutive genes, which are associated with the metabolic processes of the mandibular glands of the species studied. They are also called "house-keeping" and they would codify necessary proteins to the cells which function as RNA polymerase, DNA polymerase, transport proteins and serve the ribosomes requirements necessary to protein synthesis, as well as the RNAs and proteins involved in the splicing process.

In the mandibular glands of *A. s. rubropilosa* the class II gene fragments would be those one that probably would codify products more important in determining life stages of the individual, which could be related to the tasks performed by them in the colony, in the different castes (soldiers: colony defense; media workers: cutting and transporting of vegetation; minima workers: taking care of the offspring and fungus garden). In *Melipona scutellaris* bees the genes belonging to the class II were related to the vitelogenin synthesis, precursor protein in the reproduction processes and with the sterases enzymes responsible for the juvenile hormone hydrolysis, which production would occur in different levels according to the different development stages of the insect[19].

The gene fragments obtained from the glands of *A. s. rubropilosa* are considered as being class III, probably caste-specific. In this study they were denominated transcripts 1, 2 and 3 found respectively in the mandibular glands of soldiers (T1), media workers (T2) and minima workers (T3).

According to the *GenBank* database, the T1 presented similarity to the hialuronidase precursor protein in *P. anullaris* wasps. The hialuronidase is an enzyme that degrades hyaluronana, a glycosaminoglycane formed by regular repetitive sequences of disaccharides. Since the soldiers of *A. s. rubropilosa* perform vegetation cutting and transport sporadically, the T1, present in the mandibular glands of the individuals of this caste, would play a similar role to the hialuronidase enzyme, participating in the processes of cellulose degradation (polysaccharides) present in the vegetation.

The T2, with better markings in the mandibular glands of media workers, presented similarity with the phosphate carrier protein found in *A. mellifera.* This protein catalysis the phosphorylation and dephosphorylation reactions, making it possible for the cells to have control over the activation and de-activation of some enzymes/proteins, by the addition or not of the phosphate group. The T3, present in the minima workers, presented similarity to the mitochondrial ribosomal protein also found in *A. mellifera*, which is synthesized from the nuclear genes in free polysomes, later imported by the mitochondria to compose the rRNA, an essential element in the protein synthesis mechanism, being produced mainly by proteins belonging to the respiratory chain. With that, the T2 as well as the T3 would be considered constitutive genes, or they would be associated with metabolic processes of the mandibular glands of the species studied.

The present study highlighted the occurrence of the differential gene expression between the mandibular glands of three castes of *A. s. rubropilosa*, demonstrating a glandular gene pattern specific to each caste, as well as confirming that the DD-RTPCR technique can be one more tool to be used in gene expression analysis, permitting the establishment of inter-and intra-caste relations in the insects.

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