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Characterisation and expression analysis of UBC9 and UBS27 genes in developing gonads of cicindelids (Coleoptera: Cicindelidae)

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ABSTRACT

Ubiquitin and small ubiquitin-like modifiers (SUMO) are post-translational modifiers essential in a variety of cellular processes, including gametogenesis. SUMO-conjugating enzyme (UBC9) and the ubiquitin ribosomal fusion protein UBS27 have been characterised in several model species. However, their expression in coleopteran remains unstudied. In this study, UBC9 and UBS27 genes have been characterised in the tiger beetle *Cicindela campestris* for the first time. Bioinformatic analysis showed that the Cc-UBC9 gene encoded a 159 amino acid protein with a predicted molecular weight of 18.18 kDa, and the Cc-UBS27 gene encoded a 156 amino acid protein with a predicted molecular weight of 17.71 kDa. Selection analyses carried out in several cicindelid species revealed that both genes were affected by purifying selection. Real time quantitative PCR analysis demonstrated that Cc-UBC9 and Cc-UBS27 were expressed in different tissues. The highest expression on both genes was found in the ovary and testis, and there were differential expression levels between immature and mature stages of testis development.

The expression patterns of Cc-UBC9 and Cc-UBS27 suggest that these genes play important roles in gametogenesis in *C. campestris*. This information is relevant to better understand the reproductive process in cicindelids and the function of ubiquitin and small ubiquitin-related modifier genes in the Coleoptera.

1. Introduction

Cicindelids (tiger beetles), which belong to the suborder Adephaga (Coleoptera), are non-pest species important in ecosystems as predators and are commonly used as bioindicators (Pearson, 1988; Pearson and Cassola, 1992). Tiger beetles constitute a species-rich group with >2500 species (Pearson and Cassola, 2005) that occupy a wide range of habitats around the world (Pearson, 1988; Pearson et al., 1988; Cassola and Pearson, 2000). They have attracted attention of many scientists and therefore they have been studied at different levels (revision in Pearson and Vogler, 2001; Galián et al., 2002; Vogler et al., 2005; López-López et al., 2012, 2013, 2015, 2016). Nevertheless, there is little information on the transcriptome of this group of ecologically relevant beetles to provide information for biological questions such as adaptive evolution, the origin of new species and species richness. At present only two studies has been conducted to identify and characterise genes that might be involved in evolutionary processes in cicindelids, such as reproduction (Rodríguez-García et al., 2015) and immune response genes (Rodríguez-García et al., 2016).

Protein post-translational modifiers are essential in a variety of cellular processes. Ubiquitin is an essential protein present in all tissues, it is a highly conserved protein consisting of 76 amino acids, with only four amino acid substitutions among plants, animals and yeast (Goldstein et al., 1975; Ozkaynak et al., 1987; Jentsch et al., 1991). Two classes of genes encode ubiquitins: polyubiquitins and ubiquitin-fusion genes (Finley et al., 1989). Polyubiquitin genes encodes a precursor protein expressed in a head-to-tail form with several identical tandem repeat units. The two ubiquitin fusion repeats (ubl40 and ubl27) are fused with ribosomal proteins L40 and S27, respectively, at the C-terminus (Baker and Board, 1991; Mezquita et al., 1997; Nenoi et al., 2000). Ubiquitination requires the concatenated action of three enzymes. First, ubiquitin conjugation enzyme (E1) activates the C-terminal Gly residue of ubiquitin in an ATP-dependent process. Second, ubiquitin is transferred to a ubiquitin conjugation enzyme (E2) and finally, in the third step, ubiquitin is linked to a target protein catalysed by a ubiquitin protein ligase enzyme (E3) (Haas et al., 1982; Hershko and Ciechanover, 1998). The ubiquitin system plays important roles in the regulation of cellular processes such as cell division, apoptosis, and in conditions of cell stress. They are also involved in protein degradation, ribosomal synthesis and the immune response, and recent studies have

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demonstrated the role of ubiquitin proteins in reproductive processes (reviewed in Bebington et al., 2001; Pickart and Eddins, 2004).

Additionally, small ubiquitin–like modifiers (SUMO) are also conjugated to proteins. The amino acid similarity between sequences of ubiquitin and SUMO is only approximately 18%, but their protein structure is conserved (Bayer et al., 1998). Furthermore, SUMOylation, similar to ubiquitination, requires three enzymes, an ATP-dependent activating enzymes (E1) and a conjugating enzyme (E2, ubc9), and in some cases, requires specific SUMO ligases (E3). Therefore, SUMO proteins share similar mechanisms with ubiquitin, but there are some differences in their function. SUMO plays an important role in the regulation of transcription, nuclear transport, cell cycle, DNA replication and repair, and unlike ubiquitination, it does not intervene in degradation (Gill, 2004; Johnson, 2004).

Genes that participate in both processes are involved in reproductive function in animals. Ubiquitins are important in gametogenesis (Koken et al., 1996; Roest et al., 1996; Takagi Sawada et al., 1997), especially in spermatogenesis, where E1 and E2 enzymes, as well as ubiquitin ribosomal fusion proteins are implicated (Mitchell et al., 1991; Roest et al., 1996; Shen et al., 2009; Wang et al., 2012a). Furthermore, different studies suggest that SUMOylation also plays an important role in gametogenesis. Expression studies of the UBC9 gene in different organisms showed that it is involved in embryogenesis, gametogenesis and sex modification (Zhang et al., 2010; Wang et al., 2012b; Hu and Chen, 2013).

Many genes involved in the sexual reproduction process evolved rapidly, often as a result of adaptive evolution (Swanson and Vacquier, 2002). Evidence of positive selection has also been found for genes involved in gametogenesis, controlling both spermatogenesis and oogenesis (Civetta et al., 2006; Bauer DuMont et al., 2007). Nevertheless, not all of the reproductive proteins evolved rapidly in some linages (Metz et al., 1998), and there are genes involved in spermatogenesis, as is the case of the t-complex polypeptide 1 gene (Tcp-1), which is subjected to purifying selection in mice (Jansa et al., 2003). The ubiquitin gene family sequences are also subject to strong purifying selection (Nei et al., 2000), which may also be the case for the ubiquitin conjugating enzyme family (E2) (Ying et al., 2009). The study of reproductive genes that do not evolve rapidly could provide valuable information about why others have such high variability (Swanson and Vacquier, 2002).

Ubiquitin proteins have been identified and characterised through the use of genomic or expressed sequence tags (ESTs) in many insects, such as *Spodoptera litura* (Li et al., 2003), *Blattella germanica* (Yu et al., 2004), *Bombyx mandarina* (Chen et al., 2007) and *Musca domestica* (Jin et al., 2008). Nevertheless, there are few studies available in Coleoptera on the function of ubiquitin; only one paper is specifically related to their reproductive function (Yang et al., 2009) in a Polyphagan beetle species. In relation to SUMO, currently there are few studies in insects other than *Drosophila*, where the SUMOylation roles are well studied (reviewed in Smith et al., 2012), and there is still no information on the characterisation of these proteins in Coleoptera. Therefore, this study represents the first effort to characterise these important proteins in Adephagan beetles.

The aim of this work is to identify and characterise genes encoding post-translational modifiers and to analyse their likely involvement in the gametogenesis process in *Cicindela campestris*. The hypothesis postulates that the proteins identified as ubiquitin (UBC9) and SUMO (UBS27) in *C. campestris* are involved in the gametogenesis (spermatogenesis and oogenesis) and therefore a high expression of these genes in gonads and a high evolutionary rate (positive selection) are expected. To test this hypothesis we have performed the following: (1) obtained the full length of Ubiquitin ribosomal S27 (UBS27) and the SUMO conjugating enzyme E2 (UBC9) from *C. campestris*; (2) carried out phylogenetic analysis of UBS27 and UBC9 genes; (3) amplified a region of both genes in seven species of cicindelids and implemented purifying selection analysis; and (4) investigated the expression pattern of both genes at different developmental stages and in different tissues.

2. Material and methods

2.1. ESTs analysis and gene identification

To identify ubiquitin or ubiquitin-like genes, EST databases of *Calomera littoralis*, *Cephalota litorea* (Rodríguez-García et al., 2015) and *Cicindela campestris* (Theodorides et al., 2002) were explored. Libraries were assembled and analysed as we previously described (Rodríguez-García et al., 2015). Contigs were annotated via Blast2go v2.5.0 software (Conesa et al., 2005), and nine genes predicted to be ubiquitin or ubiquitin-like genes were identified in the libraries. Two genes annotated as the ubiquitin conjugating enzyme E2 from the *C. littoralis* database and ribosomal ubiquitin from the *C. campestris* library were selected for further analyses.

2.2. Sample preparation

Cicindela campestris adults were collected from Laguna del Arquillo (Albacete, Spain). The head, thorax, abdomen and gonads were harvested and submerged in RNAlater (Qiagen, Crawley, UK) and stored at −20 °C until RNA extraction.

The developmental stages of the gonads were discerned based on the descriptions of Paarmann (1976) for the carabid beetle *Pogonus chalceus* as follows: i) immature females, gonads containing oocytes from the undifferentiated stage to oocytes in the previtellogenesis stage; ii) mature females, containing oocytes in the vitellogenesis stage to mature eggs; iii) immature males, with undifferentiated accessory glands and very small testis that display a transparent white colour; and iv) mature males, testes full of spermatozoa with a characteristic pearl white colour.

2.3. Rapid amplification of cDNA ends (RACE)

Total RNA for the RACE reaction was extracted from an adult of *C. campestris* using TRIzol reagent (Life Technologies) following the manufacturer's protocol, and isolated RNA was treated using a TURBO DNA-free Kit (Ambion, Life Technologies) to remove DNA contamination. The 5′ and 3′ RACE were produced using the ClontechSMARTer RACE cDNA amplification Kit (Takara Bio) following the manufacturer's instructions. Gene-specific primers for 5′ and 3′ RACE were designed using as reference the original ESTs (Table 1). The synthesis of first cDNA strand was performed following PCR conditions: 30 cycles at 94 °C for 30 s, 67 °C for 30 s and 72 °C for 3 min. PCR products were sequenced in triplicate by SAI at the University of Murcia, Spain to obtain the complete cDNA sequences.

Table 1
Primer sequence used for RACE-PCR amplification (RACE), phylogenetic and selection analysis in cicindelids (RC) and real time-quantitative PCR (RT-qPCR) of the Cc-UBC9 and Cc-UBS27 genes. Arginine kinase primers used as endogenous controls in the PCR are also described.

Gene	Primer sequence
Cc-UBS27	RACE_F1 5′-TTCGCCTGGGCATTCACGTCTCAAT-3′
	RACE_R1 5′-AAGGAGTCGACTCTGCACTTGGTG-3′
	RC_F1 5′-CCTGGGCATTCACGTCTCAA-3′
	RC_R1 5′-TCGTGAAGACTTTGACGGGT-3′
	RT-qPCR_F1 5′-CGAAGTCGCAGCACCAAGT-3′
	RT-qPCR_R1–5′-CCTGACCAGCAGCGACTGAT-3′
Cc-UBC9	RACE_F1 5′-CTGCAGCACGATTAGCCGAGGAACT-3′
	RACE_R1 5′-GACGGGTACACGTTGGGGTGAAACA-3′
	RC_F1 5′ -ACACGAAATAATTCTCGCGCC-3′
	RC_R1 5′-TCCTGATGAGACATGACGCG-3′
	RT-qPCR_F1 5′-CGATTAGCCGAGGAACGTAAA-3′
	RT-qPCR_R1 5′-TCCCATGGAGTGCCTTTCTT-3′
Arg kin	F-5′-CTCGTGTGGTGCAACGAAGA-3′
	R-5′-GGTGGCTGAACGGGACTCT-3′