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Differential expression of cytochrome *c* oxidase subunit III gene in castes of the termite *Reticulitermes santonensis*

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Abstract

Social insects such as termites live in colonies in which cooperation is assumed by all individuals developing into castes to which specific tasks are allocated. Little has been reported about molecular aspects underlying termite caste-specific gene expression. Genetic regulation has recently been hypothesized to govern caste-specific traits and physiology in social insects. Cytochrome *c* oxidase (COX) has been shown to be an interesting candidate for expression study in insects. We used the cytochrome *c* oxidase subunit III gene (COXIII) that was cloned from mRNA in a lower termite, *Reticulitermes santonensis* De Feytaud (Isoptera; Rhinotermitidae). The full-length cDNA encodes a protein of 262 amino acids that shows high degree of homology with other insects COXIIIs. Reverse transcriptase-PCR and real-time PCR were performed to compare gene expression between larvae, workers, nymphs and soldiers. Analyses performed on head cDNAs revealed that COXIII is differentially expressed between castes. The level of COXIII is caste-regulated with an increase in workers (~1.9-fold) and nymphs (~2.8-fold) and a decrease in soldiers (0.8-fold) compared to the expression level in larvae (1.0-fold). These results may emphasize the physiological importance of COX in the termite brain at different developmental stages.

Keywords: Reticulitermes; *R. flavipes*; Real-time PCR; Mitochondrial DNA; COXIII

1. Introduction

Evolutionary convergence has resulted in the development of similar caste systems in Hymenoptera (bees, wasps and ants) and Isoptera (termites) (Wilson, 1971). The colony efficiency is based on labour division leading to the allocation of specific tasks and behavioural specializations between different castes. In termites of the genus *Reticulitermes,* individuals engage into specific pathways through post-embryonic development. The initial developmental branches split after two larval stages where larvae can evolve into workers (neutral path) or into nymphs (reproductive path). Workers can develop in three ways: stay as workers, become soldiers through two successive moults, or evolve into supplementary reproducers in some cases. Nymphs follow the reproductive line to develop into winged and eyed alates or eventually redifferentiate into workers (Keller, 1993; Roisin and Lenz, 1999). Genetic regulation has recently been hypothesized to govern caste-differentiation and characterize caste-specific biology after differentiation in social insects (Miura, 2005). Although the termite organization at large has been extensively documented, the physiology and molecular mechanisms underlying caste differentiation are poorly understood.

Endogenous enzymes are known to play important roles in metabolic pathways in the brain (Byers et al., 1981; Levin et al., 1992). Among those, cytochrome *c* oxidase (COX) is the terminal enzyme of the electron chain transport, reducing oxygen to water, producing ATP via oxidative phosphorylation and thus allowing energy and oxygen utilization by cells. The activity of this enzyme is linked to the metabolic demand in the brain and reflects changes in neuronal activity; as a consequence, COX is used as an endogenous marker for neuronal activity (Wong-Riley, 1989). The enzyme is constituted by a

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variable number of subunits coded by the nuclear genome and three subunits coded by the mitochondrial genome that constitute the catalytic core (COXI, COXII and COXIII) (Wikstrom and Casey, 1985; Capaldi, 1990). COXI contains the phosphorylation site, COXII interacts with cytochrome *c* in the electron transfer and COXIII is notably involved in the transmembrane proton pumping mechanism as well as protecting COX active sites (Namslauer and Brzezinski, 2004; Hosler, 2004).

In insects, differential expression of COX subunits has been found to be correlated with developmental modifications. In *Apis mellifera*, distinct larval nourishment influences the developmental differentiation between queen and worker honeybees. Queen larvae have a higher respiration rate which is reflected by an over-expression of the COXI subunit (Corona et al., 1999). Likewise, in the moth *Agrius convolvuli* COXI is highly expressed in the brain during diapause termination due to high oxygen utilization (Uno et al., 2004). In the ant species *Solenopsis invicta*, COXII has been reported to be expressed at high levels in dealate queens in association with the reproductive system development (Tian et al., 2004). These findings suggest that the expression of COX subunit genes reflect variations in COX activity which might vary during insect development. Thus, COX mitochondrial subunits seem to be interesting candidates for gene-expression studies. In social insects, brain morphological changes have been found to be age-related and task-dependent. Specific brain regions increase in size in adult ant and bee workers, as a consequence of adaptive behaviours (Withers et al., 1995; Gronenberg et al., 1996). Social organization in eusocial insects has been shown to be governed by gene-regulation; in the ant species *S. invicta,* differential expression of genes encoding a pheromone binding protein and cytochrome P450 allows the regulation of queen number by workers (Krieger and Ross, 2002; Liu and Zhang, 2004; Miura, 2005). Like ants, termite individuals develop into specific morphs, display caste-specific behaviours and may undergo morphological and physiological brain modifications upon the age.

In this study, we characterized the full-length COXIII-gene in a member of the order Isoptera for the first time and we investigated its developmental related variations in the termite species *Reticulitermes santonensis*. Reverse transcription PCR was used to examine whether COXIII in the head of different castes is caste-regulated. Real-time PCR was used to specify differences in gene expression between castes. The expression level of COXIII was found to be significantly different between castes with an up-regulation in nymph and worker heads compared to low expression level in soldier heads.

2. Material and methods

2.1. Insects

R. santonensis termites samples were supplied by CTBA (Centre Technique du Bois et de l'Ameublement, Bordeaux, France). Recent genetic evidence shows however that this termite species is most likely synonymous to *R. flavipes* (Austin et al., 2002).

Termites were raised in Petri dishes with wet filter paper and pieces of wood as complementary food sources (*Pinus* sp.) in a climate chamber maintained under constant conditions of 21 °C, 40% relative humidity and 18:6 h light: dark photoperiod. Larvae, nymphs (last stages preceding the alate stage), workers and soldiers were collected and immediately immersed in liquid nitrogen and stored at −80 °C until use.

2.2. Molecular cloning of COXIII gene

Whole body RNA was extracted from 100 frozen workers. Frozen tissues were crushed in a sterile mortar at liquid nitrogen temperature. Tissue powder was homogenized in the Trizol® reagent (Invitrogen™ Life technologies). Total RNA was stored at −20 °C until further experiments. Messenger RNA was isolated from total RNA following the detailed instructions provided in the Streptavidin Magnesphere® Paramagnetic Particles (SA-PMPs) Separation Products Protocol (Promega). For single strand cDNA synthesis, mRNA was transcribed using a M-MLV transcriptase as described by the manufacturer (Promega).

Specific cDNAs were amplified by polymerase chain reaction (PCR) in a thermocycler GeneAmp PCR system 9600. For cloning of COXIII, 10 ng of whole body cDNA from workers served as template in PCR reactions. Degenerated PCR primers were designed from conserved motifs of cytochrome *c* oxidase subunits III from different insect species. The oligonucleotide sense primer CytoSant1-s 5′-CAYCAYTTYGGNTTYGARGCNGC-3′ was designed against the conserved C-terminal motif HHFGFEAA. This primer in combination with an oligo-(dT) allowed us to clone the 3′ region of cDNA encoding COXIII from *R. santonensis*. Then, a specific antisense primer CytoSant2-as 5′-GCTGCTGCTTCAAATC CAA-3′ was designed based on the identified 3′ core of COXIII cDNA. Using CytoSant2-as in combination with a degenerated sense primer (CytoSant2-s: 5′-ARNTGGTTYCAYCART AY-3′) designed against the conserved 5′ KWFHQY motif, we amplified a 650 bp cDNA fragment that corresponded to the COXIII amino acid residues 34–239. Successful PCR programs were as follows: 94 °C during 4 min, then 36 cycles at 94 °C for 30 s, 50 °C for 40 s, and 72 °C for 1 min. A final amplification step was performed at 72 °C for 7 min.

The 5′-end of COXIII-encoding cDNA was obtained using the 5′ RACE SMART™ Technology (BD Biosciences). Two specific antisense primers CytoSantRACE1-as 5′-GTACTGATGAA ACCACTT-3′ corresponding to the amino acid motif YQHFWK and CytoSantRACE2-as 5′-GCTGTTGTTGTACTGATG-3′ corresponding to the amino acid motif SNNYQH were designed based on the 650 bp-long cDNA fragment. Double-stranded cDNA was synthesized using the Reverse Transcriptase and 5′ RACE

SMART™ Oligo Adaptators according to the detailed instructions provided in the SMART™ Technology kit. Double-stranded cDNA was amplified by PCR using cycling conditions described by the manufacturer. Successful RACE reactions were performed using CytoSantRACE2-as under the following conditions: 25 cycles at 94 °C for 30 s, 68 °C −0.5 °C/cycle for 30 s, 72 °C for 2 min then 19 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min then 72 °C for 7 min.

The PCR and RACE products were analyzed by electrophoresis on 1% agarose gel in 1 × TAE buffer and visualized under UV-light after staining with ethidium bromide. The PCR products of interest were purified using the Geneclean protocol (Q-biogene) and cloned using the pGEM®-T Easy vector system (Promega) and the One Shot® *Escherichia coli* TOP10 competent cells protocol (Invitrogen™ life technologies). Sequencing was carried out on a capillary ABI 3100 sequencer instrument (Perkin Elmer).

2.3. Reverse transcriptase PCR experiments

Total RNA samples were isolated from 100 heads (excluding antennae) of workers, larvae, nymphs and soldiers dissected from frozen whole body individuals and prepared using the Trizol® reagent. Fifty nanograms of total RNAs were used as templates for reverse transcription and PCR amplification in a single-enzyme approach using the reverse transcriptase Taq polymerase (Stratagene). Experiments were performed using specific COXIII primers (10 μM) (5′-AGTGGTTTCATCAGTAC-3′ and 5′-GCTGCTGCTTCAAAT CCAA-3′) in the automated programme: 50 °C for 1 h, 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 68 °C for 1 min and 68 °C for 2 min. For control RT–PCR, Isopteran actin primers (10 μM) were used: Actin-s: 5′-CTCAGGCGATGGTGTCTC-3′ and Actin-as 5′-GGGGTACATGGTGGTGC-3′. The amplified fragments were separated on 1% agarose gel by electrophoresis.

2.4. Real-time PCR experiments

Real-Time PCR was performed to quantify relative caste-associated gene expression. cDNA templates for real-time PCR were synthesized from 1 μg of total head RNA from worker, larvae, nymph and soldier using the Reverse Transcriptase Stratascript kit (Stratagene). Primers for use in real-time PCR were designed to provide a 120 bp-long PCR product (CytoSant3-s: 5′-GATCAACCTTCTTCATAGCC-3′; CytoSant2-as 5′-GCTGCTGC TTCAAATCCAA-3′) and actin primers (Actin-s and Actin-as) were used as reference gene in all experiments. Reactions were run for 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 1 min at 57 °C, 1 min at 72 °C, followed by a dissociation curve program from 57 to 95 °C with a heating rate of 0.1 °C and a continuous fluorescence acquisition.

Samples were triplicated in all experiments on 96-well plates with optical sealing tape and SYBR® Green I fluorescence was monitored in a Lightcycler instrument M × 3000PA (SMART Cycler II, Stratagene). Before experiments, the production of single PCR products and absence of primer-dimers was confirmed by viewing PCR products under UV-light after electrophoresis on 1% agarose gel in 1 × TAE buffer and staining in ethidium bromide. PCR products were cloned and sequenced in order to confirm for specific amplification.

Expression levels were determined using the formula $(1+E)^{-\Delta\Delta Ct}$ proposed by Liu and Saint (2002) after Livak and Schmittgen (2001) in which E is the amplification efficiency and Ct is the cycle number necessary to reach a defined fluorescence threshold. The amplification efficiency value was determined using the formula $E = [(R_a/R_b)^{1/(Ct,a-Ct,b)}] - 1$; R_a and R_b are fluorescence intensities at arbitrary thresholds a and b, respectively, and Ct(a) and Ct(b) are the corresponding cycles (Liu and Saint, 2002). The relative amounts of COXIII and Actin (reference gene) were determined for each sample at the threshold value (Ct) and the ΔCt was calculated. Normalized amounts (COXIII/Actin differences) are deduced from those of a calibrator sample (larvae) to obtain the ΔΔCt expression values. Experiments with triplicated samples were repeated to generate a set of eight Ct values for each sample. Data were analyzed using a one-way analysis of variance (ANOVA). COXIII expression levels were transformed using Box-Cox transformation prior to ANOVA analysis to ensure normality and equality of variance. The Tukey's least significant difference test was used to separate means (family error rate = 0.05). Statistical analyses were conducted using Minitab™ (release 13.20).

3. Results

3.1. Cloning of cDNA encoding COXIII gene (*COXIIIRsant*)

We amplified a 200 bp cDNA fragment of the COXIII gene comprising a non-coding poly A tail of about 100 bp (Fig. 1). Blast analysis of this coding region displayed about 25% identity with nucleotide sequences encoding the *C*-terminal motif of COXIII from different insect species: *Perga condei* (Hymenoptera), *Periplaneta fuliginosa* (Blattaria), *Triatoma dimidiata* (Hemiptera). The deduced amino acid sequence corresponded to the *C*-terminal region of COXIII: WYWHF-D–WLFLY–Y. As shown in Fig. 1, the total sequence of COXIII gene in *R. santonensis* (*COXIIIRsant*) consists of 819 bp (GenBank acc. number DQ001073) that encodes a protein with 262 amino acid residues.

The full-length COXIII deduced amino acid sequence of *R. santonensis* was compared with others using Blast analyses. It displays also highly conserved internal amino acid motifs: 58-W-RD-61, 87-FI-SE—F-SFFW-100 and 201-T-FHG-HV–G-212 (Fig. 2) with COXIII-proteins from other insect species. Distance pairwise comparison showed that *COXIIIRsant* shares 85% identity with the blattarian *P. fuliginosa* COXIII and about 75–80% identity

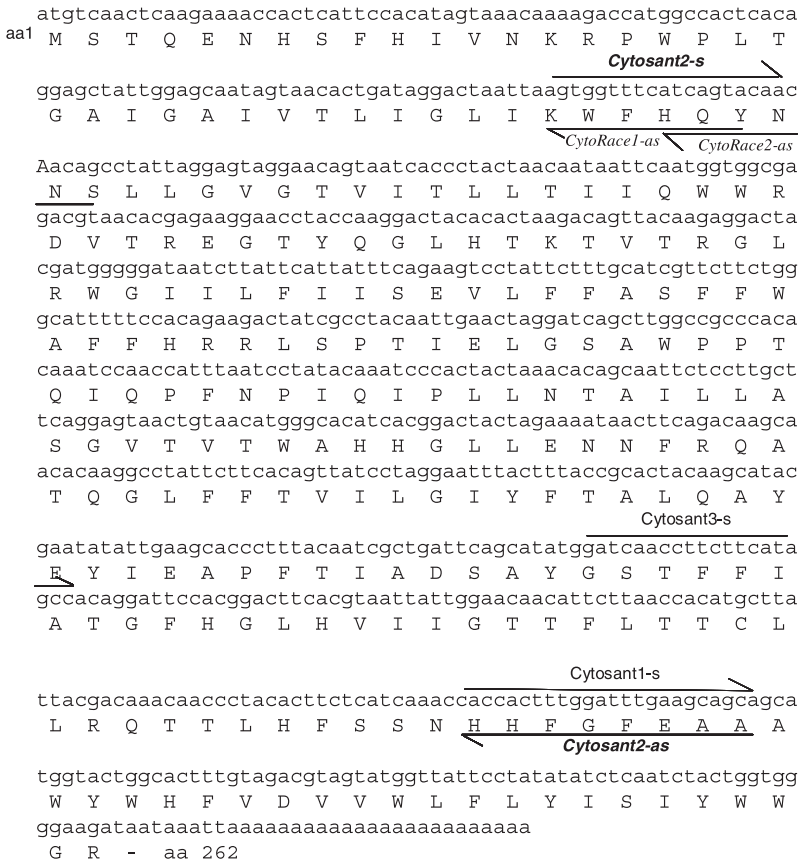

Fig. 1. cDNA and deduced amino acid sequence of COXIII identified from *Reticulitermes santonensis*. The sequence is 819 bp long encoding a protein of 262 amino acid residues. The arrows indicate the primer sites for PCR amplification designed to amplify the 3′ region (primer: Cytosant-1s), the central region (primers: Cytosant-2s and Cytosant2-as) and the 5′ region (CytoRACE1-as and CytoRACE2-as), and the primer designed for real-time PCR experiments (Cytosant3-s).

with COXIII from *Drosophila melanogaster* and other dipteran species. About 50–70% identity is found between *COXIIIRsant* and COXIII from different insect orders: 77% identity with the orthopteran *Locusta migratoria* COXIII, 70–75% identity with lepidopteran COXIIIs (*Bombyx mori* and *Ostrinia nubilalis*), 65–70% identity with coleopteran COXIIIs (*Tribolium castaneum* and *Crioceris duodecimpunctata)* and 50–70% with the hemipteran COXIII (*Schizaphis graminum)*. Only 50% identity is found between the termite *COXIIIRsant* and the COXIII from another social insect, the hymenopteran *A. mellifera*.

3.2. Reverse transcriptase PCR experiments

COXIII was expressed in all castes at different level (Fig. 3, left). The COXIII gene was found to be expressed at higher levels in nymphs and workers compared to both soldiers and larvae. Experiments performed using the control gene (Actin) revealed no difference between castes in expression levels (Fig. 3, right).

3.3. Real-time PCR experiments

Results from real-time PCR experiments showed that the expression of COXIII is different between castes. For each gene (COXIII and Actin), a single peak on the dissociation curve was observed showing that specific PCR products were amplified. Real-time-PCR products of expected size were visualized on agarose gel (data not shown). Table 1 shows the COXIII relative expression levels calculated using the $(1+E)^{-\Delta\Delta Ct}$ derivation method for each experiment. The amplification efficiencies were calculated from each amplification curve in a given experiment. No differences in efficiency were observed between target and reference genes. Mean expression levels of COXIII between workers, nymphs, soldiers and larvae were statistically different ($F = 30.59$, $p = 0.000$). Using larvae for calibration, the COXIII gene was found to be up-regulated in workers and nymphs but down-regulated in soldiers. COXIII was found to be expressed at higher levels in nymphs (2.77 ± 0.42; group a) and workers (1.89 ± 0.34; group a) compared to larvae (1.00 ± 0.00, calibrator; group b) and soldiers (0.80 ± 0.06; group c) (Fig. 4).

4. Discussion

We characterized the full-length COXIII sequence from cDNA of a member of the order Isoptera (family: Rhinotermitidae). The cDNA sequence of COXIII and its deduced amino acid sequence reflect a high degree of

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Apis mellifera                -MKKN---FPFHMVTNSPWPIILSFSFMNTLISTVIWIY-SSISMFMILNFINSILIMMLWFRDIIRESTFQGMHSMFITNFLKFSMILFILSELMFFISF 96
Melipona bicolor              -MKKN---FPFFLVKNSPRPIIMSFNLMNFLISIIMWIY-MKKYILLLMMMSNLILINSLWFRDIIREKNIP-SKSYYIKLMLKFSMIMFIISELFFFISF 95
Schizaphis graminum           MMKIN---QPYFILTLSPWPILMAFNTFNLMISNIMILN-FKMNLMSMLNLIMIISISILWWRDVIRESTFQGNHNFFIMNLIKFSMILFIISEMFLFISF 97
Tribolium castaneum           --MMN-KNHPFHLVDVSPWPIMGSISALITMSGIVKWFH-SFNSTLLFIGLTSMSLIMFQWWRDISRESTFQGHHTFIVTMGLRWGMILFITSEVFFFISF 97
Crioceris duodecimpunctata    -MSMK-QNHPFHLVDQSPWPLLSSLNAMAIMMGLIKWFH-LFELNLLLMSMISMSFIMFLWWRDVTREGTFFGYHTLKVTLGLRWGMILFIVSEIFFFISF 98
Drosophila melanogaster       -MSTH SNHPFHLVDYSPWPLTGAIGAMTTVSGMVKWFH-QYDISLFVLGNIITILTVYQWWRDVSREGTYQGLHTYAVTIGLRWGMILFILSEVLFFVSF 98
Bactrocera oleae              -MSTH-SNHPFHLVDYSPWPLTGAIGAMTSVSGLVKWFH-QYDISLFVLGNMITILTVYQWWRDVSREGTFQGLHTLPVTLGLRWGMILFILSEVLFFVSF 98
Anopheles gambiae             -MSTH-ANHPFHLVDYSPWPLTGAIGAMTTVSGLVQWFH-QYTMTLFILGNVITILTMYQWWRDISREGTFQGLHTFPVTIGLRWGMILFIVSEVFFFISF 98
Periplaneta fuliginosa        -MTSH-ANHPFHLVDQSPWPLTGAIGAMVTMTGMIKWFH-QYNQELLMIGMMIMLLTMIQWWRDIVREGTYQGLHTKMVTKGLRWGMILFIISEVFFFISF 98
Reticulitermes santonensis    -MSTQ-ENHSFHIVNKRPWPLTGAIGAIVTLIGLIKWFH-QYNNSLLGVGTVITLLTIIQWWRDVTREGTYQGLHTKTVTRGLRWGIILFIISEVLFFASF 98
Locusta migratoria            MLTNN-NNHPFHMVDYSPWPLTGAIGAMILTSGMTKWFH-TFNMNLLMIGMTVIVLTMIQWWRDVVREGTFQGLHTKLVSKGLRWGMILFIASEVLFFASF 99
Bombyx mori                   -MKMN-NNHPFHLVDYSPWPFTGAIGVLTLMTGMIKWFH-NFNFNLIIMGYIITLLTAYQWWRDISREGTYQGKHTILVNKGLRWGMILFIISEIFFFISF 98
Ostrinia nubilalis            -MKNNFYSHPYHLVDYSPWPITGAIGVLTLVTGMVEWFH-NFNMNLLMLGYLIIILTMYQWWRDVSREGTFQGKHTILVTKGLRWGMILFIISEIFFFISF 99
Thrips imaginis               ---MP--KIPFHLVTRSPWPILTSFQLFNTVFSTAKNFNGHLSNTMFFINLILLVLISWSWWRDVVRESTFEGMHTKEVLSGLKMGMILFITSEVFFFLSF 96
Bemisia tabaci                -MMMD--NHFFHLVKYSPWPILIAFNLTNTIMSLIKLILVSPSLLFFFFFFFTTIMIMFQWWRDIIRESLYEGSRSVKINYMISSGMIFFIVSEIWLFISF 98
Trialeurodes vaporariorum     -MMFN--NHFFHLVSSSPWPIVVSSNIFNVGVGLIWMIGG--EFFFFFFFTFLMLIMSFQWWRDVVYEGVYEGSHTCSITAGLDMGVVGFIISEICFFFSF 96
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Apis mellifera                FWTFFHSSISPNIEINMTWPPKNIKFFNPMEIPLLNSFILVSSGFTVTLSHYYLIINNLKLSKSYLLLTILLGIYFTILQTIEYSNSFFCFNDSIYGSIF 196
Melipona bicolor              FWAFFHSSISPSIEINMMWPPKMIKIFNYTEIPLLNTFTLITSGFFITLSHLYFLNNNFSKSIKMLLFTILMGLYFSMIQMIEYKNSYFCINDSIYGSIF 195
Schizaphis graminum           FWNFLHNSLAPSIELGLNWPPKNIMFFNPLLIPLLNTIILLTSSFTITLAHLYLLNNSKKKTIIFMNLTIILSIYFLMLQMLEYKQATFTFSDSIFGSSF 197
Tribolium castaneum           FWGFFHNSLAPAVDIGMTWPPKGIETFNPIQIPLLNTLILLTSGLTVTWAHHSLMENNFKQATHGLLLTVILGFYFTLLQGYEYFESSFSIADAAYGSSF 197
Crioceris duodecimpunctata    FWGFFHSSLAPSIEIGLNWPPKSIETFNPMEIPLLNTLILLTSGLTVTWAHHSLMENNYTQTFQGLFLTVLLGIYFTILQAYEYMEAPFTIADSVYGSSF 198
Drosophila melanogaster       FWAFFHSSLSPAIELGASWPPMGIISFNPFQIPLLNTAILLASGVTVTWAHHSLMENNHSQTTQGLFFTVLLGIYFTILQAYEYIEAPFTIADSIYGSTF 198
Bactrocera oleae              FWAFFHSSLSPAIELGAMWPPAGIQPFNPFQIPLLNTAILLSSGVTVTWAHHSLMEGNHSQATQGLFFTVLLGIYFTILQAYEYIEAPFTIADSVYGSTF 198
Anopheles gambiae             FWAFFHSSLSPTIELGMTWPPVGIAAFNPFQIPLLNTAILLASGVTVTWAHHALMEGNHSQATQGLFFTIVLGVYFTILQAYEYIEAPFTIADAVYGSTF 198
Periplaneta fuliginosa        FWAFFHSSLSPTIDIGSLWPPMGIYPFNPLQIPLLNTAILLASGVTVTWAHHGLLENNYSQALQGLFFTVILGIYFTVLQAYEYMEAPFTIADSVYGSTF 198
Reticulitermes santonensis    FWAFFHRRLSPTIELGSAWPPTQIQPFNPIQIPLLNTAILLASGVTVTWAHHGLLENNFRQATQGLFFTVILGIYFTALQAYEYIEAPFTIADSAYGSTF 198
Locusta migratoria            FWAFFNSSLAPTIELGMKWPPMGIQPFNPIQIPLLNTAILLASGVTITWAHHSIMECNHSQALQGLFFTVMLGFYFTLLQMYEYWEAPFTIADAVYGSTF 199
Bombyx mori                   FWAFFHSSLSPNIEIGSMWPPSSITPFNPFQIPLLNTIILISSGVTVTWAHHSLMENNFSQTKQSLFLTILLGFYFTILQAYEYMEASFTIADSIYGSTF 198
Ostrinia nubilalis            FWAFFHSSLSPNIEIGATWPPTNIVPFNPFQIPLLNTIILITSGVTVTWAHHAIMENNHSQMTQGLFLTIILGIYFTFLQAYEYLEAPFTIADSIYGSTF 199
Thrips imaginis               FWAFFHSCLSPDIFIGQNWPCKGIKSFNPMGIPLMNTMILLSSGITLTAAHHFIMTGNKKECNKFLFLTIILGVYFTILQYIEYKEASFTIADSIYGSTF 196
Bemisia tabaci                FWMYFYYSLSPDMTTGSIWPPYLIIQMSFMDIPLLNTLILLMSGFLITWAHYDLTMNNFKNSKTSLLMSILLGTYFLMIQGYEYLNSNFSFNDSVFGNSF 198
Trialeurodes vaporariorum     FWMFFYFSLSPDFILGFTWPPLGVFSVSFLGVPFLNTLILLSSGFFITLSHLNLVSRFSAKFFYSYFFGLALGGYFLLVQFYEYYNLSYDFSDGSFGCAF 196
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Apis mellifera                FMATGFHGLHVLIGSIFLLISLYRMMNIHFSNMHNMNFELAIWYWHFVDVIWLFLYTFIYLLI--- 259
Melipona bicolor              FLSTSFHGIHVIIGTFMLMTSYIRMYNKHFSLIHHINFELSIWYWHFVDVIWLFLYLFYYTLI--- 258
Schizaphis graminum           YMATGFHGLHVIIGTIFLIVNLMRMINLHFSHIHHISFELGAWYWHFIDIIWLFLYMTFYWWNN-- 261
Tribolium castaneum           FMATGFHGLHVIIGTTFLLVCLIRHLMITFLKFTHFGFEAAAWYWHFVDVVWLFLYISVYWWGS-- 261
Crioceris duodecimpunctata    FMATGFHGLHVIIGTTFLLTCLIRHFYGHFSSIHHFGFEAAAWYWHFVDVVWLFLYISIYWWGS-- 262
Drosophila melanogaster       FMATGFHGIHVLIGTTFLLVCLLRHLNNHFSKNHHFGFEAAAWYWHFVDVVWLFLYITIYWWGG-- 262
Bactrocera oleae              FMATGFHGIHVLIGTSFLLVCLIRHLNNHFSKTHHFGFEAAAWYWHFVDIVWLFLYISIYWWGG-- 262
Anopheles gambiae             YMATGFHGLHVLIGTTFLLICFLRHINYHFSKNHHFGFEAAAWYWHFVDVVWLFLYITIYWWGS-- 262
Periplaneta fuliginosa        FIATGFHGLHVIIGTTFLLTCMLRHLILHFSSNHHFGFEAAAWYWHFVDVVWLFLYISIYWWGS-- 262
Reticulitermes santonensis    FIATGFHGLHVIIGTTFLTTCLLRQTTLHFSSNHHFGFEAAAWYWHFVDVVWLFLYISIYWWGR-- 262
Locusta migratoria            FVATGFHGLHVIIGTTFLLTCLIRHMMNQFSSNHHFGFEAAAWYWHFVDVVWLFLYLSIYWWGS-- 263
Bombyx mori                   FMATGFHGIHVMIGTLFLLICYIRHLNNHFSKNHHFGFEAAAWYWHFVDVVWLFLYISIYWWGN-- 262
Ostrinia nubilalis            FMATGFHGLHVMIGTMFLLICLIRHINNQFSNNHHFGFEAAAWYWHFVDVVWLFLYISIYWWGN-- 263
Thrips imaginis               FMATGFHGIHVIIGTIFLSVCFARSMKNHFSSNHHFGFEAAAWYWHFVDVVWLFLYIFIYWLGK-- 260
Bemisia tabaci                FILTGFHGLHVMIGLIFLITMLNQLSMKTFSLVNLYGFEYSIWYWHFVDIVWLFLYLSIYWWGM-- 262
Trialeurodes vaporariorum     YILTGFHGFHVLLGFVFLFINFIRFYIGHLSTFFVSGFDYSVWYWHFVDVVWLFLYLFVYWWGGCS 262
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Fig. 2. Multiple alignment of deduced amino acid sequence from *Reticulitermes santonensis* COXIII (GenBank acc. number AAY17514) and other published COXIII sequences, *Apis mellifera* (NP008087), *Melipona bicolor* (NP775712), *Schizaphis graminum* (YP073301), *Tribolium* castaneum (NP203160), *Crioceris duodecimpunctata* (NP569065), *Drosophila melanogaster* (CAB91056), *Bactrocera oleae* (NP957747), *Anopheles gambiae* (NP008074), *Periplaneta fuliginosa* (YP_054488), *Locusta migratoria* (NP007295), *Bombyx mori* (NP059480), *Ostrinia nubilalis* (NP563588), *Thrips imaginis* (AAK55291), *Bemisia tabaci* (AAU14203), *Trialeurodes vaporarorium* (YP086819). Conserved amino acids in all COXIIIs are asterisked. The symbols . and : indicate partially conserved residues.

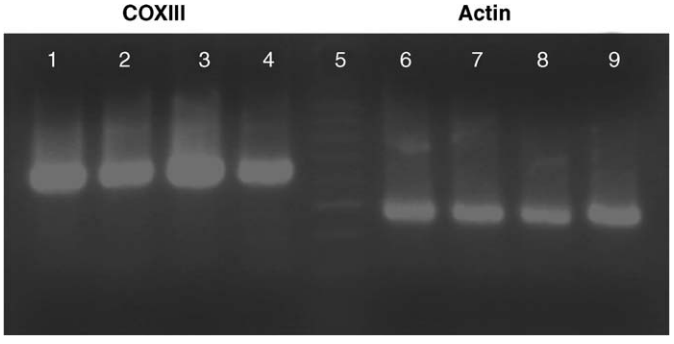

Fig. 3. Reverse transcriptase PCR analysis of COXIII (lanes 1–4; 650 bp) and Actin (lanes 6–9; control; 450 bp) in four castes of the termite species *R. santonensis*: workers (lanes 1, 6), larvae (lanes 2, 7), nymphs (lanes 3, 8), soldiers (lanes 4, 9), DNA ladder (lane 5).

homology with the full-length COXIII sequences identified from other insect orders.

In this study, we assessed whether termite castes differentially express the COXIII gene in association with development. In termites, caste-specific characteristics develop over successive moulting; termite polyphenism is based on differential gene expression associated with caste differentiation and between castes after differentiation (Miura, 2005). Workers perform various tasks from brood care, feeding other castes, prospection and location of food sources. They are also believed to play a major role in neighbouring interactions. Soldiers are seen as central components in nest defence (Kaib et al., 2002). It is known that soldier differentiation is regulated by specific gene expression that occurs during the two

Table 1
COXIII expression levels calculated for the eight independent experiments based on the $(1+E)^{-\Delta\Delta Ct}$ calculation method described by Liu and Saint (2002)

Sample	*COIIIRsant Ct*	Actin Ct	ΔCt (= Ct(*a*)− Ct(COIII))	ΔΔCt[a]	$(1+E)^{-\Delta\Delta Ctb}$
Wexp1	19.57	25.05	5.48	−1.32	1.65
Wexp2	19.40	25.74	6.34	−0.17	1.06
Wexp3	18.29	26.00	7.71	−0.37	1.15
Wexp4	17.96	23.29	5.33	−1.15	1.48
Wexp5	21.39	24.87	3.48	−1.97	1.97
Wexp6	20.04	28.38	8.34	−4.21	4.12
Wexp7	19.07	23.09	4.02	−1.20	1.52
Wexp8	19.48	25.07	5.59	−2.17	2.17
SD	1.06	1.66			
Lexp1	21.02	25.18	4.16	0.00	1.00
Lexp2	20.20	26.37	6.17	0.00	1.00
Lexp3	19.84	27.19	7.35	0.00	1.00
Lexp4	19.27	23.45	4.18	0.00	1.00
Lexp5	23.67	25.18	1.51	0.00	1.00
Lexp6	22.20	26.33	4.13	0.00	1.00
Lexp7	21.36	24.18	2.82	0.00	1.00
Lexp8	22.08	25.5	3.42	0.00	1.00
SD	1.44	1.22			
Nexp1	20.16	26.80	6.64	−2.48	2.56
Nexp2	19.66	27.68	8.02	−1.85	1.99
Nexp3	19.74	27.97	8.23	−0.88	1.39
Nexp4	19.08	25.4	6.32	−2.14	2.09
Nexp5	21.17	26.13	4.96	−3.45	3.27
Nexp6	21.17	30.12	8.95	−4.82	5.06
Nexp7	20.26	25.2	4.94	−2.12	2.10
Nexp8	21.08	28.17	7.09	−3.67	3.72
SD	0.79	1.64			
Sexp1	20.84	23.63	2.79	1.37	0.60
Sexp2	19.58	25.34	5.76	0.41	0.86
Sexp3	19.60	25.60	6.00	1.34	0.61
Sexp4	19.17	23.04	3.87	0.31	0.90
Sexp5	22.03	23.89	1.86	−0.35	1.13
Sexp6	21.35	25.07	3.72	0.41	0.87
Sexp7	20.71	22.15	1.44	1.38	0.62
Sexp8	21.36	24.24	2.88	0.54	0.82
SD	1.02	1.19			

Actin is used as internal control (normalizer). *Abbreviations*: W, L, N and S refer to workers, larvae, nymphs and soldiers, respectively. Standard deviations (SD) are calculated based on Ct values for COXIII or Actin.

[a]Calibration using corresponding values (e.g. *L* exp 1–*W* exp 1).

[b]$(1+E)$ = total efficiency (TE), $TE_1 = 1.46$; $TE_2 = 1.45$, $TE_3 = 1.45$, $TE_4 = 1.41$, $TE_5 = 1.41$, $TE_6 = 1.40$, $TE_7 = 1.42$, $TE_8 = 1.43$.

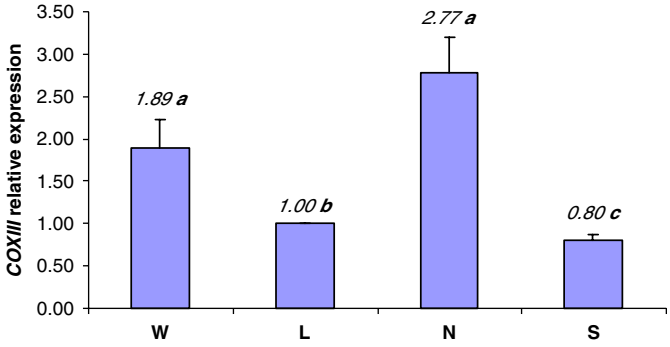

Fig. 4. Relative expression profiles (±standard error of means; $n = 8$), determined by real-time PCR, for COXIII gene transcript. Abbreviations on the *x*-axis represent different *R. santonensis* castes: W, workers; L, larvae; N, nymphs and S, soldiers. All expression levels are shown relative to expression level in larvae. Means with letters in common are not significantly different.

successive moults from the worker stage (Miura et al., 1999).

Our results clearly indicate that COXIII is differentially expressed between the four castes investigated. COXIII expression is enhanced in the head of termite workers (~1.9-fold) and nymphs (~2.8-fold) compared to larvae. Interestingly, the expression level of COXIII is found to be lower (0.8-fold) in the head of soldiers. This could potentially be due to a variation in biogenesis and in the number of mitochondria (higher/lower ATP production); but more likely due to the critical direct activation of the gene as previously shown with COXI (Sogl et al., 2000; Green-Willms et al., 2001; Uno et al., 2004). If reflecting COX activity, this may indicate that differential expression of COXIII is correlated with metabolic activity of COX, and indirectly, this would reflect variations in neuronal activity in different castes. One might consider that the observed variations of COXIII in head might not reflect variations in neuronal activity only. However, monitoring of COXI in whole body of *R. flavipes* termites have demonstrated an elevated expression in soldiers, which might result from elevated energy demands due to their large muscle mass (Scharf et al., 2003). Therefore, the observed variations in COXIII expression do not reflect variations in general metabolism but are more likely to highlight physiological variations in the termite head, in particular in the brain.

In hemimetabolous insects, the central nervous system increases during the course of individual development, which is correlated to the addition of new sensory afferents (Gronenberg et al., 1996). All neurons have a cytochrome oxidase system (Wong-Riley, 1989) and the higher expression of COXIII in nymphs and workers potentially indicates that COX activity is involved in the activity of neurons during critical phase of brain development in termite individuals. This idea is supported by a finding of memory consolidation upon upregulation of COX genes in *Periplaneta americana* (Pinter et al., 2005). Caste-specific expression may also be correlated to morphological changes in the brain as reported for hymenopteran (Fahrbach et al., 1995; Gronenberg et al., 1996). Developmental variation in the termite brain has been reported between castes. This concerns the antennal lobes, optical lobes and mushroom bodies (Richard, 1969). To extend investigations on morphological variations in the termite brain with respect to their developmental stages

could be of interest. Further research may bring elements to elucidate the down-regulation of COXIII in the head of soldiers although they undergo a more advanced post-embryonic development and may highlight interesting physiological variations during or after the process of caste-differentiation.

This study deals with the cytochrome *c* oxidase gene as a potential marker of neural activity in different castes of termites, demonstrating an increased activity in the head of nymphs and workers and a decreased activity in the head of soldiers. The finding of differential expression levels of the COXIII subunit gene in the head may emphasize molecular processes associated with termite biology. Hence, it is of interest to extend the analysis on the COX subunit genes in termites as our results raise questions about how the COX is actually involved in mechanisms that govern biological and physiological differences between termite castes.

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