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Short scientific note
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First PCR isolation of *Crithidia mellificae* (Euglenozoa: Trypanosomatidae) in *Apis mellifera* (Hymenoptera: Apidae) in Italy

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Abstract
Crithidia mellificae (Langridge & McGhee, 1967) is a trypanosomatid described in *Apis mellifera* (Linnaeus, 1758). The pathological effects of this parasite on the host are not well known. In this short communication, we report the first isolation of this pathogen in Italy, as realized in December 2013. The detection of *Crithidia* spp. was obtained by applying two PCR protocols that target the sequence of the mitochondrial cytochrome b (*Cytb*) and the sequence of small subunit ribosomal RNA (*SSUrRNA*), respectively. The PCR products were subjected to sequencing, which confirmed that the strain belonged to *Crithidia mellificae*.

Key words: *Crithidia mellificae,* honey bee, Italy.

INTRODUCTION

In recent years severe losses of honey bees (*Apis mellifera*) have been reported worldwide. Current analysis suggests that the etiology of this decline is multifactorial and *Crithidia mellificae* could be involved in it (Ravoet et al. 2013).

C. mellificae is a little studied acidophilic protozoan pathogen firstly described in Australia in 1967. It was the only one trypanosomatid species found in honey bee (Langridge & McGhee 1967; Ruiz-González & Brown 2006; Ravoet et al. 2013) until the recent description of a new genus and species by Schwarz et al. (2015).

C. mellificae, like other species of the *Trypanosomatidae* family, colonizes the honey bee by fecal-oral route (Ruiz-González & Brown 2006; Runckel et al. 2014) establishing itself at the digestive tract level. The presence of infected feces in the hive promotes the presence of parasite cells on the surface of insect body and increases the number of honey-bee workers able to transmit the disease when ingested by other healthy bees (Ruiz-González & Brown 2006).

Seasonal occurrence of this parasite seems to be significantly lower in the spring compared to summer and autumn (Schneeberger et al. 2014).

Some researchers state that *C. mellificae* does not affect the health of the colony (Runckel et al. 2011; Runckel et al. 2014), while other researchers report a positive correlation between *C. mellificae* and honey bee winter losses or colony longevity (Ravoet et al. 2013; Runckel et al. 2014).

As known, the overwintering mortality could be associated to the sinergistic effects of other honey bee pathoges, like varroa, viruses and *Nosema spp.* (Dahle 2010; Guzman-Novoa et al. 2010; Le Conte et al. 2010). According to Ravoet et al. (2013) and Runchel et al. (2014), the impact on colony health seems to be more negative with synergistic effects when *C. mellificae* and *Nosema caranae* (Fries et al. 1996) co-infect the same host simultaneously. Ravoet et al. (2014) demonstrated that, in addition to *Varroa destructor*, the presence of *C. mellificae* and *Nosema ceranae* in summer are predictive markers of winter mortality.

So far, *C. mellificae* is present in Asia, Australia, USA and Europe. Here it has been isolated in Belgium, France, Spain and Switzerland (Ravoet et al. 2013; Cepero et al. 2014; Ravoet et al. 2014; Runckel et al. 2014). Presently, there is no available information on how this trypanosomatid species is distributed in other EU Countries (refer to Schwarz et al. 2015 for some recent updates).

Actually, the isolation methods for *C. mellificae* are: cell lines cultures isolated from the dissected ileum of adult honey bees, *in vivo* inoculations, DNA purification, phylogenetic analyses, confocal laser scanning microscopy, electron microscopy scanning and transmission (Schwarz et al. 2015).

This study reports on the first PCR isolation in Italy of *C. mellificae* in *Apis mellifera*.

MATERIALS AND METHODS

In winter (December) 2013 a sample of adult honey bees taken from the experimental apiary of Rome (Central Italy) of the research institute "Istituto Zooprofilattico Sperimentale del Lazio e della Toscana" was investigated by RT-PCR for *C. mellificae*.

For the sample preparation, 20 adult honey bees were homogenized in Buffer Phosphate and 1.5ml was collected and was added to the DNA extraction performed with NucleoSpin® Tissue Kit (Macherey-Nagel; Düren, Germany). The DNA obtained was used as a template to carry out two PCR reactions using the AmpliTaqGold® with GeneAmp® Kit (Applied Biosystems; Branchburg, New Jersey, USA). The first PCR reaction amplifies a portion of 632bp of the sequence of small subunit ribosomal RNA (*SSUrRNA*) and used primers SSUrRNA-F2 (5'-CTTTTGACGAACAACTGCCCTATC-3'),SSUrRNA-B4 (5'-AACCGAACGCACTAAACCCC-3') taken from Schmid-Hempel & Tognazzo (2010). The second PCR reaction amplifies a portion of 471bp of the sequence of the mitochondrial cytochrome b (*Cytb*) using the primers CB- Cytb2-F/5'-GT(A/T)TT(G/A)TTTTT(G/A)TG(G/A)GATTTTG-3'), CB-Cytb2-B (5'-CATAAACG(T/C)TCACAATAAAATGC-3') taken from Schmid-Hendel *et al.* (2010). The PCR products were visualized after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel and stained with Gel Red 10,000X (Biotium, Hayward, CA, USA) (Figure 1). After electrophoresis, each amplicon was excised from a 1.5% agarose gel, extracted by using the QIAquick Spin (Qiagen, Duesseldolf, Germany) according to the manufacturer's protocol, and then sequenced thereafter. The obtained sequences were compared with the sequences present in GeneBank database, using BLAST.

The phylogenetic analysis was obtained using Nucleotide Blast (BLASTn) whit the sequence FASTA related to primers SSUrRNA-F and SSrRNA-B4) with the Max Sequence Difference of 0,75 and including all the sequences deposited in GenBank. To evaluate the level of infection for nosemosis and virosis, samples of adult honey bees (foragers) each constituted from 20 subjects were collected to evaluate: the *Nosema spp.* involved in the infection (*N. apis* Zander, 1909 or *N. ceranae*); the number of *N. ceranae* spores/bee; the diagnosis for ABPV, CBPV, DWV, SBV, BQCV, KBV and IAPV viruses.The qualitative detection for SBV, BQCV, KBV and IAPV viruses was realized by Reverse Trascriptase PCR (RT-PCR), as for discrimination between *N. apis* and *N. ceranae.* While for DWV, CBPV and ABPV viruses and for *N. ceranae* the quantification was realized by quantitative Real-Time PCR (qRT-PCR). *Nosema spp.* DNA extraction was realized from 20 worker honey bees homogenized in 20 ml of Buffer Phosphate; 2 ml of the homogenate were centrifuged at room temperature at 13.000 rpm for 10 min. The supernatant was removed and 50 mg of the pellet was suspended in 40 µl of Germination Buffer (75 ml of NaCl and 75 ml of NaHCO3) and incubated at 37°C at 300 rpm for 15 min. Then 180 µl of Lisozime (20 mg/ml) and 200 µl of Buffer AL both supplied by the DNA Blood Mini kit (QIAamp®, Qiagen, Hilden, Germany) were added to the sample. Subsequently, the DNA extraction was carried out according to the manufacturer instructions of the DNA Blood Mini kit.RNA viruses extraction was realized from 15 worker bees homogenized in 15 ml of Buffer Phosphate. The homogenate was diluted (1:2) in H2O-DEPC and 140 µl were used to extract total RNA by Viral Mini kit (QIAamp®, Qiagen, Hilden, Germany) according to the manufacturer instructions. The primer and probe used for qRT-PCR were:

- ABPV target in gp1 gene (data from Italian Apiculture National Reference Centre at IZSVE, Padova, Italy);
- DWV target in gp1 gene (data from Italian Apiculture National Reference Centre at IZSVE, Padova, Italy);
- CBPV target in viral RNA polymerase gene (Blanchard *et al.* 2007)
- N. ceranae target in large subunit ribosomal RNA gene sequence specie-specific (Bourgeois *et al.*, 2010).

For the viruses ABPV, CBPV and DWV, the reverse transcription was carried out using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer instructions; 5 µl of cDNA were used as template in the RT-PCRs, or Reverse Real Time -PCRs, or qReverse Real Time PCRs Master Mix.For qRT-PCR viruses was used the TaqMan® Universal PCR Master Mister kit (Applied Biosystems, Foster City, CA, USA) while for qPCR *N. ceranae* was used the TaqMan GTX pressTM Master mix kit (Applied Biosystems) and 5 µl of DNA as template. The construction of the internal standards for qRT-PCR viruses and qPCR *N. ceranae* were formed by ricombinant pCRII-TOPO vector (Invitrogen, Life Technologies, Foster City, USA) with the respective amplifiers (pCRII-TOPO-ABPV; pCRII-TOPO-DWV; pCRII-TOPO *N. ceranae*) and the LOD of the assays to cover a range of dilutions between 5 x 109 copy/µl to 3 copy/µl. In each run of the qRT-PCRs or qPCR, a quantified internal control, developed as described further on, was included for the estimation of the viral RNA or DNA copies/ml of the 2ml homogenate obtained by 15 worker bees. Amplification was carried out using ABIPRISM 7900 HT Sequence Detection System and all data were analysed using 7900HT Sequence Detection System SDS software package (Applied Biosystems).The primer for RT-PCR for viruses SBV, KBV, IAPV, BQCV and PCR for *N. apis* were selected from the literature: SBV (Grabensteiner et al. 2001), BQCV (Berènyi et al. 2006), KBV (Berènyi et al. 2006;

Table 1 – The FASTA sequence with the primers SSUrRNA-F2/SSrRNA-B4>gb|GU321196.1|:114-610 *Crithidia mellificae* small subunit ribosomal RNA gene, partial sequence.

***Crithidia mellificae* small subunit ribosomal RNA gene, partial sequence**
TCAAAACAAAACGATGAGGCAGCGAAAAGAAATAGAGTTGTCAGTCCATTTGGATTGTCATTTCAATGAG
GGATATTTAAACCCATCGAAAATCTAGTAACAATTGGAGGACAAGTCTGGTGCCAGCACCCGCGGTAATT
CCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAGGGTTCGTAGTTGAACTGTGGGCTGTGTAGG
TTTGTTCCTGGTCGTCCCGTCCATGTCGGATTTTGGTGGCCCAGGCCCTTGCAGCCCGTGAACATTCAAA
GAAACAAGAAACACGGGAGTGGTTCCTTTCCTGACTCACGCATGTCATGCATGCTGCAGGGGGCGTCCGT
GATTTTTACTGTGACTAAAGAAGTGTGACTAAAGCAGTCATTTGACTTGAATTAGCAAGCATGGGATAAC
AAAGGAGCAGCCTTTAGGCTACCGTTTCGGCTTTTGTTGGTTTTAAAGGTCTATTGGAGATTATGGAGCT
GTGCGAC

Cersini et al. 2013) and *N. apis* (Martin-Hernàndez et al. 2007). The size of PCR products were: 570bp for CBPV, 604bp for SBV, 609bp for BQCV, 395bp for KBV and 321bp for *N. apis*. For IAPV was used a nested-PCR protocol. The first couple of primers amplified a 381bp fragment of Coat Protein region of the IAPV virus (Blanchard et al. 2008); while the second couple of primers amplified a 137 fragment internal at the Coat Protein region of the IAPV virus (Formato et al. 2011). For SBV, KBV, IAPV and BQCV RT-PCR, the reverse transcription was carried out using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and then 5 µl of cDNA were used as template in the specific PCRs to prepared using Platinum®Taq DNA Polymerase (Invitrogen). For PCR N. apis, the PCR reaction carried out using Ampli Gold® Taq Polymerase kit (Applied Biosystems). The amplifications were performed with GeneAmp®PCR System 9700 (Applied Biosystems) and the PCR products were visualized after electrophoresis in a 1,5% Tris-Boric Acid-EDTA agarose gel and stained with Gel Red 10,000X (Biotium, Hayward, CA, USA) using the 100bp molecular ladder (Invitrogen).

RESULT AND DISCUSSION

The analysis of sequences showed an identity of 100% with the sequence *C. mellificae* small subunit ribosomal RNA gene (GenBank GU321196.1). The same result was obtained with primers CB-Cytb2-F and Cytb2-R (Table 1, Fig. 1 and Fig. 2).

The results of the phylogenetic analysis for the *Crithidia mellificae*_Italy 2013 strain are shown in figure 2.

The same adult honey bee sample showed co-infection of *C. mellificae* with: *Nosema ceranae* (low level of infection at Real time PCR: 4.65 DNA target/µl = 1.16 spore/honey bee), *Acute Bee Paralysis Virus (ABPV)* with 10.9 DNA copies/µl (Ct = 33,7 and R^2 = 0,98), *Deformed Wing Virus (DWV)* with 1.520.000 DNA copies/µl (Ct = 20 and R^2 = 0,99), *Sac Brood Virus (SBV), Black Queen Cell Virus (BQCV)* and *Israeli Acute Paralysis Virus (IAPV)*. On the other hand, the honey bee sample was negative for: *Nosema apis*, Chronic *Bee Paralysis Virus (CBPV*) (R^2 = 0,99) and *Kashmire Bee Virus (KBV)*.

The affected honey bee colony on December 2013 was developed on 6 frames and no clinical symptoms of *Varroa* mite, neither virosis, nor other pathologies could be observed in it.

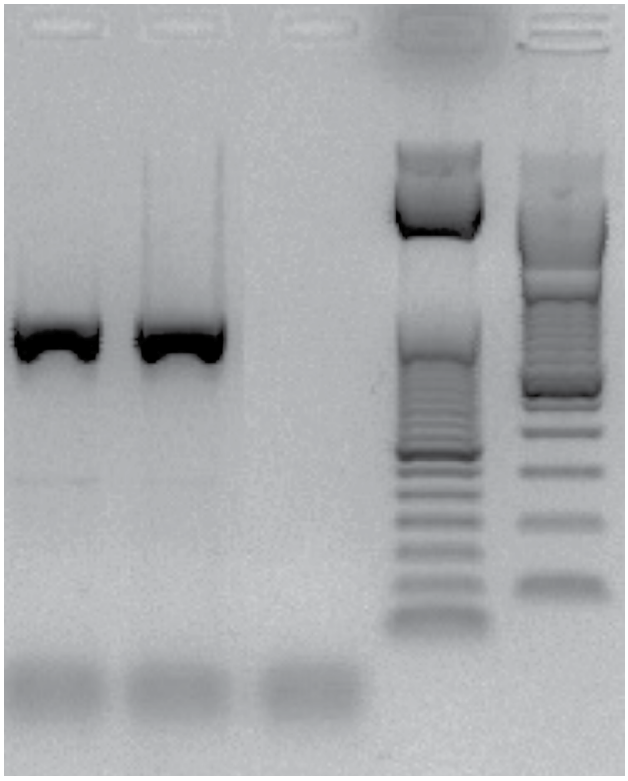

Fig. 1 – Amplification obtained using SSUrNA primer. From left: positive sample, positive control, negative control, 50 bp and 100 bp.

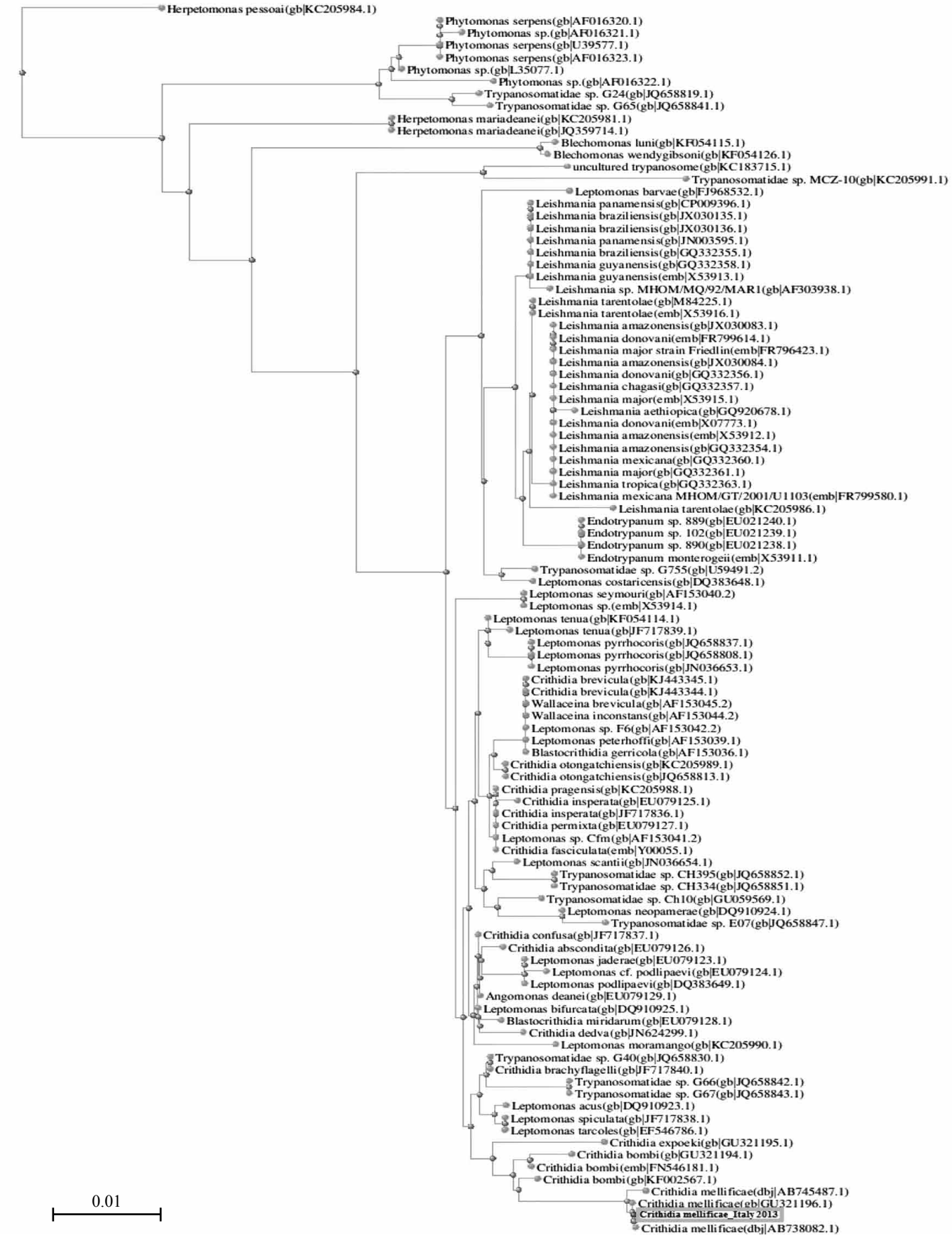

Fig. 2 – The phylogenetic analysis for the *Crithidia mellificae*_Italy 2013 strain.

Even though the contemporary presence of *C. mellificae* and *Nosema ceranae,* the hive could overwinter without any kind of healthy problem. The PCR methods used to identify *Crithidia* can not establish the viability of the pathogen, however we consider our *C. mellificae* diagnosis an important goal. In fact, literature data show *C. mellificae* infection can cause an increase of host mortality, alters the intestinal microbiota population, implies behavioral change and immune responses that involve the production of defensin antimicrobial peptides (Schwarz & Evans 2013). Although further studies are needed to better define the host-parasite relationship and the pathogenic role of *Crithidia mellificae* in honey bees, our work could help to better understand the distribution of this little studied honey bee pathogen.

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