An improved DNA method to unambiguously detect small hive beetle *Aethina tumida*, an invasive pest of honeybee colonies

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The small hive beetle (SHB) *Aethina tumida* Murray (Coleoptera: Nitidulidae) is a scavenger native to sub-Saharan Africa and is a pest of honey bees without provoking significant damage within its endemic range.\(^1\,^2\) Since the first report in 1996 out of its native range\(^3\) in North Carolina, USA, the beetle became an invasive species in Australia, and in central and North America. It was introduced in Europe in 2004 in Portugal where an eradication program was effective and then in Italy in 2014 where infestation is still ongoing\(^4\) probably originating from an African population.\(^5\) Its life cycle is intimately linked to the honey bee where it mates and reproduces inside the colony and where the larvae feed on bee bread, honey and brood causing destruction.\(^1\,^6\,^7\) Besides honey bees, it can also affect bumble bees and stingless bees (see review in reference\(^2\)).

The economic damage to the beekeeping industry can therefore be substantial thereby explaining why the SHB is a statutory notifiable pest in the European Union (EU). After its introduction in Italy, the EU authorities (Commission Implementing Decision 2014/909/EU of 12 December 2014) established new protective measures to prevent SHB spread including the goal to eradicate it if possible. It is consequently of the utmost importance to have an easy, reliable and cheap technique for diagnostics. However, the eggs and larvae stage are extremely difficult to identify with conventional taxonomic techniques bearing a too high risk of incorrect results. A previous PCR assay is based on work published in 2007,\(^8\) where an amplification system targeting the cytochrome oxidase subunit I (COI) gene was proposed. However, the sequence of the reverse primer contains an important internal mismatch of three nucleotides with all currently published *A. tumida* COI sequences, rendering its application tedious and susceptible to false negative diagnostic. In this study, we propose a new SHB diagnostics using a multiplex PCR approach targeting COI gene and a common region of 18S ribosomal gene as internal control.

The DNA extraction method was chosen according to a previous study comparing different extraction procedures of genomic DNA from ticks providing material allowing maximal recovery and good quality for a consistent amplification.\(^9\) Briefly, insects stored in ethanol 70% (v/v) were air-dried prior to dissection into four quarters and then DNA was extracted using GeneJet Genomic Kit (Thermo Fisher, Waltham, MA, USA) following the manufacturer’s instructions and eluted into a final volume of 100 μL. Then, 4 μL (0.8–10 ng) of insect genomic DNA were used for amplification. PCR was performed in a 20 μL reaction volume containing 1x

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Table 1. Sequences of primers and probes targeting COI and 18S genes. Amplicon lengths were of 396 bp for COI and 80 bp for 18S

| COI gene | 18S gene |
|----------|----------|
| Forward primers | 5'-CGACCCTCAGGCATAACCTT-3' |
| Reverse primers | 5'-AGGCTCGAGTAGTATCAAGTCTA-3' |
| Probes | 5'-HEX-GGAAGCCTTTGGAACTTTAGG-BHQ-3' |
| | 5'-FAM-GTAACCCGCTGAACCTCCTT-BHQ-3' |

Table 2. Results of multiplex *Aethina tumida* PCR system application to 49 different insect DNA

| Species with GBOLD accession | Origin | Development stage | Atum Cq | 18S Cq |
|------------------------------|--------|------------------|---------|--------|
| 1 Aethina tumida | Italy | Adult | 28.7 | 28.6 |
| 2 Aethina tumida | Italy | Larvae | 30.9 | 26.4 |
| 3 Aethina tumida | Italy | Larvae | 32.5 | 26.1 |
| 4 Aethina tumida | Italy (Calabria) | Adult | 21 | 22.8 |
| 5 Aethina tumida | Italy (Calabria) | Adult | 20.2 | 22.1 |
| 6 Aethina tumida | United Kingdom (breeding from an US strain) | Larvae | 35.2 | 28.2 |
| 7 Aethina tumida | United Kingdom (breeding from an US strain) | Adult | 30.3 | 26.2 |
| 8 Aethina tumida | Mexico | Adult | 18.8 | 21.4 |
| 9 Aethina tumida | South Africa | Adult | 27.9 | 34.1 |
| 10 Aethina tumida | South Africa | Adult | 28 | 33.4 |
| 11 Aethina tumida | South Africa | Larvae | 28 | 31.5 |
| 12 Aethina tumida | South Africa | Larvae | 22.4 | 30 |
| 13 Harmonia axyridis | Switzerland | Adult | ND | 16.1 |
| 14 Harmonia axyridis | Switzerland | Adult | ND | 23.5 |
| 15 Muscidae | Switzerland | Adult | ND | 42.1 |
| 16 Forficula auricularia | Switzerland | Adult | ND | 38.5 |
| 17 Leptoglossus occidentalis | Switzerland | Adult | ND | 37.1 |
| 18 Galleria mellonella | Switzerland | Larvae | ND | 21.4 |
| 19 Galleria mellonella | Switzerland | Larvae | ND | 22.8 |
| 20 Lepidoptera | Switzerland | Larvae | ND | 32.7 |
| 21 Varroa destructor | Switzerland | Adult | ND | 31 |
| 22 Meligethes viridescens | Switzerland | Adult | ND | 35 |
| 23 Cychramus luteus (Nitidulidae) | ZFMK-TIS-2504554 | Germany | Adult | ND | 34.9 |
| 24 Cychramus luteus (Nitidulidae) | ZFMK-TIS-2503863 | Germany | Adult | ND | 33.3 |
| 25 Cychramus luteus (Nitidulidae) | ZFMK-TIS-2506747 | Italy | Adult | ND | 34.7 |
| 26 Epuraea aestiva (Nitidulidae) | ZFMK-TIS-13931 | Germany | Adult | ND | 24.3 |
| 27 Epuraea aestiva (Nitidulidae) | ZFMK-TIS-2504534 | Germany | Adult | ND | 29.4 |
| 28 Epuraea aestiva (Nitidulidae) | ZFMK-TIS-2504535 | Germany | Adult | ND | 28.4 |
| 29 Glischrochilus hortensis (Nitidulidae) | ZFMK-TIS-2522755 | Germany | Adult | ND | 30.6 |
| 30 Glischrochilus hortensis (Nitidulidae) | ZFMK-TIS-11274 | Germany | Adult | ND | 37.5 |
| 31 Glischrochilus hortensis (Nitidulidae) | ZFMK-TIS-11650 | Germany | Adult | ND | 29.5 |
| 32 Glischrochilus quadriguttatus (Nitidulidae) | ZFMK-TIS-2515238 | Germany | Adult | ND | 27.9 |
were positive for 18S, with Cq ≤ 6.4 cycles (Table 2). All the other 37 insect samples analyzed by this assay had Cq for the two systems never diverged for the 18S and COI gene amplification. Interestingly, the COI reverse primer and at 5′ extremity of the COI probe were designed completely complementary to any known sequences and probes with Primer3 version 0.4.0.11 Both primers and probes were synthesized by Microsynth (Microsynth, Balgach, Switzerland). Amplification results were analyzed with Eco Study v. 5.0 (PCRmax). Size and sequence of the COI amplicon (∼650 bp) were further verified on a DNA extracted from a beetle individual from ZFMK-TIS-251033, isolated in Italy (data not shown).

After optimization of the concentration of the two probes, to further confirm the specificity of the primers the multiplex assay was tested on a total of 49 DNAs extracted from different insects of different geographical origins and relatives from the Nitidulidae family. All the DNA extracted from 12 A. tumida individuals from Germany (individuals 33–34 and 38–41) were obtained from BOLD Germany, details can be found at https://doi.org/10.5883/DS-AETHINA. Some DNA from insects (individuals 23–34 and 38–41) were obtained from BOLD Germany, details can be found at https://doi.org/10.5883/DS-AETHINA. The DNA test set included 12 A. tumida individuals from Germany and 37 different insect species from other geographical origins. These observations prove the reliability of our multiplex PCR system for a rapid, reliable and specific diagnostics of SHB that may be found in the hive and thus facilitate for example early detection programs.

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