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Introduction of *Aethina tumida* (Coleoptera: Nitidulidae) in the regions of Calabria and Sicily (southern Italy)

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Abstract – *Aethina tumida* (small hive beetle, SHB) was first detected in September 2014 in Calabria region, southern Italy, and in a single apiary in Sicily in November 2014. In September 2015, SHB was again recorded in Calabria, and in 2016, only sentinel honey bee nucleus colonies were found to be infested. Its phylogenetic relationship and possible origin were investigated comparing the *cox1* sequences with the corresponding region available in the GenBank database. The neighbour-joining method revealed that the first Italian specimen belonged to a group also containing an African specimen from Cameroon. The Italian specimens differ from the SHBs spread worldwide and are split into two different groups: group B1 includes the AfricCam3 sequence and the first SHB identified in Calabria; group B2 includes specimens from Calabria and the only one from Sicily which share identical *cox1* sequences. SHB in Italy appears to have been introduced from Africa and includes independent or contemporary incursions in the two concerned regions. The most likely scenario is that SHB was introduced into Calabria followed by man-mediated migration to Sicily.

Aethina tumida / *Apis mellifera* / *cox1* gene / exotic bee infestation / southern Italy

1. INTRODUCTION

Aethina tumida (small hive beetle, SHB), Murray 1867 (Coleoptera: Nitidulidae) is a free-living predator, parasite and scavenger of honey bee colonies (species of the genera *Apis* and *Bombus* and also stingless bees) (OIE 2013) and is

responsible for their infestation. Both adults and larvae feed on the honey bee brood, honey and pollen, causing the death of the brood, honey fermentation and comb destruction, often resulting in the complete structural collapse of the nest and the absconding of the colony. For this reason, *A. tumida* severely impacts on honey bee colonies and strongly affects the international trade of bees and bee products (OIE 2013).

A. tumida is native to sub-Saharan Africa (Lundie 1940; Neumann and Ellis 2008; Neumann et al. 2016) and is currently reported in the USA (Elzen et al. 2000), Canada (Clay 2006), Australia (Gillespie et al. 2003), the Philippines (Brion 2015), Mexico, some countries of

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Central America (Neumann and Ellis 2008; OIE 2015a; Neumann et al. 2016) and Brazil (OIE 2016).

In 2004, during an official import inspection, larvae morphologically attributable to SHB were intercepted in cages carrying *Apis mellifera ligustica* queens and accompanying workers, legally imported into Portugal from Texas, USA (Murilhas 2004). The further spread of SHB was prevented by the total destruction, under official supervision, of all honey bees and hive materials in the apiary that had received the queen bees (Valerio da Silva 2014).

A. tumida was exotic to Europe until September 2014, when Italy reported an outbreak in the south of the country (Mutinelli et al. 2014; Palmeri et al. 2015). The pest was reported for the first time on 5 September 2014 (Palmeri et al. 2015) in the municipality of Gioia Tauro, in the region of Calabria, southern Italy (38.4547 N, 15.9411 E). The infested hives were located close to Gioia Tauro, an international port through which more than two million containers pass in transit per year from all over the world. A further outbreak outside the region of Calabria was later detected on 7 November in a migratory apiary located in the municipality of Melilli (37.27224 N, 15.06722 E), in the region of Sicily, southern Italy. This apiary has been in the Gioia Tauro area from April to August 2014 and was returned to Sicily at the end of the productive season (Mutinelli et al. 2014). Until now, this was the only apiary found to be infested by SHBs outside Calabria.

Intensive surveillance activity was immediately initiated by official veterinarians in collaboration with beekeepers to try and determine how far the beetle had already spread in the regions of Calabria and Sicily (Mutinelli et al. 2014; Mutinelli 2016a). In Calabria, 60 infested apiaries were detected between September and December 2014 within a radius of 20 km of the infested sites, identified as the protection zone. According to the surveillance program, all apiaries in the protection zone were visited and beehives were selected and clinically inspected based on an expected prevalence of SHB infestation of 5 % (95 % CI). Apiaries were randomly selected in the rest of the region of Calabria, based on an expected

prevalence of 2 % (95 % CI), and beehives were selected and clinically inspected according to an expected prevalence of SHB infestation of 5 % (95 % CI). All apiaries (= 282, including the 60 positive cases) within the protection zone were inspected up to eight times by the official veterinarians from September to December 2014, and 808 apiaries in the rest of the region of Calabria were repeatedly inspected in the same time frame. No infested apiaries were detected outside the protection zone. A similar surveillance program was implemented in the region of Sicily after the first and only infested apiary was detected in November 2014. In the protection zone, 185 apiaries were recorded and beehives were selected and clinically inspected by the end of the year, according to an expected prevalence of SHB infestation of 5 % (95 % CI). No further infested apiaries were detected in 2014 (<http://www.izsvenezie.com/documents/reference-laboratories/beekeeping/aethina-tumida/2014/final-figure-3-monitoring-area-calabria-sicily.pdf>).

Despite the ongoing surveillance activity, over 9 months after the pest had previously been identified, SHB was again detected on 16 September 2015 in the municipality of Taurianova, in the region of Calabria, which had been infested in 2014 (<http://www.izsvenezie.com/documents/reference-laboratories/beekeeping/aethina-tumida/2015/table-1-confirmed-cases.pdf>). Twenty-nine infested apiaries were detected in the protection zone between September and December 2015. According to the Italian national surveillance program carried out in 2015 (Mutinelli 2016a), no other cases were detected in any Italian territory outside Calabria. Briefly, based on the size of the Italian national apiary registry, 164 non-migratory apiaries were randomly selected in northern, central and southern Italy, excluding Calabria and Sicily, giving a total of 492 apiaries based on an expected prevalence of 2 % (95 % CI). Beehives were selected and clinically inspected according to the same expected prevalence of SHB infestation (Mutinelli 2016b). In 2015, the competent authorities of the regions of Calabria and Sicily conducted the same surveillance program as in 2014.

A possible relationship was hypothesised between the introduction of honey bees from

infested countries and territories and the close proximity of SHB detection sites to the port of Gioia Tauro (38.4605 N, 15.9095 E) (region of Calabria), considering the intense transit of containers and ships from all over the world. SHB was introduced into the USA in similar circumstances (Hood 2004; Neumann and Elzen 2004).

In recent decades, the frequency of biological invasions (Nentwig 2007) has largely been increased by the worldwide trade of live animals, honey bees and their products (Mutinelli 2011), agricultural commodities (Bacon et al. 2012) and travel. Furthermore, gaps in border inspections, and gaps between trade pathways that ought to be controlled and actual number of interceptions could contribute to the dispersal of alien insects around the world (Bacon et al. 2012).

Brown et al. (2002) investigated the risks to UK beekeeping from SHB and the parasitic mite *Tropilaelaps clareae*, considering both of them to be serious threats to apiculture and their spread around the world to be of great concern. EFSA (2013) subsequently performed a qualitative risk assessment for both SHB and *Tropilaelaps* spp.—bee parasites considered exotic in the EU—taking into account current legislation but ruling out the implementation of risk reduction options. A worst-case scenario assessment was performed for each risk factor. The risk pathways with a high risk of pest entry were the “import of bee products used in apiculture” in the case of SHB, and “the accidental import of bees” (the unintended presence of bees in a non-bee consignment) in the case of both pests. The other risk pathways were associated with a moderate or low risk of entry of SHB or *Tropilaelaps* into the risk assessment area. More recently, EFSA (2015a, b) has carefully considered SHB diagnosis and risk reduction options as well as the survival, spread and establishment of SHB in Europe following its detection in southern Italy. Since being found in southern Italy, three EU decisions have been issued (EU 2014; EU 2015a, b) to enforce protective measures that will apply until 31 March 2017, depending on the epidemiological development of the SHB infestation. An EU surveillance program to detect SHB was also implemented in spring 2015 to sample and inspect at-risk apiaries, apiaries in at-risk zones, and apiaries throughout Italy,

in accordance with the guidelines issued by the European Union Reference Laboratory for honey bee health (Chauzat et al. 2015).

To trace the spread of new pathogens and pests, it is critically important to establish their geographical origins (Nentwig 2007). The mitochondrial cytochrome oxidase I (*cox1*) gene, used to classify and identify insect species, was sequenced (Evans et al. 2000) to determine the relationship between hive beetles collected in the USA and those collected in South Africa, showing that SHB introduced into the USA was closely related to SHB from South Africa (Evans et al. 2000; Evans et al. 2003; Evans et al. 2008; Lounsberry et al. 2010).

Following the worldwide spread of *A. tumida* from its native range, its first detection in Europe (Portugal 2004) and the first reported SHB population build-up in the same continent (southern Italy 2014), it is important to understand the possible source of the population, its movements, and genetic features, as has been determined in other continents (Evans et al. 2003; Lounsberry et al. 2010). Establishing the origin of the Italian *A. tumida* population could contribute to updating maps of the worldwide spread of the parasite, survival and establishment in new continents (OIE 2015b), as well as provide information on the risks associated with potential invasion pathways.

Here, we report on the putative origin of SHB samples collected in the infested areas of southern Italy, based on mitochondrial DNA (mtDNA) sequence analysis.

2. MATERIAL AND METHODS

2.1. Sample collection and DNA isolation

All SHB adult and larvae specimens, manually collected in the infested apiaries from 2014 to the present and submitted to the National Reference Laboratory, were morphologically investigated according to the OIE Manual (2013). SHB adults were collected in the beehives (Dadant-Blatt type) from the top bars and frames of the nest box as well as from the bottom board. Larvae were found on the nest frames and bottom board. Adults were found in both strong and weak queenright colonies with no sign of infestation. Larvae were found

on the nest frames in a very limited number of infested apiaries. We observed typical signs of beetle-associated damage in only one infested, abandoned apiary (Mutinelli et al. 2014; Mutinelli 2016a).

We analysed 18 adult *A. tumida* specimens collected in 2014 from 16 different sites within the protection zone of the region of Calabria (Figure 1), and one adult specimen from the one positive site in Sicily (no. 53 in Figure 1). We also analysed one larva and three adult *A. tumida* specimens collected in the first outbreak of 2015.

Total DNA was extracted from individual beetles. The samples were homogenized using 5-mm stainless steel beads in a Tissue Lyser II (Qiagen GmbH, Hilden, Germany) and the DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Negative controls (water for molecular biology applications in place of the sample) were processed in parallel to detect any contamination. The extracted DNA yield and purity (260/280 and 260/230 nm absorbance ratios) were checked using a Nanodrop N1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). DNA was stored at -20°C prior to use.

2.2. PCR amplification and sequencing

A 1091-bp fragment of the *cox1* gene was amplified using primers AT1904S (5'-GGTGGATCTTCAGT TGATTTAGC-3') and AT2953A (5'-TCAG CTGGGGGATAAAATTG-3'), as described by Evans et al. (2000). PCR was performed in a total volume of 50 μL containing a final concentration of $1\times$ PCR Buffer, 3 mM MgCl_2 , 0.4 mM dNTPs, 0.4 μM of each primer, 2 U AmpliTaq Gold (Applied Biosystem, Foster City, CA, USA) and 50 ng of DNA. PCR was carried out using a Veriti[®] Thermal Cycler (Applied Biosystem, Foster City, CA, USA). The thermal cycling profile consisted of an initial step at 95°C for 10 min for AmpliTaq Gold activation, followed by 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min.

Positive (DNA obtained from *A. tumida* samples identified morphologically and by PCR and sequencing, as the template) and negative (water for molecular biology applications instead of the DNA template) controls were included in each PCR. PCR products were

analysed for purity and size by electrophoresis in 7 % acrylamide gel after silver staining. Amplicons were subsequently purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) through binding to magnetic beads, then treated with ExoSAP-IT[®] (USB Corporation, Cleveland, OH) and sequenced in both directions. Two internal primers, Aet-int-F (5'-CTTCTGCTACAATAATTATTGC-3') and Aet-int-R (5'-TTGTGTACCATGAAGAGTAGC-3'), were designed using Gene Runner 3.05 and added to the sequencing reactions, in order to obtain better coverage of the 1091-bp fragment to be sequenced. Samples were processed using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and cleaned using Performa DTR Ultra 96-well kit (Edge BioSystems, Gaithersburg, MD). The sequencing process was carried out in a 16-capillary ABI PRISM[®] 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were deposited in the GenBank database (see Online Resource).

2.3. Phylogenetic analyses

Sequencing data were assembled and edited with SeqScape[®] software v2.5 (Applied Biosystems, Foster City, CA, USA). The consensus sequences obtained were aligned and compared to reference nucleotide sequences available in the GenBank using the MEGA 5 package and the ClustalW alignment algorithm (Tamura et al. 2011).

To determine the phylogenetic relationship and origin of the Italian *A. tumida* samples, the *cox1* gene sequences were compared to the corresponding region of representative beetles ($n = 41$) available in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>). The alignment was performed on sequences of different lengths, ranging from 757 to 1091 bp: the longest sequences (1091 bp) were obtained from the Italian samples, whereas the 41 *A. tumida cox1* sequences in the GenBank database were all shorter (from 757 to 1038 bp) and included in the 1091-bp fragment.

Phylogenetic analyses were performed according to the neighbour-joining method (Saitou and Nei 1987) implemented in MEGA version 5 (Tamura et al. 2011). The maximum composite likelihood evolutionary model (Tamura et al. 2004) was used to compute the phylogenetic distances. To test the robustness of the tree topology, 1000 bootstrap replicates were conducted

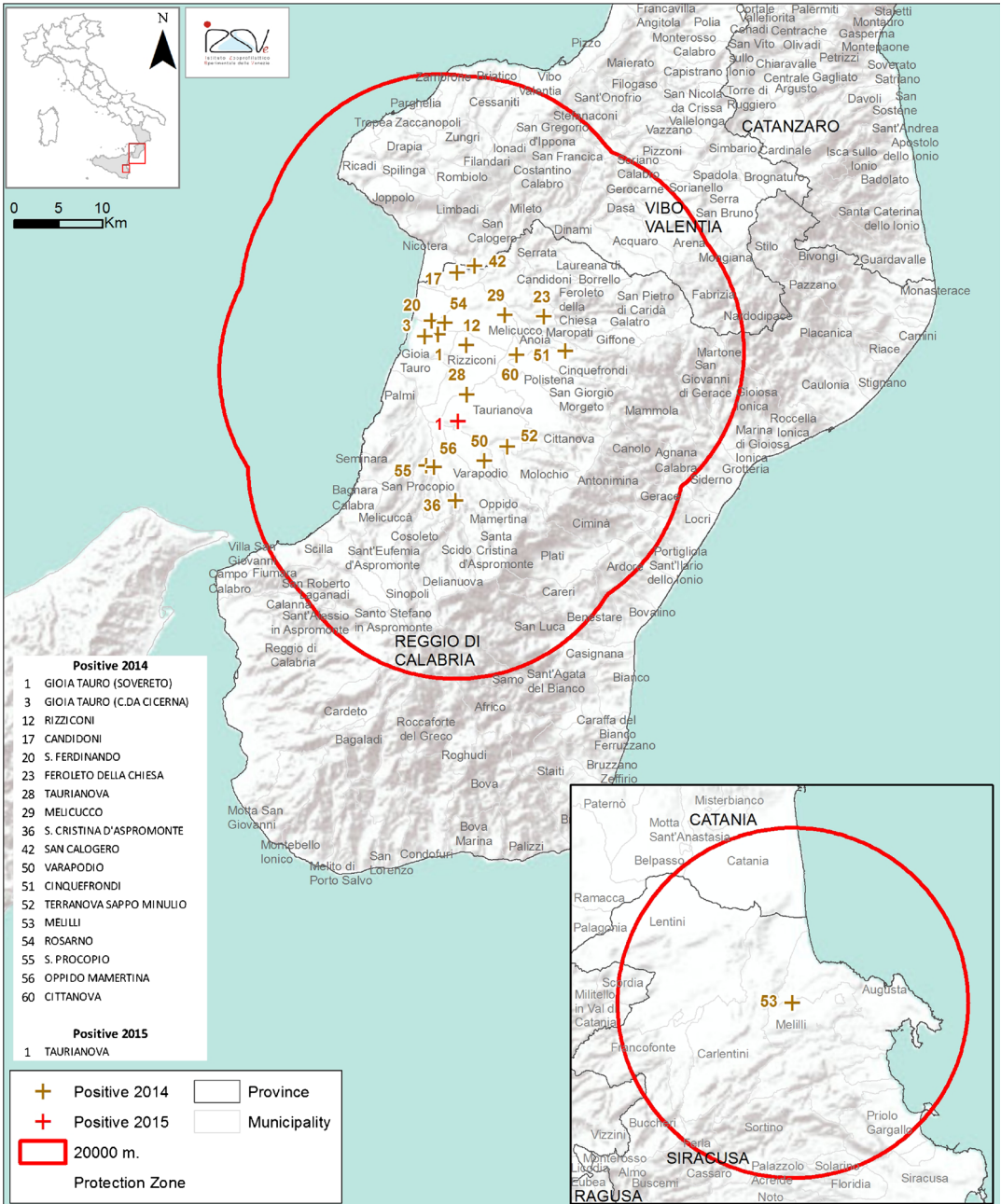


Figure 1. *A. tumida* infested sites of the regions of Calabria (left) and Sicily (right) where the samples used for molecular biology investigation originated. The numbers identify the municipality where each specimen was detected. The numbers correspond to those in the phylogenetic tree

(Felsenstein 1985). The phylogenetic analyses were performed both on the dataset composed by sequences of different length (from 757 to 1091 bp) and on the alignment of the region shared by all the sequences (721 bp). The topology of the tree was confirmed by both analyses.

A *cox1* sequence from *Leptura aurosericans* (KF737783) was used as an outgroup to root the tree.

2.4. Cloning and sequencing

To confirm the results of the phylogenetic analyses, the amplification products, obtained with AT1904S and AT2953A primers, were cloned into the PCR[®] II-TOPO[®] vector using a dual-promoter TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Plasmid DNAs were isolated from positive *Escherichia coli* colonies using a GenElute[™] Plasmid Miniprep Kit (Sigma-Aldrich Co, St. Louis, MO, USA). The inserted fragment was sequenced after PCR using M13 forward and M13 reverse primers, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

3. RESULTS

Phylogenetic analyses performed on *cox1* multiple alignments provided the tree depicted in Figure 2.

The analysis of the topology identified two principal groups of *A. tumida*, labelled A and B in Figure 2. Furthermore, the B lineage can be divided into two groups (B1 and B2). Groups A and B are supported by high bootstrap values (>94 %) and present a genetic distance (p-distance method) of 4 %. Groups B1 and B2 also exhibit a distance of 4 %. Group A reflects global spread and includes the SHB from North America, Australia and South Africa. The Italian specimens are all included in group B and differ from the beetles that have spread worldwide. Group B1 contains the AfricCam3 sequence and the first SHB identified in Italy [Calabria, no. 1 (2014) in Figure 1]. Interestingly, the Italian specimens form two distinct clusters within this group (identity of 98.4 %), which respectively show 98.5 and 98.3 % similarity with the AfricCam3 sequence. Group B2 includes six Italian specimens from Calabria and the only one from Sicily. All members of group B2 share an identical *cox1* haplotype. The AfricCam3 shows 96.3 % similarity with the specimen from Sicily.

Two samples belonging to group B1 (GenBank accession numbers KT380628 and KT380629) and all the sequences of samples belonging to group B2 were confirmed by cloning and sequencing.

4. DISCUSSION

The analyses performed on *cox1* genes revealed that the Italian specimens belong to clade B, which is distinct from clade A encompassing the beetles that have spread worldwide. The "Italian" clade is further split into the two clusters B1 and B2. Group B1 includes the AfricCam3 sequence and the first SHB identified in Italy. Group B2 is very homogeneous (100 % identity among the sequences) and includes specimens from Calabria and the only sample from Sicily.

The presence of AfricCam3 within clade B supports the view that the Italian SHB isolates originate from an African stock. This result differs markedly from what was observed by Lounsberry et al. (2010) in the USA, where a sample from Cameroon was identified as the outgroup, differing from the next closest isolate by 4.6 %. Hence, the occurrence of SHB in Italy appears to be due to the independent introduction from Africa and not from USA or Australia.

The phylogenetic analysis suggests that there may have been a single introduction of SHB into southern Italy. However, the subdivision of the Italian specimens into two different clusters (B1 and B2) could also suggest two separate introductions. The findings reported herein prompt different hypotheses about how SHB came to be in Italy. In the first scenario, SHB was detected for the first time, in September 2014, in the region of Calabria, following one or two separate introductions (groups B1 and B2 in the phylogenetic tree) in the same region. It was then transported to the municipality of Melilli in Sicily (as detected on 7 November 2014), through migration of the apiary that had been in the infested area of Calabria (Figure 1) between April and August 2014, before being returned to its original location (Mutinelli

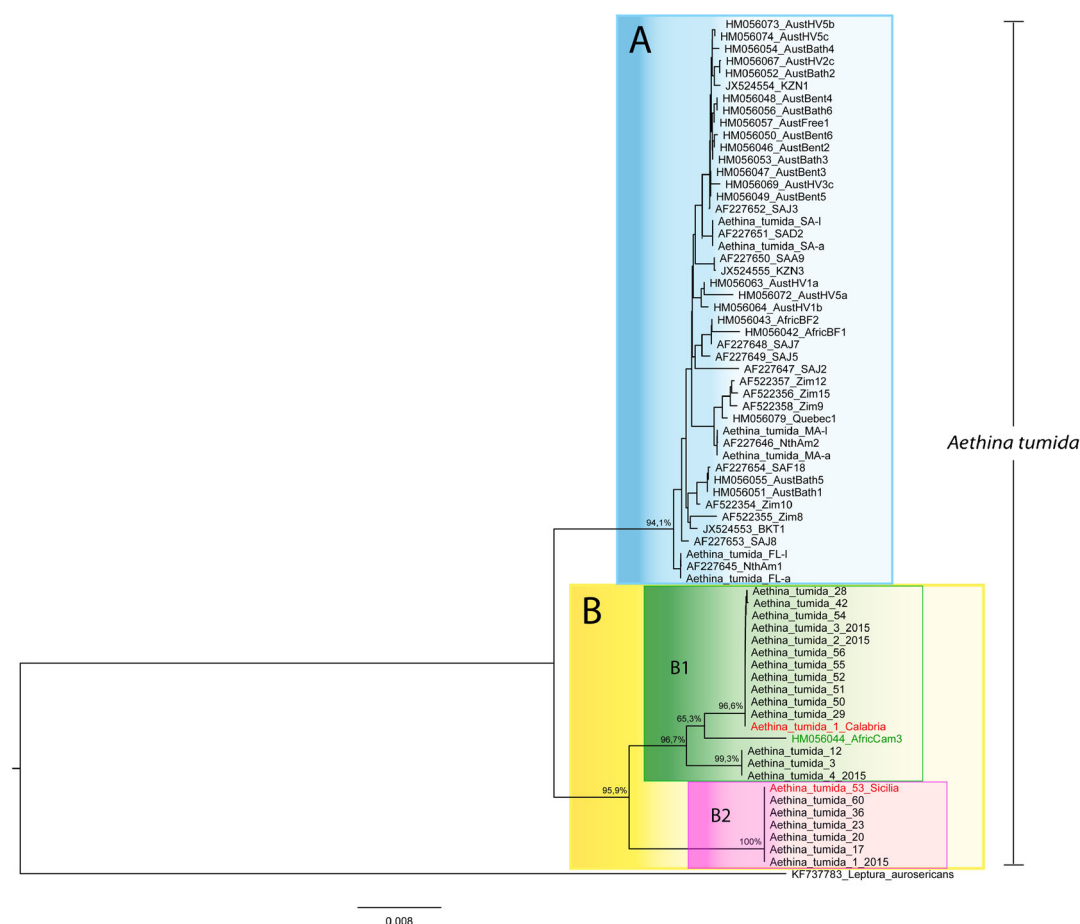


Figure 2. Neighbour-joining phylogenetic tree for the partial sequence of the *cox1* gene of *A. tumida*. All specimens dated 2015 were collected in Taurianova municipality, in the region of Calabria (from number 1_2015 to 4_2015), as indicated in Figure 1. Sequences of clades B1 and B2 are coloured as follows: *red* for the first specimen identified in Calabria (Italy) and the only specimen from Sicily (Italy) and *black* for all the other Italian specimens obtained in this study, *green* for the African sequence (AfricCam3). Bootstrap values are shown above nodes with over 70 % support

et al. 2014). Five of the six sequenced *cox1* genes of the SHBs belonging to group B2, associated with the SHB found in Sicily, appear to be grouped in a limited area north of Gioia Tauro, at a distance of between 1684 and 12,125 m from the site of first detection in 2014. One, detected between 3 and 15 October 2014, was instead located, at a distance of 18,875 m south of Gioia Tauro. The first 2015 specimen clustered in this same location (Figure 1).

The second scenario postulates two separate introductions of SHB in the regions of Calabria

and Sicily, respectively, followed by a further introduction of the SHB into Calabria through the migration of an infested apiary from the municipality of Melilli to the Gioia Tauro area between April and August 2014, before being returned to its original location. This apiary was only found positive on 7 November 2014. Since the surveillance carried out in 2014, 2015 and 2016 (present time) in the region of Sicily (<http://www.izsvenezie.com/documents/reference-laboratories/beekeeping/aethina-tumida/2015/figure-2-protection-area-sicily.pdf>) did not reveal any other infested apiaries, it seems most

unlikely that this second scenario could have occurred.

The third possible scenario is that SHB was independently introduced into both regions. Moreover, as the beetle was found to have massively reproduced at the time of the first finding in Calabria, it could have been introduced some time before its detection in September 2014. However, according to the results of epidemiological investigations and surveillance programs carried out in both regions, the most plausible scenario seems to be the introduction of SHB into Calabria followed by its man-mediated migration to Sicily, corroborating the lack of SHB detection in Sicily until November 2014.

More specimens, if available, particularly from Sicily, are needed to confirm our results, in addition to further investigations to better characterise the *A. tumida* found in Italy. A relationship cannot be ruled out between the introduction of honey bees from infested countries and the close proximity of the port of Gioia Tauro to the SHB detection sites in Calabria. Introduction to the USA occurred in similar circumstances (Hood 2000, 2004; Neumann and Elzen 2004). The results obtained from mtDNA sequence analyses of the SHB samples collected in southern Italy can be used to investigate possible further epidemiological developments of the infestation, according to the origin and characteristics of the SHB, and any impact on established diversity and genetic traits of SHB circulation across new honey bee populations. The findings can also contribute to tracking future movements of this honey bee pest, particularly through migratory beekeeping and trade, considering the existing restrictions on movements within the two affected regions of Italy and between these regions and the rest of the European Union (EU 2015b). These genetic markers have already proven useful in the detection and tracing of new incursions of pests leading to economic losses in a variety of cropping systems around the world (Ehler et al. 2004; Díaz et al. 2015; Blacket et al. 2015; Wu et al. 2015; Arnemann et al. 2016). mtDNA sequence analysis should also facilitate identification of the origins of any further beetle introductions to Europe within the framework of an EU surveillance program (Chauzat et al. 2015).

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Introduction d'*Aethina tumida* (Coleoptera: Nitidulidae) dans les régions de Calabre et de Sicile (Italie du sud)

petit coléoptère des ruches / *Apis mellifera* / gène *cox1* / infestation d'origine exotique / Italie

Einschleppung von *Aethina tumida* (Coleoptera: Nitidulidae) in die Regionen Kalabrien und Sizilien in Süditalien

***Aethina tumida* / *Apis mellifera* / *cox1* Gen / exotische Bienenpathogene / Italien**

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