



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE
DELLA RICERCA

Alma Mater Studiorum Università di Bologna Archivio istituzionale della ricerca

Analysis of honey environmental DNA indicates that the honey bee (*Apis mellifera* L.) trypanosome parasite *Lotmaria passim* is widespread in the apiaries of the North of Italy

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Ribani, A., Utzeri, V.J., Taurisano, V., Galuppi, R., Fontanesi, L. (2021). Analysis of honey environmental DNA indicates that the honey bee (*Apis mellifera* L.) trypanosome parasite *Lotmaria passim* is widespread in the apiaries of the North of Italy. *JOURNAL OF INVERTEBRATE PATHOLOGY*, 184(September 2021), 1-5 [10.1016/j.jip.2021.107628].

Availability:

This version is available at: <https://hdl.handle.net/11585/862521> since: 2022-02-21

Published:

DOI: <http://doi.org/10.1016/j.jip.2021.107628>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

1 **Short Communication**

2

3 **Analysis of honey environmental DNA indicates that the honey bee (*Apis mellifera* L.)**
4 **trypanosome parasite *Lotmaria passim* is widespread in the apiaries of the North of Italy**

5

6 Anisa Ribani^a, Valerio Joe Utzeri^a, Valeria Taurisano^a, Roberta Galuppi^b, Luca Fontanesi^{a,*}

7

8 ^a Department of Agricultural and Food Sciences, University of Bologna, Viale Giuseppe Fanin 46,
9 40127 Bologna, Italy

10 ^b Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064
11 Ozzano Emilia, Bologna, Italy

12

13 * Corresponding author: Tel: +39 051 2096535

14 Email: luca.fontanesi@unibo.it (L. Fontanesi)

15

16 **Highlights**

17

18 • Axenic culture of *L. passim* from Italian isolates was established

19 • We analysed environmental DNA extracted from honey samples to detect the presence of *L.*

20 *passim*

21 • *L. passim* was present in 78% of the honey samples collected in the North of Italy

22

23 **Abstract**

24 *Lotmaria passim* is a trypanosomatid that infects honey bees. In this study, we established an
25 axenic culture of *L. passim* from Italian isolates and then used its DNA as a control in subsequent
26 analyses that investigated environmental DNA (eDNA) to detect this trypanosomatid. The source
27 of eDNA was honey, which has been already demonstrated to be useful to detect honey bee parasites.
28 DNA from a total of 164 honey samples collected in the North of Italy was amplified with three *L.*
29 *passim* specific PCR primers and 78% of the analysed samples gave positive results. These results
30 indicated a high prevalence rate of this trypanosomatid in the North of Italy, where it might be
31 considered another threat to honey bee health.

32

33 **Keywords:** health/monitoring/parasite/PCR/Trypanosomatidae.

34 **1. Introduction**

35 Analysis of environmental DNA (eDNA), defined as DNA obtained directly from
36 environmental-related matrixes or samples, is considered an efficient, non-invasive and easy-to-
37 standardize methodology that has been proposed to facilitate the detection and monitoring of cryptic
38 or invasive organisms, that would be expensive or difficult to be sampled and identified with other
39 methods (Taberlet et al., 2012; Bohmann et al., 2014; Bass et al., 2015; Ribani et al., 2020).
40 Environmental DNA can extend and facilitate the possibility to evaluate the distribution of elusive
41 organisms, including parasites, even over time and geographic areas (Thomsen and Willerslev, 2015).
42 Different analytical approaches based on eDNA have been tested according to the objective, the
43 methods of specimen sampling and the targeted organisms (Jerde et al., 2011; Jain et al., 2013; Wilcox
44 et al., 2013).

45 Honey is a natural matrix produced by honey bees (*Apis mellifera* L.) from two main types of
46 secretions derived directly (i.e. nectar) or indirectly (i.e. honeydew) from plants. This product is
47 mainly made by sugars with other minor components, including DNA, a component that is usually
48 neglected even if it includes interesting information that can provide environment-derived
49 fingerprints (Utzeri et al., 2018a, 2018b; Bovo et al., 2018, 2020). That means that honey DNA
50 derives from all organisms that directly or indirectly are involved in its production or that are part of
51 the ecological niche in which it is produced, including parasites and pathogens of *A. mellifera*
52 (Bakonyi et al., 2003; Lauro et al., 2003; McKee et al., 2003; D'Alessandro et al., 2007; Ribani et al.,
53 2020). We recently demonstrated that this eDNA can be used for monitoring purposes of some main
54 health threats of honey bees, simplifying the possibility to obtain information on the incidence and
55 distribution of honey bee pathogens and parasites (Bovo et al., 2018, 2020; Utzeri et al., 2019; Ribani
56 et al., 2020).

57 The trypanosomatid *Lotmaria passim* is a unicellular obligate parasite that infects honey bees.
58 *Lotmaria passim* has been only recently well characterized and distinguished from other
59 trypanosomatids, particularly from *Crithidia mellificae* (Schwarz et al., 2015). In-depth molecular

60 analyses have contributed to clarify that previous studies and published DNA sequences assigned to
61 *C. mellifica* actually belonged to *L. passim*, that should be also considered the predominant and
62 widespread trypanosomatid of *A. mellifera* (Cepero et al., 2014; Ravoet et al., 2015; Schwarz et al.,
63 2015). Based on these studies, a few molecular methods have been proposed to detect *L. passim*
64 (Arismendi et al., 2016; 2020; Stevanovic et al., 2016; Vojnovic et al., 2018). Few studies have,
65 however, reported detailed information about the distribution and prevalence of this trypanosomatid
66 in different parts of the world. Recent reports have evaluated the presence and prevalence of *L. passim*
67 in some European countries, USA and South America (Arismendi et al., 2016; Stevanovic et al., 2016;
68 Vargas et al., 2017; Castelli et al., 2019; Williams et al., 2019; Michalczyk et al., 2020). At present
69 there is no detailed information in Italy, apart from a preliminary investigation that we carried out
70 using honey eDNA (Ribani et al., 2020).

71 In this study, after having characterized an axenic culture of *L. passim* that was used as source
72 of control DNA in PCR analyses, we took advantage from the possibility to use honey eDNA to obtain
73 a more detailed and specific analysis of the distribution of *L. passim* in the North of Italy.

74

75 **2. Materials and methods**

76 **2.1. Isolation and characterization of *Lotmaria passim***

77 Sixty *A. mellifera* workers were collected from an apiary in the province of Bologna (Italy),
78 where in the past the presence of intestinal flagellates had been preliminarily microscopically
79 observed. Each honey bee was immobilized by chilling at – 20 °C for 4-5 min, briefly washed in 99%
80 ethanol, and decapitated prior to dissection in a sterile environment. The intestine was removed with
81 sterile tools, submerged in 0.5 mL of supplemented DS2 medium [Insectagro DS2 serum free/protein
82 free medium without L glutamine (Corning™, NY, USA) plus 5% fetal bovine serum (Sigma Aldrich,
83 St. Louis, MO, USA) and 1% Antibiotic/Antimycotic solution (Sigma Aldrich, St.Louis, MO, USA)]
84 in a 1.5-mL microtube, gently macerated with a sterile pestle and incubated at 26 °C. Ten µL of each
85 culture was observed on wet mount slide, at light microscope with 400× objective, after 24-48 and

86 72 hours to verify the presence of free active flagellates. One out of 60 honey bees examined
87 individually was positive for flagellates. The established active culture was expanded in
88 supplemented DS2 medium and maintained by subculture steps every 4-10 days in fresh medium
89 (ratio 1:5). The procedure was a modification of the protocols reported by Runckel et al. (2011) and
90 Schwartz et al. (2015).

91 Morphological observations and image acquisition were performed on active cultures, both on
92 wet and stained slides, at 400× and 1000× magnification through Leica DMLS light microscope
93 (Leica, Wetzlar, Germany), equipped with digital camera Nikon DS-Fi2 with imaging software NIS
94 Elements 4.10.01 (Nikon, Tokyo, Japan). The first type of observations was obtained from a drop of
95 culture (10 µL) under coverslip whereas the second type was derived from a thin film of the culture
96 that was air-dried quickly and May-Grunwald Giemsa or Giemsa stained.

97 Cultures at peak density were centrifuged at 2900 rpm for 15 min. The obtained pellet was
98 washed twice in Phosphate Buffered Saline (PBS), resuspended in 200 µL of PBS and cryopreserved
99 at -20 °C until DNA extraction.

100

101 **2.2 Honey samples**

102 A total of 164 honey samples, produced in 2018, were directly provided by beekeepers. The
103 samples derived from apiaries located in all regions of the North of Italy (Liguria, n. 6; Piedmont, n.
104 10; Valle d'Aosta, n. 8; Lombardia, n. 10; Emilia-Romagna, n. 100; Trentino Alto Adige, n. 10;
105 Veneto, n. 10; and Friuli Venezia Giulia, n. 10). Geographic coordinates were used to locate the
106 production sites. Detailed information on the analysed samples is reported in Table S1.

107

108 **2.3. DNA extraction from axenic cultures and from honey samples**

109 DNA was extracted from the cultivated flagellates, subsequently identified as being from *L.*
110 *passim*, using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA)
111 following the Tissue Culture Cells manufacturer's protocol, starting from 300 µL of the axenic culture

112 containing about 1×10^6 cells. Isolated DNA from cell culture was resuspended in 30 μL of sterile
113 water and stored at $-20\text{ }^\circ\text{C}$ for further analyses. The same protocol was used to extract DNA from a
114 standard cell culture of *Crithidia mellificae*, purchased from ATCC (ATCC 30254).

115 DNA extraction from honey samples was based on the protocol described by Utzeri et al.
116 (2018c). Briefly, starting from a total of 50 g of honey divided in four 50 mL tubes, 40 mL of ultrapure
117 water was added to each tube, vortexed and incubated at $40\text{ }^\circ\text{C}$ for 30 minutes. Then tubes were
118 centrifuged for 25 min at 5000 g at room temperature and the supernatant was discarded. The pellet
119 was resuspended in 5 mL of ultrapure water and the content of the four tubes was merged in one and
120 then diluted with ultrapure water. After centrifugation for 25 min at 5000 g at room temperature, the
121 supernatant was discarded, and the pellet was resuspended in 0,5 mL of ultrapure water. The DNA
122 extraction protocol included the following steps: 1) 1 mL of CTAB extraction buffer [2% (w/v)
123 cetyltrimethylammoniumbromide; 1.4 M NaCl; 100 mM Tris-HCl; 20 mM EDTA; pH 8] and 5 μL of
124 RNase A solution (10 mg/mL) were added to each honey pre-treated sample; 2) samples were
125 incubated for 10 min at $60\text{ }^\circ\text{C}$ and, after the incubation, 30 μL of proteinase K (20 mg/mL) were
126 added; 3) subsequently, samples were incubated at $65\text{ }^\circ\text{C}$ for 90 min with gentle mixing; 4) then,
127 samples were cooled at room temperature and centrifuged for 10 min at 16,000 g; 5) 700 μL of the
128 resulting supernatant was transferred in a tube containing 500 μL of chloroform/isoamyl alcohol
129 (24:1), mixed by vortexing and centrifugated for 15 min (16,000 g at room temperature); 6) the
130 supernatant was transferred in a new 1.5 mL tube and the DNA was precipitated with 500 μL of
131 isopropanol and washed with 500 μL of ethanol 70%; 7) the resulting DNA pellets were rehydrated
132 with 30 μL of sterile H_2O and stored at $-20\text{ }^\circ\text{C}$ until PCR analyses.

133 Extracted DNA was quality checked using the nanophotometer IMPLEN P300 (Implen GmbH,
134 Munchen, Germany) and visually inspected by 1% agarose gel electrophoresis in TBE 1X buffer after
135 staining with 1X GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA).

136

137 **2.4. Primer pairs, PCR analyses, sequencing and sequence data analyses**

138 Primer pairs used in this study and PCR conditions are listed in Table S2. To assess the
139 possibility to successfully amplify the extracted DNA from honey, we first verified if amplification
140 could occur for honey bee DNA using primers designed on the mitochondrial DNA (mtDNA) region
141 of *Apis mellifera* to amplify a short fragment that had an amplification success rate from this source
142 of DNA equal to 100% (Utzeri et al., 2018c). Three primer pairs were then used to specifically
143 amplify *L. passim* DNA. One pair that targets the cytochrome b (*CYTB*) gene of *L. passim* was from
144 Stevanovic et al. (2016). Two other primer pairs were re-defined from the work of Arismendi et al.
145 (2016) to amplify two shorter DNA fragments from *L. passim* than those reported in the mentioned
146 study. This was needed to facilitate the amplification of the DNA from the degraded honey DNA. Of
147 the two new primer pairs derived from that work, the pair that amplified the *18S* fragment included
148 the same reverse primer of Arismendi et al. (2016) and a new forward primer whereas the pair that
149 amplified the *GAPDH* fragment included the same forward primer of Arismendi et al. (2016) and a
150 new reverse primer. The new primers were designed to have a 100% match with the corresponding
151 *L. passim* sequence (accession number KM066244) and to have several mismatches and frameshifts
152 in the homologous gene regions of *C. mellifica*e (e.g. accession number KJ713345). Primers were
153 selected using PRIMER3 (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>).
154 Additional PCR primers derived from studies that specifically targeted *Crithidia spp.* were also used:
155 a pair of primers that specifically amplifies a short region of the *C. mellifica*e *GAPDH* gene and a
156 pair of primers that specifically targets a short fragment of the *C. bombi TOPII* locus (Bartolomé et
157 al., 2018; Table S2).

158 PCR were performed on a 2700 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) in a
159 total volume of 20 μ L using KAPA HiFi HotStart Mastermix (Roche, Basel, Switzerland) with the
160 following PCR profile: initial denaturation step at 95 °C for 3 min, then 35 cycles of alternate
161 temperatures (20 s at 98 °C, 15 s at the specific annealing temperature for the different primer pairs
162 as indicated in Table S2, 30 s at 72 °C), followed by a final extension step at 72 °C for 1 min.
163 Amplified DNA fragments were electrophoresed in 2.5 % agarose gels in TBE 1 \times buffer and stained

164 with 1× GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA). Primer specificity was
165 tested using DNA extracted from axenic cultures of *L. passim* (reported above) and *C. mellifica*
166 (ATCC 30254). All two primer pairs specific for one of the two targeted *Crithidia spp.* (Table S2)
167 amplified this *C. mellifica* DNA.

168 Amplified fragments from pure cell culture DNA and from 30 honey DNA samples (7 µL of
169 PCR product) were purified with 1 µL of ExoSAP-IT® (USB Corporation, Cleveland, OH, USA) for
170 15 min at 37 °C and then sequenced using the BrightDye® Terminator Cycle Sequencing Kit
171 (NIMAGEN, Nijmegen, the Netherlands). Sequencing reactions were purified using EDTA 0.125 M,
172 ethanol 100% and ethanol 70%, following a standard protocol, and then were loaded on an ABI3100
173 Avant Genetic Analyzer sequencer for detection of DNA sequences (Life Technologies, Carlsbad,
174 CA, USA). Obtained electropherograms were visually inspected using MEGA 7 (Kumar et al., 2016)
175 and the BLASTn algorithm was run on the online platform BLAST
176 (<http://www.ncbi.nlm.nih.gov/BLAST/>) in order to compare and validate the assignment of the
177 obtained DNA sequences to the correct organism.

178

179 **3. Results and discussion**

180 We first established and characterized an axenic culture of a trypanosomatid, that according to
181 its features (Figure 1) was clearly consistent with *L. passim*, as described by Schwartz et al. (2015).
182 However, while the morphology of the flagellated stage of *C. mellifica* and *L. passim* can be
183 generally useful to discriminate these two species within honey bees, cryptic species may be present
184 that cannot be distinguished from one another by cell morphology, supporting the need for a genetic
185 confirmation (Schwartz et al., 2015), that was subsequently carried out by PCR analysis and
186 sequencing with primer pairs specifically designed and here tested for this purpose (Table S2). All
187 three primer pairs designed on *L. passim* (Table S2) produced the expected amplicons in the PCR
188 analyses of this newly established isolate, with 100% identity with the corresponding GenBank
189 entries (cytb: Accession no. MG494247, E-values = 8×10^{-115} ; 18S: MG182398, E-values = 9×10^{-74} ;

190 GAPDH: KX953207, E-values = 2×10^{-63}). The established axenic culture was then used to obtain
191 DNA useful for the validation of PCR tests designed to identify the presence of *L. passim* in Italian
192 colonies using its DNA footprint recovered from honey.

193 The same PCR primers were used to amplify the DNA extracted from honey produced in all
194 regions of the North of Italy by 164 different beekeepers (Figure 2; Table S1). For all samples, honey
195 bee DNA was always amplified confirming that the extracted DNA was not completely degraded and
196 could be used for subsequent analyses (Ribani et al., 2020).

197 Positive samples for *L. passim* were then considered those honey samples from which at least
198 two out of three primer pairs obtained a clearly visible amplified fragment of the expected size,
199 according to the criteria applied for general pathogen diagnosis based on PCR detection assays
200 (Sachse, 2004). A total of 128 honey samples (78%) gave positive results for this trypanosomatid
201 using the tested primer pairs. For 90% of the 128 positive samples (115 honey samples), all three
202 primer pairs produced the expected amplicons. Among the remaining 13 honey samples from which
203 only two primer pairs tested returned an amplified fragment, the largest fragment of 247 bp was not
204 amplified in 10 of them, indicating that the lack of amplification could be due to a highly degraded
205 DNA that was isolated from these problematic samples. All sequenced fragment obtained from
206 amplification of honey DNA had the same sequence already reported from the DNA of the established
207 axenic cell culture. None of the other two primer pairs designed on *Crithidia spp.* sequences produced
208 any amplified fragment from honey extracted DNA, further confirming that *L. passim* is the prevalent
209 trypanosomatid parasite in this part of Italy. Table 1 reports the percentage of positive honey samples
210 divided by administrative regions of the North of Italy. Even if the number of tested samples was not
211 proportional to the geographic areas, the frequency of positive samples ranged from 33 to 100% in
212 Liguria and Friuli Venezia Giulia regions, respectively. In the region with the highest number of
213 samples (Emilia-Romagna, n. 100), 88% were positive. These results are in line with annual
214 frequencies reported for Serbia (38.9% to 83.3%) for period 2007-2015 (Stevanovic et al., 2016).

215 Other studies, that however were based on DNA assays applied on individual bees, detected

216 frequently *C. melliferae* and *C. bombi* on *Apis mellifera* (Graystock et al., 2015; Bartolomè et al.,
217 2018) even if *L. passim* resulted the prevalent trypanosomatid in all parts of the world where the
218 prevalence of this parasite has been evaluated at large scale (e.g. Castelli et al., 2019; William et al.,
219 2019). The results we obtained for the North of Italy support the global and almost housekeeping
220 presence of *L. passim* also in the Italian Peninsula.

221

222 **4. Conclusions**

223 The trypanosome *L. passim* has been suggested as an emerging potential contributor to honey
224 bee health decline even if its role has not been completely clarified yet. In this study we first
225 established an axenic culture of *L. passim*. Then, we demonstrated the usefulness of honey eDNA in
226 monitoring studies and established the first prevalence map of *L. passim* in the North of Italy. The
227 study indicated that this trypanosomatid was present in almost all apiaries from which honey samples
228 were collected.

229

230 **5. Declaration of interest**

231 The authors declare that they have no competing financial interests or personal relationships
232 that could have appeared to influence the work reported in this paper.

233

234 **6. Acknowledgements**

235 The authors thank Alberto Contessi and Gianfranco Naldi (Osservatorio Nazionale del Miele,
236 Italy), Lucia Piana (Piana Ricerca e Consulenza srl, Italy) and all beekeepers for providing honey
237 samples. This study was funded by University of Bologna RFO program and by the BEE-RER-2
238 project, supported by Regione Emilia-Romagna (Progetto BEE- RER 2 - CUP E39J21000260007 -
239 del Regolamento (UE) n. 1308/2013 – OCM Apicoltura).

240

241 **References**

242 Arismendi, N., Bruna, A., Zapata, N., Vargas, M., 2016. PCR-specific detection of recently described
243 *Lotmaria passim* (Trypanosomatidae) in Chilean apiaries. J. Invertebr. Pathol. 134, 1–5.
244 <https://doi.org/10.1016/j.jip.2015.12.008>.

245 Arismendi, N., Caro, S., Castro, M.P., Vargas, M., Riveros, G., Venegas, T., 2020. Impact of mixed
246 infections of gut parasites *Lotmaria passim* and *Nosema ceranae* on the lifespan and immune-
247 related biomarkers in *Apis mellifera*. Insects 11, 420. <https://doi.org/10.3390/insects11070420>.

248 Bakonyi, T., Derakhshifar, I., Grabensteiner, E., Nowotny, N., 2003. Development and evaluation of
249 PCR assays for the detection of *Paenibacillus larvae* in honey samples: comparison with
250 isolation and biochemical characterization. Appl. Environ. Microb. 69, 1504-1510.
251 <https://doi.org/10.1128/AEM.69.3.1504-1510.2003>.

252 Bartolomé, C., Buendía, M., Benito, M., De la Rúa, P., Ormosa, C., Martín-Hernández, R., Higes, M.,
253 Maside, X. 2018. A new multiplex PCR protocol to detect mixed trypanosomatid infections in
254 species of *Apis* and *Bombus*. J. Invertebr. Pathol. 154, 37-41.
255 <https://doi.org/10.1016/j.jip.2018.03.015>.

256 Bass, D., Stentiford, G.D., Littlewood, D.T.J., Hartikainen, H., 2015. Diverse applications of
257 environmental DNA methods in parasitology. Trends Parasitol, 31, 499-513.
258 <https://doi.org/10.1016/j.pt.2015.06.013>.

259 Bohmann, K., Evans, A., Gilbert, M.T.P., Carvalho, G.R., Creer, S., Knapp, M., Yu D.W., De Bruyn,
260 M. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends Ecol.
261 Evol, 29, 358-367. <https://doi.org/10.1016/j.tree.2014.04.003>.

262 Bovo, S., Ribani, A., Utzeri, V.J., Schiavo, G., Bertolini, F., Fontanesi, L., 2018. Shotgun
263 metagenomics of honey DNA: Evaluation of a methodological approach to describe a multi-
264 kingdom honey bee derived environmental DNA signature. PLoS One 13, e0205575.
265 <https://doi.org/10.1371/journal.pone.0205575>.

266 Bovo, S., Utzeri, V.J., Ribani, A., Cabbri, R., Fontanesi, L., 2020. Shotgun sequencing of honey DNA
267 can describe honey bee derived environmental signatures and the honey bee hologenome
268 complexity. *Sci. Rep.* 10, 1-17. <https://doi.org/10.1038/s41598-020-66127-1>.

269 Castelli, L., Branchiccela, B., Invernizzi, C., Tomasco, I., Basualdo, M., Rodriguez, M., Zunino, P.,
270 Antúnez, K. 2019. Detection of *Lotmaria passim* in Africanized and European honey bees from
271 Uruguay, Argentina and Chile. *J. Invertebr. Pathol.* 160, 95-97.
272 <https://doi.org/10.1016/j.jip.2018.11.004>.

273 Cepero, A., Ravoet, J., Gómez-Moracho, T., Bernal, J. L., Del Nozal, M. J., Bartolomé, C., Maside,
274 X., Meana, A., González-Porto, A. V., De Graaf, D. C., Martín-Hernández, R., Higes, M. 2014
275 Holistic screening of collapsing honey bee colonies in Spain: a case study. *BMC Res. Notes* 7,
276 649. <https://doi.org/10.1186/1756-0500-7-649>

277 D'Alessandro, B., Antúnez, K., Piccini, C., Zunino, P., 2007. DNA extraction and PCR detection of
278 *Paenibacillus larvae* spores from naturally contaminated honey and bees using spore-decoating
279 and freeze-thawing techniques. *World J. Microbiol. Biotechnol.* 23, 593-597.
280 <https://doi.org/10.1007/s11274-006-9261-y>.

281 Graystock, P., Goulson, D., Hughes, W. O. 2015. Parasites in bloom: flowers aid dispersal and
282 transmission of pollinator parasites within and between bee species. *Proc. Biol. Sci.* 282,
283 20151371. <https://doi.org/10.1098/rspb.2015.1371>.

284 Jain, S.A., Jesus, F.T.D., Marchioro, G.M., Araújo, E.D.D. 2013. Extraction of DNA from honey and
285 its amplification by PCR for botanical identification. *Food Sci. Technol. (Campinas)* 33, 753-
286 756. <https://doi.org/10.1590/S0101-20612013000400022>.

287 Jerde, C. L., Mahon, A. R., Chadderton, W. L., Lodge, D. M., 2011. "Sight-unseen" detection of rare
288 aquatic species using environmental DNA. *Conserv. Lett.* 4, 150-157.
289 <https://doi.org/10.1111/j.1755-263X.2010.00158.x>.

290 Langridge, D.F., McGhee, R.B., 1967. *Crithidia mellifica* n. sp. an acidophilic trypanosomatid of
291 the honey bee *Apis mellifera*. J. Protozool, 14, 485-487. <https://doi.org/10.1111/j.1550->
292 7408.1967.tb02033.x

293 Lauro, F.M., Favaretto, M., Covolo, L., Rassa, M., Bertoloni, G., 2003. Rapid detection of
294 *Paenibacillus larvae* from honey and hive samples with a novel nested PCR protocol. Int J
295 Food Microbiol, 81, 195-201. [https://doi.org/10.1016/S0168-1605\(02\)00257-X](https://doi.org/10.1016/S0168-1605(02)00257-X).

296 Michalczyk, M., Bancercz-Kisiel, A., Sokół, R. 2020. *Lotmaria passim* as third Parasite
297 gastrointestinal tract of honey bees living in tree trunk. J. Apic. Sci., 64, 143-151.
298 <https://doi.org/10.2478/jas-2020-0012>

299 McKee, B.A., Djordjevic, S.P., Goodman, R.D., Hornitzky, M.A., 2003. The detection of
300 *Melissococcus pluton* in honey bees (*Apis mellifera*) and their products using a hemi-nested
301 PCR. Apidologie 34, 19-27. <https://doi.org/10.1051/apido:2002047>.

302 Ravoet, J., Schwarz, R.S., Descamps, T., Yañez, O., Tozkar, C.O., Martin-Hernandez, R., Bartolomé,
303 C., De Smet, L., Higes, M., Wenseleers, T., Schmid-Hempel, R., Neumann, P., Kadowaki, T.,
304 Evans, J.D., de Graaf, D., 2015. Differential diagnosis of the honey bee trypanosomatids
305 *Crithidia mellifica* and *Lotmaria passim*. J. Invertebr. Pathol. 130, 21–27.
306 <http://dx.doi.org/10.1016/j.jip.2015.06.007>.

307 Ribani, A., Utzeri, V.J., Taurisano, V., Fontanesi, L. 2020. Honey as a source of environmental DNA
308 for the detection and monitoring of honey bee pathogens and parasites. Vet. Sci. 7, 113.
309 <https://doi.org/10.3390/vetsci7030113>.

310 Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D., Andino, R., DeRisi, J.L., 2011.
311 Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal
312 prevalence of known viruses, *Nosema*, and *Crithidia*. PLoS One 6, e20656.
313 <https://doi.org/10.1371/journal.pone.0020656>

314 Sachse, K., 2004. Specificity and performance of PCR detection assays for microbial pathogens. Mol
315 Biotechnol, 26, 61-79. <https://doi.org/10.1385/MB:26:1:61>.

316 Schwarz, R.S., Bauchan, G., Murphy, C., Ravoet, J., de Graaf, D.C., Evans, J.D., 2015.
317 Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera*:
318 *Crithidia mellificae* Langridge and McGhee, 1967 and *Lotmaria passim* n. gen., n. sp. J.
319 Eukaryot. Microbiol. 62, 567–583. <http://dx.doi.org/10.1111/jeu.12209>.

320 Stevanovic, J., Schwarz, R.S., Vejnovic, B., Evans, J.D., Irwin, R.E., Glavinic, U., Stanimirovic, Z.,
321 2016. Species-specific diagnostics of *Apis mellifera* trypanosomatids: A nine-year survey
322 (2007–2015) for trypanosomatids and microsporidians in Serbian honey bees. J. Invertebr.
323 Pathol. 139, 6-11. <https://doi.org/10.1016/j.jip.2016.07.001>.

324 Taberlet, P., Coissac, E., Hajibabaei, M., Rieseberg, L.H., 2012. Environmental DNA. Towards next-
325 generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 1789-1793.
326 <https://doi.org/10.1111/j.1365-294X.2012.05470.x>.

327 Thomsen, P.F., Willerslev, E., 2015. Environmental DNA—An emerging tool in conservation for
328 monitoring past and present biodiversity. Biol. Conserv. 183, 4-18.
329 <https://doi.org/10.1016/j.biocon.2014.11.019>

330 Utzeri, V.J., Ribani, A., Fontanesi, L., 2018a. Authentication of honey based on a DNA method to
331 differentiate *Apis mellifera* subspecies: Application to Sicilian honey bee (*A. m. siciliana*) and
332 Iberian honey bee (*A. m. iberiensis*) honeys. Food Control 91, 294-301.
333 <https://doi.org/10.1016/j.foodcont.2018.04.010>.

334 Utzeri, V.J., Ribani, A., Schiavo, G., Bertolini, F., Bovo, S., Fontanesi, L., 2018b. Application of next
335 generation semiconductor based sequencing to detect the botanical composition of monofloral,
336 polyfloral and honeydew honey. Food Control 86, 342-349.
337 <https://doi.org/10.1016/j.foodcont.2017.11.033>.

338 Utzeri, V.J., Schiavo, G., Ribani, A., Tinarelli, S., Bertolini, F., Bovo, S., Fontanesi, L., 2018c.
339 Entomological signatures in honey: an environmental DNA metabarcoding approach can
340 disclose information on plant-sucking insects in agricultural and forest landscapes. Sci. Rep. 8,
341 1-13. <https://doi.org/10.1038/s41598-018-27933-w>.

- 342 Utzeri, V.J., Schiavo, G., Ribani, A., Bertolini, F., Bovo, S., Fontanesi, L., 2019. A next generation
343 sequencing approach for targeted *Varroa destructor* (Acari: Varroidae) mitochondrial DNA
344 analysis based on honey derived environmental DNA. J. Invertebr. Pathol. 161, 47-53.
345 <https://doi.org/10.1016/j.jip.2019.01.005>.
- 346 Vargas, M., Arismendi, N., Riveros, G., Zapata, N., Bruna, A., Vidal, M., Rodríguez, M., Gerding,
347 M., 2017. Viral and intestinal diseases detected in *Apis mellifera* in Central and Southern Chile.
348 Chilean J. Agr. Res. 77, 243-249. <http://dx.doi.org/10.4067/S0718-58392017000300243>.
- 349 Vejnovic, B., Stevanovic, J., Schwarz, R.S., Aleksic, N., Mirilovic, M., Jovanovic, N.M.,
350 Stanimirovic, Z., 2018. Quantitative PCR assessment of *Lotmaria passim* in *Apis mellifera*
351 colonies co-infected naturally with *Nosema ceranae*. J. Invertebr. Pathol, 151, 76-81.
352 <https://doi.org/10.1016/j.jip.2017.11.003>.
- 353 Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R., Schwartz,
354 M.K., 2013. Robust detection of rare species using environmental DNA: The importance of
355 primer specificity. PLoS One 8, e59520. <https://doi.org/10.1371/journal.pone.0059520>.
- 356 Williams, M.K.F., Tripodi, A.D., Szalanski, A.L. 2019. Molecular survey for the honey bee (*Apis*
357 *mellifera* L.) trypanosome parasites *Crithidia mellificae* and *Lotmaria passim*. J. Apicul. Res.
358 58, 553-558. <https://doi.org/10.1080/00218839.2019.1568956>.
- 359

360 **Table 1.** Frequency of honey samples produced in different regions of the North of Italy that were
361 positive for *Lotmaria passim*.

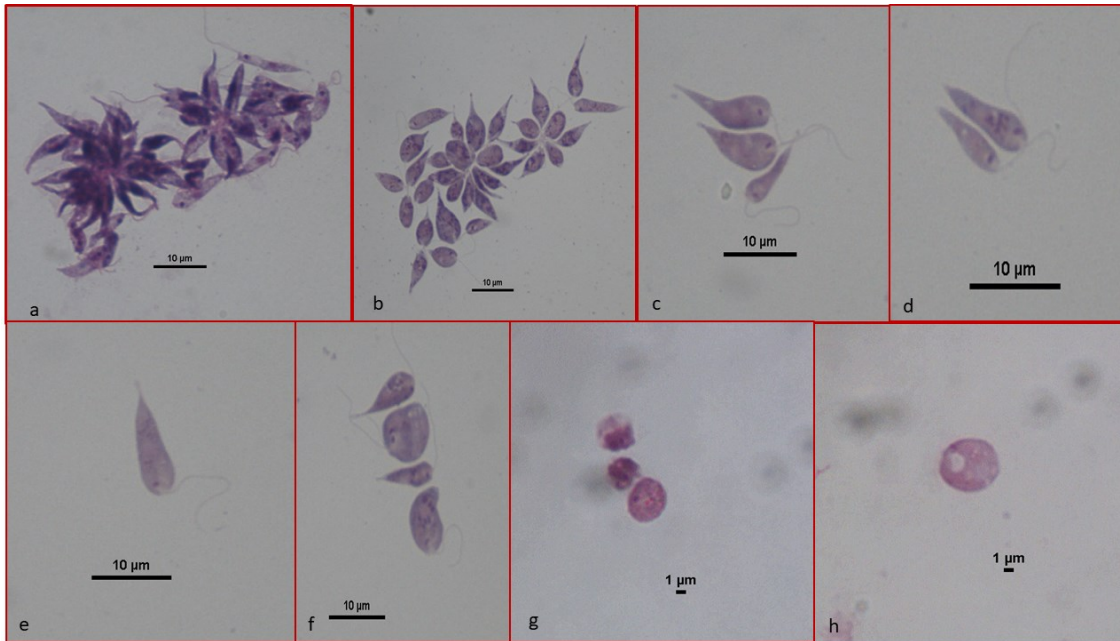
362

Region	No. of samples	Samples positive to <i>L. passim</i>	% of positive samples
Valle D'Aosta	8	5	62.5%
Piedmont	10	6	60.0%
Liguria	6	2	33.3%
Lombardia	10	5	50.0%
Emilia-Romagna	100	88	88.0%
Trentino-Alto Adige	10	7	70.0%
Veneto	10	5	50.0%
Friuli-Venezia Giulia	10	10	100.0%
Total	164	128	78.0%

363

364

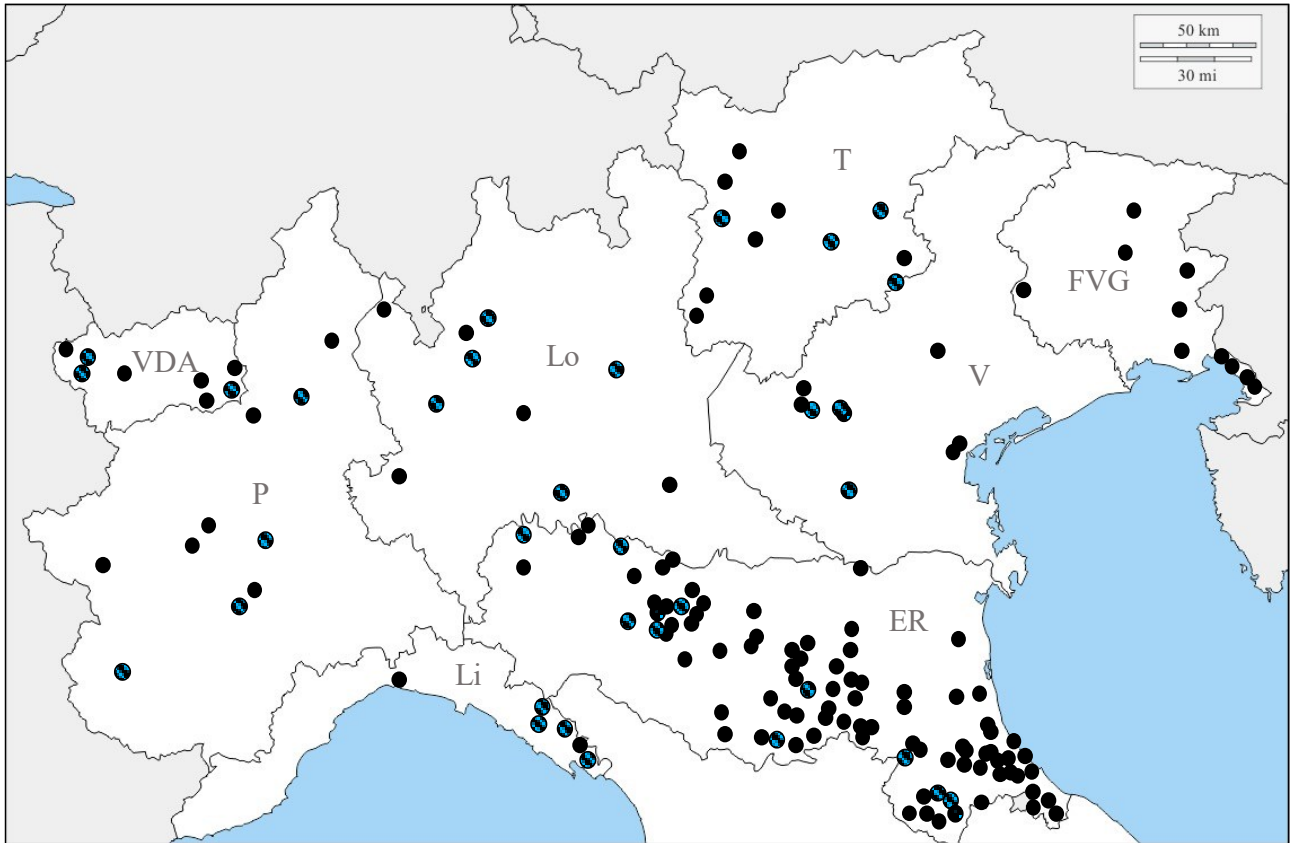
365 **Figure 1.** May Grunwald-Giemsa stained slides from culture: a) cells aggregates known as “rosettes”;
366 b) morphological polymorphism; c, d, e) promastigote morphotype tear-drop shaped, with short
367 caudate (tail-like) posterior extension; f) transitional variants and spheroid stage; g, h) spheroids
368 forms in old cultures.



369

370

371 **Figure 2.** Geographic distribution of the analysed honey samples that were tested for the presence
372 of *L. passim* DNA. Full black dots: samples that were positive; black and blue striped dots: samples
373 that were negative. The initial of the region name are shown: VDA = Valle D'Aosta; P = Piedmont;
374 Lo = Lombardia; Li= Liguria; ER = Emilia-Romagna; V = Veneto; TAA = Trentino-Alto Adige;
375 FVG = Friuli-Venezia Giulia.



376

377

378 **Supplementary Material**

379

380 **Table S1.** List of honey samples used in this study: regions, localities and provinces of origin, year of production and presence/absence of *Lotmaria*
 381 *passim* DNA.

382

Sample	Honey	Region	Locality	Municipality	Province	Year of production	<i>Lotmaria passim</i> presence
1	Mixed flower from Alpes	Valle D'Aosta	La Thuile (Mont du Parc)	La Thuile	Aosta	2018	Absent
2	Silver fir honeydew	Valle D'Aosta	Les Combes	Introd	Aosta	2018	Present
3	Rhododendron	Valle D'Aosta	La Joux	La Salle	Aosta	2018	Absent
4	Mixed flower from Alpes	Valle D'Aosta	Barasson	St Oyen	Aosta	2018	Present
5	Mixed flower from Alpes	Valle D'Aosta	Fontaney	Challand Saint Victor	Aosta	2018	Present
6	Rhododendron	Valle D'Aosta	Niel	Gaby	Aosta	2018	Present
7	Rhododendron	Valle D'Aosta	Fontainemore	Fontainemore	Aosta	2018	Absent
8	Mixed flower	Valle D'Aosta	Breil	Perloz	Aosta	2018	Present
9	Mixed flower	Piedmont	Parco delle Vallere	Moncalieri	Torino	2018	Present
10	Acacia (<i>Robinia</i>)	Piedmont	Bra	Bra	Cuneo	2018	Absent
11	Honeydew (Forest)	Piedmont	Pocapaglia	Pocapaglia	Cuneo	2018	Present
12	Mixed flower	Piedmont	Mongrando	Mongrando	Biella	2018	Present
13	Acacia (<i>Robinia</i>)	Piedmont	Vinovo (Fraz. Tetti Rosa)	Vinovo	Torino	2018	Present
14	Rhododendron	Piedmont	Fraz. Chiotti	Castelmagno	Cuneo	2018	Absent
15	Chestnut	Piedmont	Ameno	Ameno	Novara	2018	Present
16	Cherry	Piedmont	Mondoni	San Germano Chisoni	Torino	2018	Present
17	Acacia (<i>Robinia</i>)	Piedmont	Vallone	Cossato	Biella	2018	Absent
18	Acacia (<i>Robinia</i>)	Piedmont	Roncaglia	Pralormo	Torino	2018	Absent
19	Mixed flower	Liguria	Beverino	Beverino	La Spezia	2018	Absent
20	Mixed flower	Liguria	Usurana	Calice al Cornoviglio	La Spezia	2018	Absent
21	Acacia (<i>Robinia</i>)	Liguria	Sesta a Godano	Sesta a Godano	La Spezia	2018	Absent

22	Chestnut	Liguria	Santa Maria	Calice al Cornoviglio	La Spezia	2018	Present
23	Acacia (<i>Robinia</i>)	Liguria	Ziona	Carro	La Spezia	2018	Absent
24	Ailanthus	Liguria	Genova	Genova	Genova	2018	Present
25	Acacia (<i>Robinia</i>)	Lombardia	Rengione	Missaglia	Lecco	2018	Absent
26	Linden/Lime tree (<i>Tilia</i>)	Lombardia	Onno	Oliveto Lario	Lecco	2018	Present
27	Mixed flower	Lombardia	Diga nel Parco Adda Nord	Vaprio d'Adda	Milano	2018	Present
28	Acacia (<i>Robinia</i>)	Lombardia	Oggiono	Oggiono	Lecco	2018	Absent
29	Acacia (<i>Robinia</i>)	Lombardia	Iseo	Iseo	Brescia	2018	Absent
30	Mixed flower	Lombardia	Casalmoro	Casalmoro	Mantova	2018	Present
31	Acacia (<i>Robinia</i>)	Lombardia	Missaglia	Missaglia	Lecco	2018	Absent
32	Mixed flower	Lombardia	Cascina Pirolo Casello	Pizzighettone	Cremona	2018	Absent
33	Mixed flower	Lombardia	Graglio (Val Veddasca)	Maccagno - Pino - Veddasca	Varese	2018	Present
34	Acero	Lombardia	Parco del Ticino	Vigevano	Pavia	2018	Present
35	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Madonna dei Prati	Zola Predosa	Bologna	2018	Present
36	Acacia (<i>Robinia</i>)	Emilia-Romagna	Zola Vecchia	Zola Predosa	Bologna	2018	Present
37	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Zola Vecchia	Zola Predosa	Bologna	2018	Present
38	Mixed flower	Emilia-Romagna	Osteria Nuova di Monte Colombo	Montescudo - Monte Colombo	Rimini	2018	Present
39	Acacia (<i>Robinia</i>)	Emilia-Romagna	Borgo Pedrosa	Montefiore Conca	Rimini	2018	Present
40	Acacia (<i>Robinia</i>)	Emilia-Romagna	Valle del Limenta	Castel di Casio	Bologna	2018	Absent
41	Mixed flower	Emilia-Romagna	Valle del Limenta	Castel di Casio	Bologna	2018	Present
42	Chestnut	Emilia-Romagna	Sant'Andrea	Caste del Rio	Bologna	2018	Present
43	Acacia (<i>Robinia</i>)	Emilia-Romagna	Sant'Andrea	Castel del Rio	Bologna	2018	Present
44	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	San Gabriele	Minerbio	Bologna	2018	Present
45	Sulla	Emilia-Romagna	Casoni di Romagna	Monterenzio	Bologna	2018	Present
46	Acacia (<i>Robinia</i>)	Emilia-Romagna	Neviano degli Arduini	Neviano degli Arduini	Parma	2018	Absent

47	Acacia (<i>Robinia</i>)	Emilia-Romagna	Pisignano	Cervia	Ravenna	2018	Present
48	Mixed flower	Emilia-Romagna	Pisignano	Cervia	Ravenna	2018	Present
49	Mixed flower	Emilia-Romagna	Fellicarolo	Fanano	Modena	2018	Present
50	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Soliera	Soliera	Modena	2018	Present
51	Acacia (<i>Robinia</i>)	Emilia-Romagna	Mercato Saraceno	Mercato Saraceno	Forlì - Cesena	2018	Absent
52	Mixed flower	Emilia-Romagna	Bertinoro	Bertinoro	Forlì - Cesena	2018	Present
53	Mixed flower	Emilia-Romagna	Perticara	Novafeltria	Rimini	2018	Present
54	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Forlì	Forlì	Forlì - Cesena	2018	Present
55	Mixed flower	Emilia-Romagna	Castiglione di Ravenna	Castiglione di Ravenna	Ravenna	2018	Present
56	Mixed flower	Emilia-Romagna	Forlimpopoli	Forlimpopoli	Forlì - Cesena	2018	Present
57	Mixed flower	Emilia-Romagna	da Cervia a Rontagnano Sogliano	Cervia - Rontagnano Sogliano	Ravenna	2018	Present
58	Acacia (<i>Robinia</i>)	Emilia-Romagna	Mercato Saraceno	Mercato Saraceno	Forlì - Cesena	2018	Absent
59	Mixed flower	Emilia-Romagna	Longiano	Longiano	Forlì - Cesena	2018	Present
60	Acacia (<i>Robinia</i>)	Emilia-Romagna	Sorrivoli	Roncofreddo	Forlì - Cesena	2018	Present
61	Chestnut	Emilia-Romagna	Bagno di Romagna	Bagno di Romagna	Forlì - Cesena	2018	Present
62	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Bellaria	Rimini	Rimini	2018	Present
63	Mixed flower	Emilia-Romagna	San Mauro Mare	San Mauro Pascoli	Forlì - Cesena	2018	Present
64	Chestnut	Emilia-Romagna	Alfero	Alfero	Forlì - Cesena	2018	Present
65	Mixed flower	Emilia-Romagna	Gatteo	Gatteo	Forlì - Cesena	2018	Present
66	Mixed flower	Emilia-Romagna	Olmo	Gattatico	Reggio Emilia	2018	Present

67	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Pianura	Gattatico	Reggio Emilia	2018	Absent
68	Honeydew (Forest)	Emilia-Romagna	Gattatico	Gattatico	Reggio Emilia	2018	Present
69	Acacia (<i>Robinia</i>)	Emilia-Romagna	Marazzano	Montescudo	Rimini	2018	Present
70	Acacia (<i>Robinia</i>)	Emilia-Romagna	Az. Masèra	Modigliana	Forlì - Cesena	2018	Present
71	Honeydew (Forest)	Emilia-Romagna	Celle - Pergola	Faenza	Ravenna	2018	Present
72	Mixed flower	Emilia-Romagna	Monte Trebbio	Modigliana	Forlì - Cesena	2018	Present
73	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Parco della Contessa	Castel Bolognese	Ravenna	2018	Present
74	Mixed flower	Emilia-Romagna	Cà Corradini	Monterenzio	Bologna	2018	Present
75	Mixed flower	Emilia-Romagna	Antica Miniera di Bisano	Monterenzio	Bologna	2018	Present
76	Mixed flower	Emilia-Romagna	S. Maria Ripoetra	Sogliano al Rubicone	Forlì - Cesena	2018	Present
77	Acacia (<i>Robinia</i>)	Emilia-Romagna	San Polo d'Enza	San Polo d'Enza	Reggio Emilia	2018	Present
78	Bastard indago (<i>Amorpha fruticosa</i>)	Emilia-Romagna	Bodriazzo	Zibello	Parma	2018	Absent
79	Mixed flower	Emilia-Romagna	Castel San Pietro	Castel San Pietro	Bologna	2018	Present
80	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Val di Zena	Pianoro	Bologna	2018	Present
81	Mixed flower	Emilia-Romagna	Gallo Bolognese	Castel San Pietro	Bologna	2018	Present
82	Mixed flower	Emilia-Romagna	Gallo Bolognese	Castel San Pietro	Bologna	2018	Present
83	Honeydew (Forest)	Emilia-Romagna	Montechiarugolo	Montechiarugolo	Parma	2018	Present
84	Mixed flower	Emilia-Romagna	Monticelli	Montechiarugolo	Parma	2018	Absent
85	Acacia (<i>Robinia</i>)	Emilia-Romagna	Lesignano de' Bagni	Lesignano de' Bagni	Parma	2018	Present
86	Chestnut	Emilia-Romagna	San Darmiano	Camugnano	Bologna	2018	Present

87	Mixed flower	Emilia-Romagna	San Darmiano	Camugnano	Bologna	2018	Present
88	Acacia (<i>Robinia</i>)	Emilia-Romagna	Montalbano	Zocca	Modena	2018	Present
89	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	Le Budrie	San Giovanni in Persiceto	Bologna	2018	Present
90	Chestnut	Emilia-Romagna	Montalbano	Zocca	Modena	2018	Present
91	Alfalfa	Emilia-Romagna	Balsemano	Villanova sull'Arda	Piacenza	2018	Present
92	Mixed flower	Emilia-Romagna	San Polo	Torrile	Parma	2018	Present
93	Honeydew (Forest)	Emilia-Romagna	Montecchio Emilia	Montecchio Emilia	Reggio Emilia	2018	Present
94	Ailanthus	Emilia-Romagna	San Giovanni	Cavriago	Reggio Emilia	2018	Present
95	Mixed flower	Emilia-Romagna	Bertinoro	Bertinoro	Forlì - Cesena	2018	Present
96	Bastard indago (<i>Amorpha fruticosa</i>)	Emilia-Romagna	Zona Fiume Po	Villanova sull'Arda	Piacenza	2018	Present
97	Honeydew (Forest)	Emilia-Romagna	Castelnuovo Rangone	Castelnuovo Rangone	Modena	2018	Present
98	Mixed flower	Emilia-Romagna	San Polo d'Enza	San Polo d'Enza	Reggio Emilia	2018	Present
99	Acacia (<i>Robinia</i>)	Emilia-Romagna	Campremoldo Sotto	Gragnano Trebbiene	Piacenza	2018	Absent
100	Mixed flower	Emilia-Romagna	Frassineta	Monghidoro	Bologna	2018	Present
101	Honeydew (Forest)	Emilia-Romagna	Montebabbio	Castellarano	Reggio Emilia	2018	Present
102	Linden/Lime tree (<i>Tilia</i>)	Emilia-Romagna	San Polo d'Enza	San Polo d'Enza	Reggio Emilia	2018	Present
103	Honeydew (Forest)	Emilia-Romagna	Monteveglia (Parco Abbazia)	Valsamoggia	Bologna	2018	Present
104	Coriander	Emilia-Romagna	Ca' de Fabbri	Minerbio	Bologna	2018	Present
105	Sulla	Emilia-Romagna	Montecalderaro	Castel S Pietro Terme	Bologna	2018	Present
106	Mixed flower	Emilia-Romagna	San Polo d'Enza	San Polo d'Enza	Reggio Emilia	2018	Present

107	Mixed flower	Emilia-Romagna	Covignano	Rimini	Rimini	2018	Present
108	Acacia (<i>Robinia</i>)	Emilia-Romagna	Saludecio	Saludecio	Rimini	2018	Present
109	Mixed flower	Emilia-Romagna	Lama Mocogno	Lama Mocogno	Modena	2018	Present
110	Mixed flower	Emilia-Romagna	Colline di Predappio	Predappio	Forlì - Cesena	2018	Present
111	Coriander	Emilia-Romagna	Bertinoro	Bertinoro	Forlì - Cesena	2018	Present
112	Mixed flower	Emilia-Romagna	Montiano	Montiano	Forlì - Cesena	2018	Present
113	Acacia (<i>Robinia</i>)	Emilia-Romagna	Vignale	Traversetolo	Parma	2018	Absent
114	Mixed flower	Emilia-Romagna	Cesata	Tredozio	Forlì - Cesena	2018	Absent
115	Alfalfa	Emilia-Romagna	Taglio Corelli	Alfonsine	Ravenna	2018	Present
116	Mixed flower	Emilia-Romagna	Montesasso	Mercato Saraceno	Forlì - Cesena	2018	Absent
117	Acacia (<i>Robinia</i>)	Emilia-Romagna	Sasso Marconi	Sasso Marconi	Bologna	2018	Absent
118	Chestnut	Emilia-Romagna	Castelnuovo di Vergato	Vergato	Bologna	2018	Present
119	Mixed flower	Emilia-Romagna	Monte Acuto Ragazza	Grizzana Morandi	Bologna	2018	Present
120	Mixed flower	Emilia-Romagna	Acqua Partita	Bagno di Romagna	Forlì - Cesena	2018	Present
121	Acacia (<i>Robinia</i>)	Emilia-Romagna	Selbagnone	Meldola	Forlì - Cesena	2018	Present
122	Mixed flower	Emilia-Romagna	Ozzano dell'Emilia	Ozzano dell'Emilia	Bologna	2018	Present
123	Honeydew (Forest)	Emilia-Romagna	Lungo il Torrente Tiepido	Castelnuovo Rangone	Modena	2018	Present
124	Acacia (<i>Robinia</i>)	Emilia-Romagna	Tibbio	Sarsina	Forlì - Cesena	2018	Present
125	Mixed flower	Emilia-Romagna	S. Andrea in Fiume	Cesena	Forlì - Cesena	2018	Present
126	Sulla	Emilia-Romagna	Castel del Rio	Castel del Rio	Bologna	2018	Present

127	Honeydew (Forest)	Emilia-Romagna	Castal San Pietro Terme	Castal San Pietro Terme	Bologna	2018	Present
128	Mixed flower	Emilia-Romagna	Boncellino	Bagnacavallo	Ravenna	2018	Present
129	Mixed flower	Emilia-Romagna	Carpineti	Carpineti	Reggio Emilia	2018	Present
130	Ailanthus	Emilia-Romagna	Colorno	Colorno	Parma	2018	Present
131	Acacia (<i>Robinia</i>)	Emilia-Romagna	Noceto	Noceto	Parma	2018	Present
132	Honeydew (Forest)	Emilia-Romagna	Colorno	Colorno	Parma	2018	Present
133	Honeydew (Forest)	Emilia-Romagna	Ravalle	Ferrara	Ferrara	2018	Present
134	Acacia (<i>Robinia</i>)	Emilia-Romagna	Celleri	Carpaneto	Piacenza	2018	Present
135	Rhododendron	Trentino-Alto Adige	Malga Vallina d'Amola	Giustino	Trento	2018	Absent
136	Rhododendron	Trentino-Alto Adige	Malga Bissina	Val Daone	Trento	2018	Present
137	Rhododendron	Trentino-Alto Adige	Malga Lavazzè	Rumo	Trento	2018	Present
138	Dandelion	Trentino-Alto Adige	Taio	Taio	Trento	2018	Present
139	Rhododendron	Trentino-Alto Adige	Tognola	Primiero di San Martino di Castrozza	Trento	2018	Present
140	Dandelion	Trentino-Alto Adige	Fraz. Piazzola	Rabbi	Trento	2018	Present
141	Raspberry	Trentino-Alto Adige	Bellamonte	Predazzo	Trento	2018	Absent
142	Rhododendron	Trentino-Alto Adige	Valfloriana	Valfloriana	Trento	2018	Absent
143	Mixed flower from Alpes	Trentino-Alto Adige	Malga Sadron	Croviana	Trento	2018	Present
144	Rhododendron	Trentino-Alto Adige	Malga Bissina	Daone	Trento	2018	Present
145	Acacia (<i>Robinia</i>)	Veneto	Castello	Arzignano	Vicenza	2018	Absent
146	Acacia (<i>Robinia</i>)	Veneto	Arzignano	Arzignano	Vicenza	2018	Absent
147	Mixed flower	Veneto	Montagnana	Montagnana	Padova	2018	Absent

148	Mixed flower	Veneto	Val d'Alpone	Bolca	Verona	2018	Present
149	Acacia (<i>Robinia</i>)	Veneto	Volpago del Montello	Volpago del Montello	Treviso	2018	Present
150	Acacia (<i>Robinia</i>)	Veneto	Chiampo	Chiampo	Vicenza	2018	Absent
151	Rhododendron	Veneto	Passo Valles	Falcade	Belluno	2018	Absent
152	Honeydew (Forest)	Veneto	Chiampo	Chiampo	Vicenza	2018	Present
153	Mixed flower	Veneto	Pianura Bassa Padovana	Correzzola	Padova	2018	Present
154	Mixed flower	Veneto	Pianura Bassa Padovana	Correzzola	Padova	2018	Present
155	Linden/Lime tree (<i>Tilia</i>)	Friuli-Venezia Giulia	Trebiciano	Trieste	Trieste	2018	Present
156	Bastard indago (<i>Amorpha fruticosa</i>)	Friuli-Venezia Giulia	Pianura Medio Friuli	Buttrio	Udine	2018	Present
157	Mixed flower	Friuli-Venezia Giulia	Poscolle	Cavazzo Carnico	Udine	2018	Present
158	Mixed flower	Friuli-Venezia Giulia	Piscianzi-Sottomonte	Trieste	Trieste	2018	Present
159	Morello Cherry (<i>Prunus mahaleb</i>)	Friuli-Venezia Giulia	Carso triestino Santa Croce	Trieste	Trieste	2018	Present
160	<i>Erica carnea</i>	Friuli-Venezia Giulia	Monte Corno	Trasaghis	Udine	2018	Present
161	Linden/Lime tree (<i>Tilia</i>)	Friuli-Venezia Giulia	San Martino	Terzo di Aquileia	Udine	2018	Present
162	Mixed flower	Friuli-Venezia Giulia	Caneva	Caneva	Pordenone	2018	Present
163	Linden/Lime tree (<i>Tilia</i>)	Friuli-Venezia Giulia	San Pietro al Natisone	San Pietro al Natisone	Udine	2018	Present
164	Mixed flower	Friuli-Venezia Giulia	Barcola	Trieste	Trieste	2018	Present

384 **Table S2.** PCR primer pairs used in this study.

Species	Original primer pair name	Primer sequences (5'-3'): forward and reverse	Size of the amplified DNA in bp	Amplified DNA regions	Ann. T. (°C)	References
<i>Apis mellifera</i>	Apis_trnL_group_F Apis_trnL_group_R	GGCAGAATAAGTGCATTG TTAATATGAATTAAGTGGGG	C 85, M 139, A 153	mtDNA COI- COII	51	Utzeri et al. (2018)
<i>Lotmaria passim</i>	LpCytb_F1 LpCytb_R	CGAAGTGCACATATATGCTTTAC GCCAAACACCAATAACTGGTACT	247	mtDNA cytb	59	Stevanovic et al. (2016)
<i>Lotmaria passim</i>	Lp_163_F Lp2R	CATTTGACTTGAATTAGCAAGC ACCACAAGAGTACGGAATGC	163	18S	55	Arismendi et al. (2016) – Reverse – Forward: this study.
<i>Lotmaria passim</i>	Lp-gF Lp_140_R	TTGCGAAGAGCTCGCCTGAGGT GGTCGACTCGATCACGTACT	140	GAPDH	60	Arismendi et al. (2016) – Forward - Reverse: this study.
<i>Crithidia mellificae</i>	CmGAPDH-F4 CmGAPDH-R1b	CGGCGTGGACTACGTGATT ACGACGTGGTGCTTGGAC	177	GAPDH	60	Bartolomè et al. (2018)
<i>Crithidia bombi</i>	CbTOP-F1 CbTOP-R1	CGAGGTGCGGCTCAACA GATGCAGCCATTTCGGGCT	133	<i>TOPII</i>	62	Bartolomè et al. (2018)

385