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Short Communication

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

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Highlights

- Axenic culture of *L. passim* from Italian isolates was established
- We analysed environmental DNA extracted from honey samples to detect the presence of *L. passim*
- *L. passim* was present in 78% of the honey samples collected in the North of Italy

Abstract

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

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

1. Introduction

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

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

The trypanosomatid *Lotmaria passim* is a unicellular obligate parasite that infects honey bees. *Lotmaria passim* has been only recently well characterized and distinguished from other trypanosomatids, particularly from *Crithidia mellificae* (Schwarz et al., 2015). In-depth molecular analyses have contributed to clarify that previous studies and published DNA sequences assigned to *C. mellificae* actually belonged to *L. passim,* that should be also considered the predominant and widespread trypanosomatid of *A. mellifera* (Cepero et al., 2014; Ravoet et al., 2015; Schwarz et al., 2015). Based on these studies, a few molecular methods have been proposed to detect *L. passim* (Arismendi et al., 2016; 2020; Stevanovic et al., 2016; Vejnovic et al., 2018). Few studies have, however, reported detailed information about the distribution and prevalence of this trypanosomatid in different parts of the world. Recent reports have evaluated the presence and prevalence of *L. passim* in some European countries, USA and South America (Arismendi et al., 2016; Stevanovic et al., 2016; Vargas et al., 2017; Castelli et al., 2019; Williams et al., 2019; Michalczyk et al., 2020). At present there is no detailed information in Italy, apart from a preliminary investigation that we carried out using honey eDNA (Ribani et al., 2020).

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

2. Materials and methods

2.1. Isolation and characterization of *Lotmaria passim*

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

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

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

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

2.2 Honey samples

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

2.3. DNA extraction from axenic cultures and from honey samples

DNA was extracted from the cultivated flagellates, subsequently identified as being from *L. passim*, using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) following the Tissue Culture Cells manufacturer's protocol, starting from 300 µL of the axenic culture containing about 1 × 10^6 cells. Isolated DNA from cell culture was resuspended in 30 µL of sterile water and stored at -20 °C for further analyses. The same protocol was used to extract DNA from a standard cell culture of *Crithidia mellificae*, purchased from ATCC (ATCC 30254).

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

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

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

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

PCR were performed on a 2700 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) in a total volume of 20 μL using KAPA HiFi HotStart Mastermix (Roche, Basel, Switzerland) with the following PCR profile: initial denaturation step at 95 °C for 3 min, then 35 cycles of alternate temperatures (20 s at 98 °C, 15 s at the specific annealing temperature for the different primer pairs as indicated in Table S2, 30 s at 72 °C), followed by a final extension step at 72 °C for 1 min. Amplified DNA fragments were electrophoresed in 2.5 % agarose gels in TBE 1× buffer and stained with 1× GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA). Primer specificity was tested using DNA extracted from axenic cultures of *L. passim* (reported above) and *C. mellificae* (ATCC 30254). All two primer pairs specific for one of the two targeted *Crithidia spp*. (Table S2) amplified this *C. mellificae* DNA.

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

3. Results and discussion

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

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

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

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

Other studies, that however were based on DNA assays applied on individual bees, detected frequently *C. mellificae* and *C. bombi* on *Apis mellifera* (Graystock et al., 2015; Bartolomè et al., 2018) even if *L. passim* resulted the prevalent trypasonomatid in all parts of the world where the prevalence of this parasite has been evaluated at large scale (e.g. Castelli et al., 2019; William et al., 2019). The results we obtained for the North of Italy support the global and almost housekeeping presence of *L. passim* also in the Italian Peninsula.

4. Conclusions

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

5. Declaration of interest

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

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

Region	**No. of samples**	**Samples positive to *L. passim***	**% 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%**

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

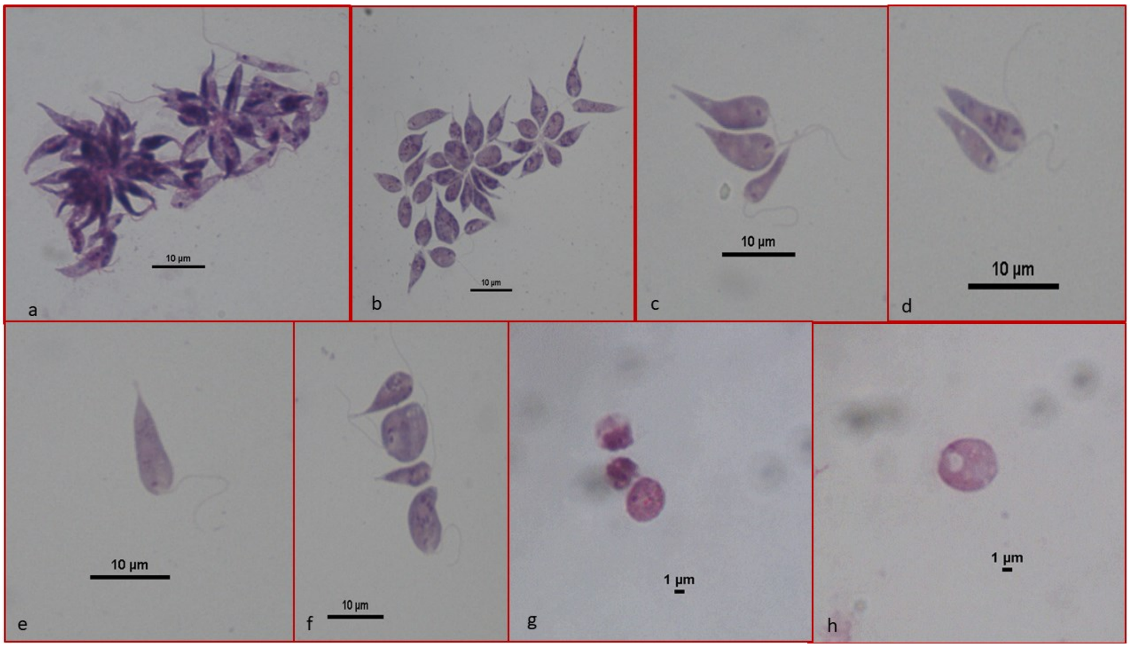
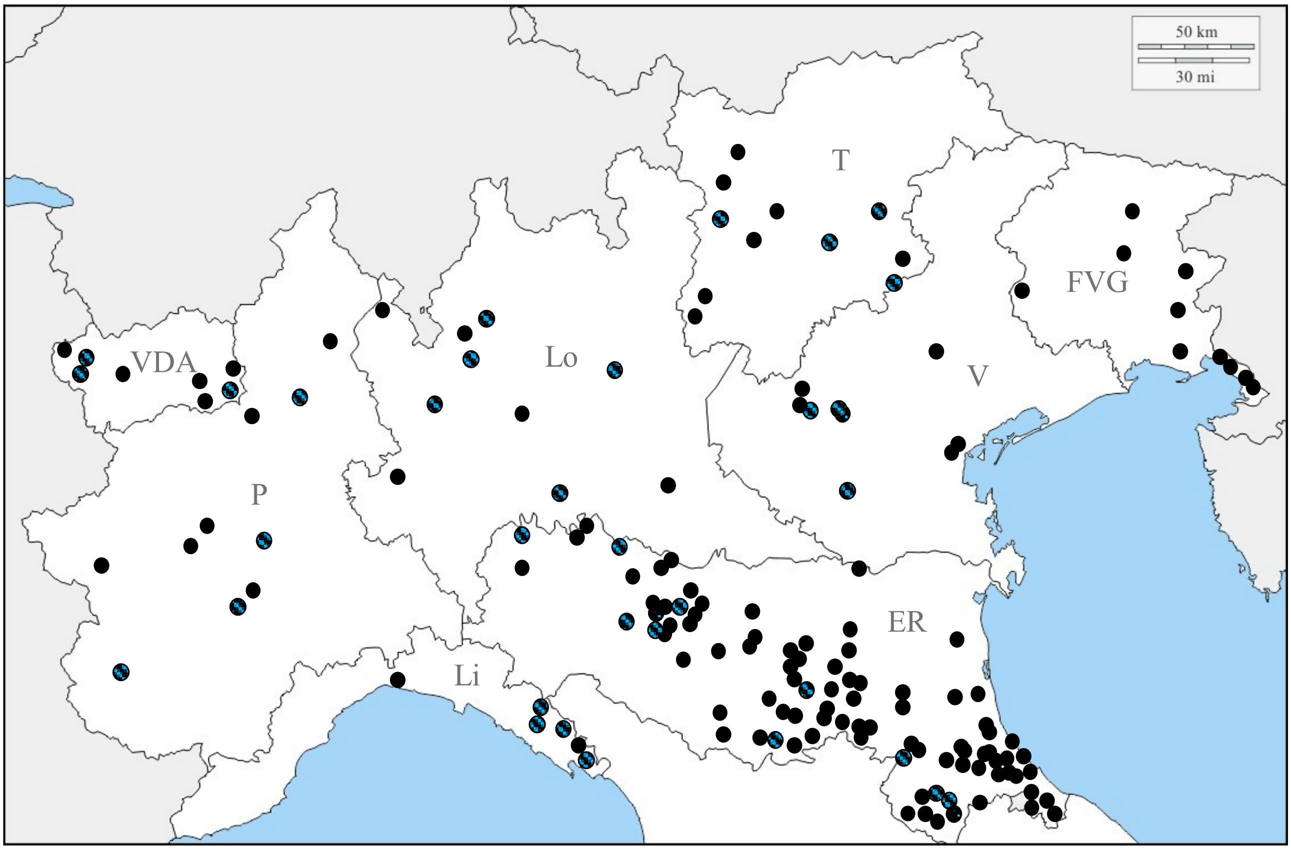


Figure 2. Geographic distribution of the analysed honey samples that were tested for the presence of *L. passim* DNA. Full black dots: samples that were positive; black and blue striped dots: samples that were negative. The initial of the region name are shown: VDA = Valle D'Aosta; P = Piedmont; Lo =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



Supplementary Material

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

Sample	Honey	Region	Locality	Municipality	Province	Year of production	*Lotmaria passim* 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 (*Robinia*)	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 (*Robinia*)	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 (*Robinia*)	Piedmont	Vallone	Cossato	Biella	2018	Absent
18	Acacia (*Robinia*)	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 (*Robinia*)	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 (*Robinia*)	Liguria	Ziona	Carro	La Spezia	2018	Absent
24	Ailanthus	Liguria	Genova	Genova	Genova	2018	Present
25	Acacia (*Robinia*)	Lombardia	Rengione	Missaglia	Lecco	2018	Absent
26	Linden/Lime tree (*Tilia*)	Lombardia	Onno	Oliveto Lario	Lecco	2018	Present
27	Mixed flower	Lombardia	Diga nel Parco Adda Nord	Vaprio d'Adda	Milano	2018	Present
28	Acacia (*Robinia*)	Lombardia	Oggiono	Oggiono	Lecco	2018	Absent
29	Acacia (*Robinia*)	Lombardia	Iseo	Iseo	Brescia	2018	Absent
30	Mixed flower	Lombardia	Casalmoro	Casalmoro	Mantova	2018	Present
31	Acacia (*Robinia*)	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 (*Tilia*)	Emilia-Roma-gna	Madonna dei Prati	Zola Predosa	Bologna	2018	Present
36	Acacia (*Robinia*)	Emilia-Roma-gna	Zola Vecchia	Zola Predosa	Bologna	2018	Present
37	Linden/Lime tree (*Tilia*)	Emilia-Roma-gna	Zola Vecchia	Zola Predosa	Bologna	2018	Present
38	Mixed flower	Emilia-Roma-gna	Osteria Nuova di Monte Co-lombo	Montescudo - Monte Colombo	Rimini	2018	Present
39	Acacia (*Robinia*)	Emilia-Roma-gna	Borgo Pedrosa	Montefiore Conca	Rimini	2018	Present
40	Acacia (*Robinia*)	Emilia-Roma-gna	Valle del Limenta	Castel di Casio	Bologna	2018	Absent
41	Mixed flower	Emilia-Roma-gna	Valle del Limenta	Castel di Casio	Bologna	2018	Present
42	Chestnut	Emilia-Roma-gna	Sant'Andrea	Caste del Rio	Bologna	2018	Present
43	Acacia (*Robinia*)	Emilia-Roma-gna	Sant'Andrea	Castel del Rio	Bologna	2018	Present
44	Linden/Lime tree (*Tilia*)	Emilia-Roma-gna	San Gabriele	Minerbio	Bologna	2018	Present
45	Sulla	Emilia-Roma-gna	Casoni di Romagna	Monterenzio	Bologna	2018	Present
46	Acacia (*Robinia*)	Emilia-Roma-gna	Neviano degli Arduini	Neviano degli Arduini	Parma	2018	Absent

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

67	Linden/Lime tree (*Tilia*)	Emilia-Romagna	Pianura	Gattatico	Reggio Emilia	2018	Absent
68	Honeydew (Forest)	Emilia-Romagna	Gattatico	Gattatico	Reggio Emilia	2018	Present
69	Acacia (*Robinia*)	Emilia-Romagna	Marazzano	Montescudo	Rimini	2018	Present
70	Acacia (*Robinia*)	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 (*Tilia*)	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 (*Robinia*)	Emilia-Romagna	San Polo d'Enza	San Polo d'Enza	Reggio Emilia	2018	Present
78	Bastard indago (*Amorpha fruticosa*)	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 (*Tilia*)	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 (*Robinia*)	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 (*Robinia*)	Emilia-Romagna	Montalbano	Zocca	Modena	2018	Present
89	Linden/Lime tree (*Tilia*)	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 (*Amorpha fruticosa*)	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 (*Robinia*)	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 (*Tilia*)	Emilia-Romagna	San Polo d'Enza	San Polo d'Enza	Reggio Emilia	2018	Present
103	Honeydew (Forest)	Emilia-Romagna	Monteveglio (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 (*Robinia*)	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 (*Robinia*)	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 (*Robinia*)	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 (*Robinia*)	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 (*Robinia*)	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-Roma-gna	Castal San Pietro Terme	Castal San Pietro Terme	Bologna	2018	Present
128	Mixed flower	Emilia-Roma-gna	Boncellino	Bagnacavallo	Ravenna	2018	Present
129	Mixed flower	Emilia-Roma-gna	Carpineti	Carpineti	Reggio Emilia	2018	Present
130	Ailanthus	Emilia-Roma-gna	Colorno	Colorno	Parma	2018	Present
131	Acacia (*Robinia*)	Emilia-Roma-gna	Noceto	Noceto	Parma	2018	Present
132	Honeydew (Forest)	Emilia-Roma-gna	Colorno	Colorno	Parma	2018	Present
133	Honeydew (Forest)	Emilia-Roma-gna	Ravalle	Ferrara	Ferrara	2018	Present
134	Acacia (*Robinia*)	Emilia-Roma-gna	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 Ca-strozza	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 (*Robinia*)	Veneto	Castello	Arzignano	Vicenza	2018	Absent
146	Acacia (*Robinia*)	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 (*Robinia*)	Veneto	Volpago del Montello	Volpago del Montello	Treviso	2018	Present
150	Acacia (*Robinia*)	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 (*Tilia*)	Friuli-Venezia Giulia	Trebiciano	Trieste	Trieste	2018	Present
156	Bastard indago (*Amorpha fruticosa*)	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 (*Prunus mahaleb*)	Friuli-Venezia Giulia	Carso triestino Santa Croce	Trieste	Trieste	2018	Present
160	*Erica carnea*	Friuli-Venezia Giulia	Monte Corno	Trasaghis	Udine	2018	Present
161	Linden/Lime tree (*Tilia*)	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 (*Tilia*)	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

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
Apis mellifera	Apis_trnL_group_F Apis_trnL_group_R	GGCAGAATAAGTGCATTG TTAATATGAATTAAGTGGGG	C 85, M 139, A 153	mtDNA COI-COII	51	Utzeri et al. (2018)
Lotmaria passim	LpCytb_F1 LpCytb_R	CGAAGTGCACATATATGCTTTAC GCCAAACACCAATAACTGGTACT	247	mtDNA cytb	59	Stevanovic et al. (2016)
Lotmaria passim	Lp_163_F Lp2R	CATTTGACTTGAATTAGCAAGC ACCACAAGAGTACGGAATGC	163	18S	55	Arismendi et al. (2016) – Reverse – Forward: this study.
Lotmaria passim	Lp-gF Lp_140_R	TTGCGAAGAGCTCGCCTGAGGT GGTCGACTCGATCACGTACT	140	GAPDH	60	Arismendi et al. (2016) – Forward - Reverse: this study.
Crithidia mellificae	CmGAPDH-F4 CmGAPDH-R1b	CGGCGTGGACTACGTGATT ACGACGTGGTGCTTGGAC	177	GAPDH	60	Bartolomè et al. (2018)
Crithidia bombi	CbTOP-F1 CbTOP-R1	CGAGGTGCGGCTCAACA GATGCAGCCATTCGGGCT	133	*TOPII*	62	Bartolomè et al. (2018)