



OPEN

A novel and non-invasive method for DNA extraction from dry bee specimens

Giovanni Cilia, Simone Flaminio & Marino Quaranta

In recent years molecular techniques have been used on museum material as integrative support for classic taxonomy. This cumulative systematics approach is especially for rare or extinct specimens, and genetic analysis may be useful to discern information that is not possible to glean from live materials or morphology. To date, the extraction of DNA required at least a partial destruction of the specimens, which is not possible for all individuals, especially the types. In this study, we described a novel method to extract mitochondrial DNA (mtDNA) from pinned museum bee individuals to avoid any external morphological damage. This method was able to amplify the mtDNA *Cytochrome C oxidase subunit I (COI)* gene in bee samples collected up to 27 years ago. We tested the efficacy of this method on 72 preserved bee specimens belonging to nine species among four families, it could be used on many museums' rare and/or extinct bee species because it does not provide external morphological damages. The method could be helpful for providing ecological, taxonomic, and phylogenetic information about specimens preserved in museum collections.

Insects are currently facing global decline¹, to better understand their historic patterns and assess biodiversity without further depleting populations, there is a necessity to turn to museums. Genetic and genomic techniques and their applications have proved to be a useful complement to morphological taxonomy². The array of molecular approaches is effective in delineating evolutionary boundaries, it does not replace the critical role of classic morphological taxonomy³. Indeed, classic taxonomy remains a fundamental discipline at the basis of biological sciences, which the advent of molecular approaches has expanded upon and enriched. For example, the molecular taxonomy has facilitated the description of delimitating species boundaries and for phylogenetic reconstruction⁴, understanding the species boundaries^{5,6} and reconstructing the species lineage⁷⁻⁹.

Insects represent 40% of all living species, this massive biodiversity has made museums a critical place for studying them, as they foster historic material on a global scale¹⁰. Molecular taxonomy, as mentioned above, has been identified as a valid instrument for museum material, making the scope of knowledge more accessible¹¹. Recently, the molecular studies to support classical taxonomy have constantly increased but they are focused especially on individuals collected for genetic analysis and preserved in a specific way to not degrade the DNA (i.e. avoid the use of ethyl acetate)⁷. Unfortunately, this does not allow the use of rare or extinct species and type material. The current approaches to DNA extraction require the total or, at least, partial destruction of the individuals^{3,7,12}, approaches that should not be used for unique specimens or specimens attributable to voucher collections. To defeat these limitations, in 2007 Gilbert and colleagues developed a method to extract DNA on museum specimens without external morphological damages¹³. This method is very successful for beetles because they are covered by a robust, hairless exoskeleton, but is unsuitable for bees, due to the treatment with digestion buffer which leads to depigmentation of hair, an important morphological character for bee taxonomy. Our project aimed to test the above-mentioned Gilbert et al.¹³ method of non-invasive DNA extraction on bee specimens, a group that has 20,000 species and is critical for biodiversity and ecosystem functions.

Results

mtDNA was successfully amplified (Fig. 1) and sequenced from 8 of the 9 bee species investigated, regardless of when they were collected (Table 1). The only species we could not amplify DNA from was *Ceratina cucurbitina* (Rossi, 1792).

No amplification was obtained from the negative control and no DNA sequence highlighted evidence of contamination or alteration, and their identity was confirmed through the BLAST analysis and the alignment

CREA Research Centre for Agriculture and Environment, Via di Corticella 133, 40128 Bologna, Italy. email: simone.flaminio@crea.gov.it

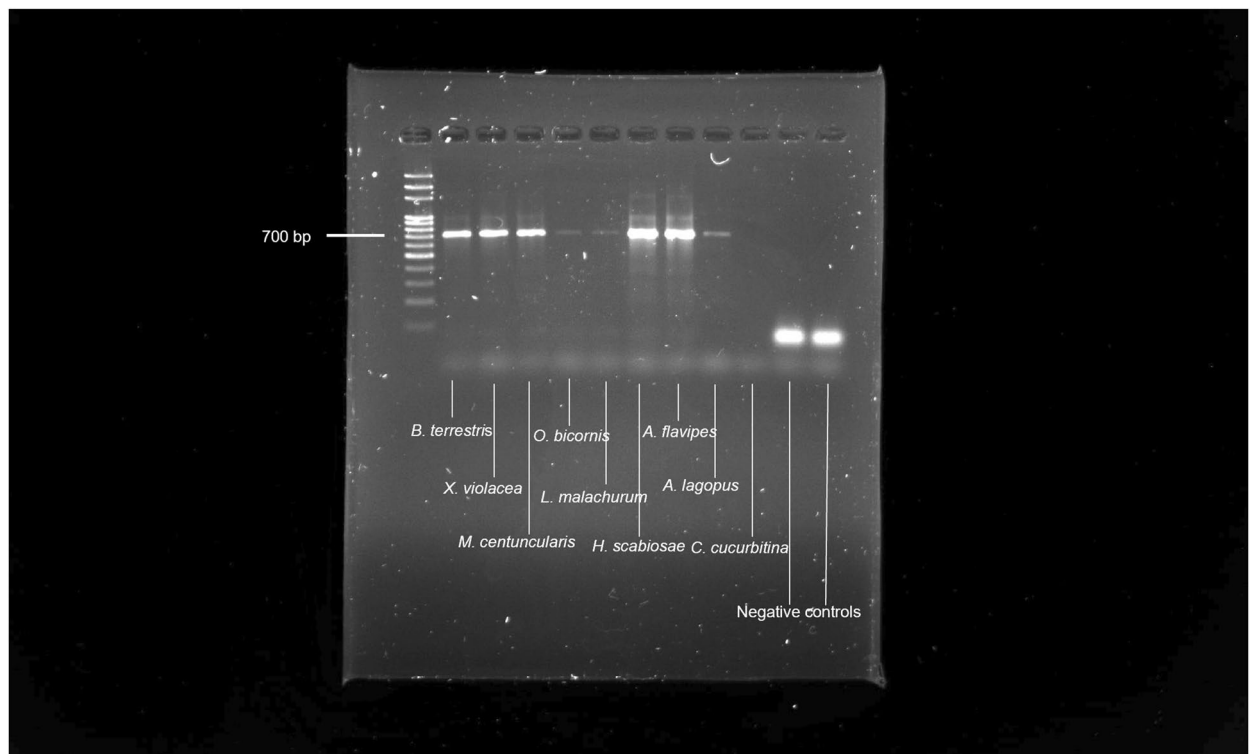


Figure 1. Gel electrophoresis of PCR performed on DNA amplified from the investigated bee species. As a negative control double distilled RNase-DNase-free water. Light bands also could mean low DNA concentration.

with sequences deposited in the Barcoding of Life Data system (BOLD system) (<https://www.boldsystems.org/index.php/>).

Sequences were deposited in GenBank (OL961135, OL966966, OL966999, OL967010, OL979169-OL979174, OL979211-OL979218, OL982531-OL982538, OL984023-OL984030; OL986022-OL986029).

Current non-destructive methods of total immersion of the individual in buffer (Fig. 2) alters the colouration of the bee's hair and diagnostic characters. Whereas our extraction method using swabs does not exhibit significant external damage or colour change, thus validating its use on important specimens' the remainder is not necessary as is implied (Fig. 3). To confirm that, all investigated specimens retained all diagnostic characters for recognition at the species level, after careful post-analysis microscopic examination. This supports the potential of the proposed method.

Discussion

Before this investigation, only a few studies showed evidence for the possibility of extracting DNA from bee specimens non-destructively. These methods required puncturing the exoskeleton, grinding body parts, or immersion in a buffer, all of which result in the destruction of the specimen in some capacity¹⁴, while the full immersion of the specimens in the digestion buffer¹³. This method did not confer any external damages to the specimens, but is not applicable for hairy insects, like bees, because the digestion buffer causes the depigmentation of hairs. We proposed here a non-invasive method that does not cause any external damages and can be done without removing the entomological pin, decreasing further damages. The method consisting of the full immersion of bee individuals in the buffer was just used to perform the DNA barcoding of a new Megachilidae species, *Trachusa vietnamensis*¹⁵. Although the extraction was successful, it was very complicated to restore the individual to an acceptable initial condition. Several drying processes were necessary to avoid changing the appearance and diagnostic characteristics, which took a total of 3 days. This makes the proposed method an important aid for the molecular identification of bee specimens.

As previously reported, in beetles the digestion buffer acts to liberate the DNA from the mouth, anus, spiracles, sclerites, ectodermal glands, and possibly broken setae, in pinned beetles, the man-made opening in the left elytron and pterothorax¹³. It remains unclear why it has not been possible to extract DNA from *C. cucurbitina* individuals, though it could be related to the size of bee individuals, as it is the smallest investigated species. The fragility along with size could also pose a problem, as in the attempt to not break the specimens, the microbiological swab was passed too gently which did not produce the required results. It is necessary to investigate this question on as many bee species as possible to know the feasibility of the proposed method across taxa.

Although DNA degradation increases over time^{16,17}, the mitochondrial DNA remain amplifiable through PCR for a long period^{13,17}. For this reason, we were able to extract DNA from investigated individuals, promoting non-destructive methods for bees. On the other hand, the possible DNA degradation can be caused by chemical reactions with ethyl acetate or ethyl alcohol (compounds usually used to kill bees upon collection)^{18–20}, which

| ID | Species | Geographic origin | Year of sampling | Amplification mtDNA | Sequence length | Accession number |
|----|---|-----------------------|------------------|---------------------|-----------------|------------------|
| 1 | <i>Bombus terrestris</i> (Linneaus., 1758) | Italy, Emilia Romagna | 2021 | Yes | 268 bp | OL986022 |
| 2 | <i>Bombus terrestris</i> (Linneaus., 1758) | Italy, Emilia Romagna | 2021 | Yes | 244 bp | OL986023 |
| 3 | <i>Bombus terrestris</i> (Linneaus., 1758) | Italy, Emilia Romagna | 2018 | Yes | 241 bp | OL986024 |
| 4 | <i>Bombus terrestris</i> (Linneaus., 1758) | Italy, Emilia Romagna | 2017 | Yes | 220 bp | OL986025 |
| 5 | <i>Bombus terrestris</i> (Linneaus., 1758) | Italy, Emilia Romagna | 2011 | Yes | 254 bp | OL986026 |
| 6 | <i>Bombus terrestris</i> (Linneaus., 1758) | Italy, Emilia Romagna | 2011 | Yes | 220 bp | OL986027 |
| 7 | <i>Bombus terrestris</i> (Linneaus., 1758) | Italy, Umbria | 1994 | Yes | 245 bp | OL986028 |
| 8 | <i>Bombus terrestris</i> (Linneaus., 1758) | Italy, Sicily | 2002 | Yes | 213 bp | OL986029 |
| 9 | <i>Xylocopa violacea</i> (L., 1758) | Italy, Campania | 2021 | Yes | 413 bp | OL966999 |
| 10 | <i>Xylocopa violacea</i> (L., 1758) | Italy, Emilia Romagna | 2021 | Yes | 317 bp | OL967010 |
| 11 | <i>Xylocopa violacea</i> (L., 1758) | Italy, Sardinia | 2017 | Yes | 367 bp | OL979169 |
| 12 | <i>Xylocopa violacea</i> (L., 1758) | Italy, Emilia Romagna | 2018 | Yes | 354 bp | OL979170 |
| 13 | <i>Xylocopa violacea</i> (L., 1758) | Italy, Emilia Romagna | 2011 | Yes | 329 bp | OL979171 |
| 14 | <i>Xylocopa violacea</i> (L., 1758) | Italy, Emilia Romagna | 2011 | Yes | 340 bp | OL979172 |
| 15 | <i>Xylocopa violacea</i> (L., 1758) | Italy, Liguria | 2003 | Yes | 355 bp | OL979173 |
| 16 | <i>Xylocopa violacea</i> (L., 1758) | Greece, Thessaly | 1992 | Yes | 335 bp | OL979174 |
| 17 | <i>Ceratina cucurbitina</i> (Rossi, 1792) | Italy, Campania | 2021 | No | nd | na |
| 18 | <i>Ceratina cucurbitina</i> (Rossi, 1792) | Italy, Campania | 2021 | No | nd | na |
| 19 | <i>Ceratina cucurbitina</i> (Rossi, 1792) | Italy, Emilia Romagna | 2017 | No | nd | na |
| 20 | <i>Ceratina cucurbitina</i> (Rossi, 1792) | Italy, Emilia Romagna | 2016 | No | nd | na |
| 21 | <i>Ceratina cucurbitina</i> (Rossi, 1792) | Italy, Emilia Romagna | 2011 | No | nd | na |
| 22 | <i>Ceratina cucurbitina</i> (Rossi, 1792) | Italy, Emilia Romagna | 2011 | No | nd | na |
| 23 | <i>Ceratina cucurbitina</i> (Rossi, 1792) | Italy, Latium | 2006 | No | nd | na |
| 24 | <i>Ceratina cucurbitina</i> (Rossi, 1792) | Italy, Latium | 2002 | No | nd | na |
| 25 | <i>Osmia bicornis</i> (Linneaus, 1758) | Italy, Veneto | 2021 | Yes | 480 bp | OL979211 |
| 26 | <i>Osmia bicornis</i> (Linneaus, 1758) | Italy, Veneto | 2021 | Yes | 367 bp | OL979212 |
| 27 | <i>Osmia bicornis</i> (Linneaus, 1758) | Italy, Tuscany | 2017 | Yes | 322 bp | OL979213 |
| 28 | <i>Osmia bicornis</i> (Linneaus, 1758) | Italy, Tuscany | 2017 | Yes | 370 bp | OL979214 |
| 29 | <i>Osmia bicornis</i> (Linneaus, 1758) | Italy, Emilia Romagna | 2011 | Yes | 211 bp | OL979215 |
| 30 | <i>Osmia bicornis</i> (Linneaus, 1758) | Italy, Emilia Romagna | 2011 | Yes | 288 bp | OL979216 |
| 31 | <i>Osmia bicornis</i> (Linneaus, 1758) | Italy, Umbria | 2003 | Yes | 308 bp | OL979217 |
| 32 | <i>Osmia bicornis</i> (Linneaus, 1758) | Italy, Umbria | 2000 | Yes | 335 bp | OL979218 |
| 33 | <i>Megachile centuncularis</i> (Linneaus, 1758) | Italy, Emilia Romagna | 2020 | Yes | 261 bp | OL981351 |
| 34 | <i>Megachile centuncularis</i> (Linneaus, 1758) | Italy, Emilia Romagna | 2020 | Yes | 238 bp | OL981352 |

Continued

| ID | Species | Geographic origin | Year of sampling | Amplification mtDNA | Sequence length | Accession number |
|----|---|-----------------------|------------------|---------------------|-----------------|------------------|
| 35 | <i>Megachile centuncularis</i> (Linnaeus, 1758) | Italy, Emilia Romagna | 2018 | Yes | 291 bp | OL981353 |
| 36 | <i>Megachile centuncularis</i> (Linnaeus, 1758) | Italy, Emilia Romagna | 2018 | Yes | 314 bp | OL981354 |
| 37 | <i>Megachile centuncularis</i> (Linnaeus, 1758) | Italy, Emilia Romagna | 2011 | Yes | 303 bp | OL981355 |
| 38 | <i>Megachile centuncularis</i> (Linnaeus, 1758) | Italy, Emilia Romagna | 2011 | Yes | 236 bp | OL981356 |
| 39 | <i>Megachile centuncularis</i> (Linnaeus, 1758) | Italy, Latium | 1997 | Yes | 300 bp | OL981357 |
| 40 | <i>Megachile centuncularis</i> (Linnaeus, 1758) | Italy, Umbria | 1996 | Yes | 311 bp | OL981358 |
| 41 | <i>Andrena flavipes</i> (Panzer, 1799) | Italy, Emilia Romagna | 2021 | Yes | 215 bp | OL982531 |
| 42 | <i>Andrena flavipes</i> (Panzer, 1799) | Italy, Campania | 2021 | Yes | 180 bp | OL982532 |
| 43 | <i>Andrena flavipes</i> (Panzer, 1799) | Italy, Emilia Romagna | 2018 | Yes | 205 bp | OL982533 |
| 44 | <i>Andrena flavipes</i> (Panzer, 1799) | Italy, Emilia Romagna | 2018 | Yes | 209 bp | OL982534 |
| 45 | <i>Andrena flavipes</i> (Panzer, 1799) | Italy, Emilia Romagna | 2011 | Yes | 186 bp | OL982535 |
| 46 | <i>Andrena flavipes</i> (Panzer, 1799) | Italy, Emilia Romagna | 2011 | Yes | 196 bp | OL982536 |
| 47 | <i>Andrena flavipes</i> (Panzer, 1799) | Italy, Umbria | 1996 | Yes | 203 bp | OL982537 |
| 48 | <i>Andrena flavipes</i> (Panzer, 1799) | Italy, Tuscany | 1994 | Yes | 172 bp | OL982538 |
| 49 | <i>Andrena lagopus</i> (Fabricius, 1775) | Italy, Emilia Romagna | 2020 | Yes | 291 bp | OL981466 |
| 50 | <i>Andrena lagopus</i> (Fabricius, 1775) | Italy, Emilia Romagna | 2020 | Yes | 286 bp | OL981467 |
| 51 | <i>Andrena lagopus</i> (Fabricius, 1775) | Italy, Emilia Romagna | 2018 | Yes | 207 bp | OL981468 |
| 52 | <i>Andrena lagopus</i> (Fabricius, 1775) | Italy, Emilia Romagna | 2018 | Yes | 205 bp | OL981469 |
| 53 | <i>Andrena lagopus</i> (Fabricius, 1775) | Italy, Emilia Romagna | 2011 | Yes | 224 bp | OL981470 |
| 54 | <i>Andrena lagopus</i> (Fabricius, 1775) | Italy, Emilia Romagna | 2011 | Yes | 215 bp | OL981471 |
| 55 | <i>Andrena lagopus</i> (Fabricius, 1775) | Italy, Latium | 1996 | Yes | 214 bp | OL981472 |
| 56 | <i>Andrena lagopus</i> (Fabricius, 1775) | Italy, Latium | 1996 | Yes | 230 bp | OL981473 |
| 57 | <i>Lasioglossum malachurum</i> (Kirby, 1802) | Italy, Piedmont | 2021 | Yes | 236 bp | OL984023 |
| 58 | <i>Lasioglossum malachurum</i> (Kirby, 1802) | Italy, Piedmont | 2021 | Yes | 226 bp | OL984024 |
| 59 | <i>Lasioglossum malachurum</i> (Kirby, 1802) | Italy, Emilia Romagna | 2018 | Yes | 197 bp | OL984025 |
| 60 | <i>Lasioglossum malachurum</i> (Kirby, 1802) | Italy, Emilia Romagna | 2018 | Yes | 236 bp | OL984026 |
| 61 | <i>Lasioglossum malachurum</i> (Kirby, 1802) | Italy, Tuscany | 2011 | Yes | 204 bp | OL984027 |
| 62 | <i>Lasioglossum malachurum</i> (Kirby, 1802) | Italy, Tuscany | 2011 | Yes | 218 bp | OL984028 |
| 63 | <i>Lasioglossum malachurum</i> (Kirby, 1802) | Italy, Sicily | 2000 | Yes | 201 bp | OL984029 |
| 64 | <i>Lasioglossum malachurum</i> (Kirby, 1802) | Italy, Latium | 1996 | Yes | 208 bp | OL984030 |
| 65 | <i>Halictus scabiosae</i> (Rossi, 1790) | Italy, Emilia Romagna | 2021 | Yes | 204 bp | OL984254 |
| 66 | <i>Halictus scabiosae</i> (Rossi, 1790) | Italy, Emilia Romagna | 2021 | Yes | 195 bp | OL984255 |
| 67 | <i>Halictus scabiosae</i> (Rossi, 1790) | Italy, Emilia Romagna | 2018 | Yes | 214 bp | OL984256 |
| 68 | <i>Halictus scabiosae</i> (Rossi, 1790) | Italy, Emilia Romagna | 2017 | Yes | 214 bp | OL984257 |

Continued

| ID | Species | Geographic origin | Year of sampling | Amplification mtDNA | Sequence length | Accession number |
|----|---|-----------------------|------------------|---------------------|-----------------|------------------|
| 69 | <i>Halictus scabiosae</i> (Rossi, 1790) | Italy, Emilia Romagna | 2011 | Yes | 193 bp | OL984258 |
| 70 | <i>Halictus scabiosae</i> (Rossi, 1790) | Italy, Emilia Romagna | 2011 | Yes | 207 bp | OL984259 |
| 71 | <i>Halictus scabiosae</i> (Rossi, 1790) | Italy, Liguria | 2004 | Yes | 207 bp | OL984260 |
| 72 | <i>Halictus scabiosae</i> (Rossi, 1790) | Italy, Liguria | 2004 | Yes | 217 bp | OL9842561 |

Table 1. Details of bee individuals analyzed in this investigation. *nd* not detected, *na* not available.

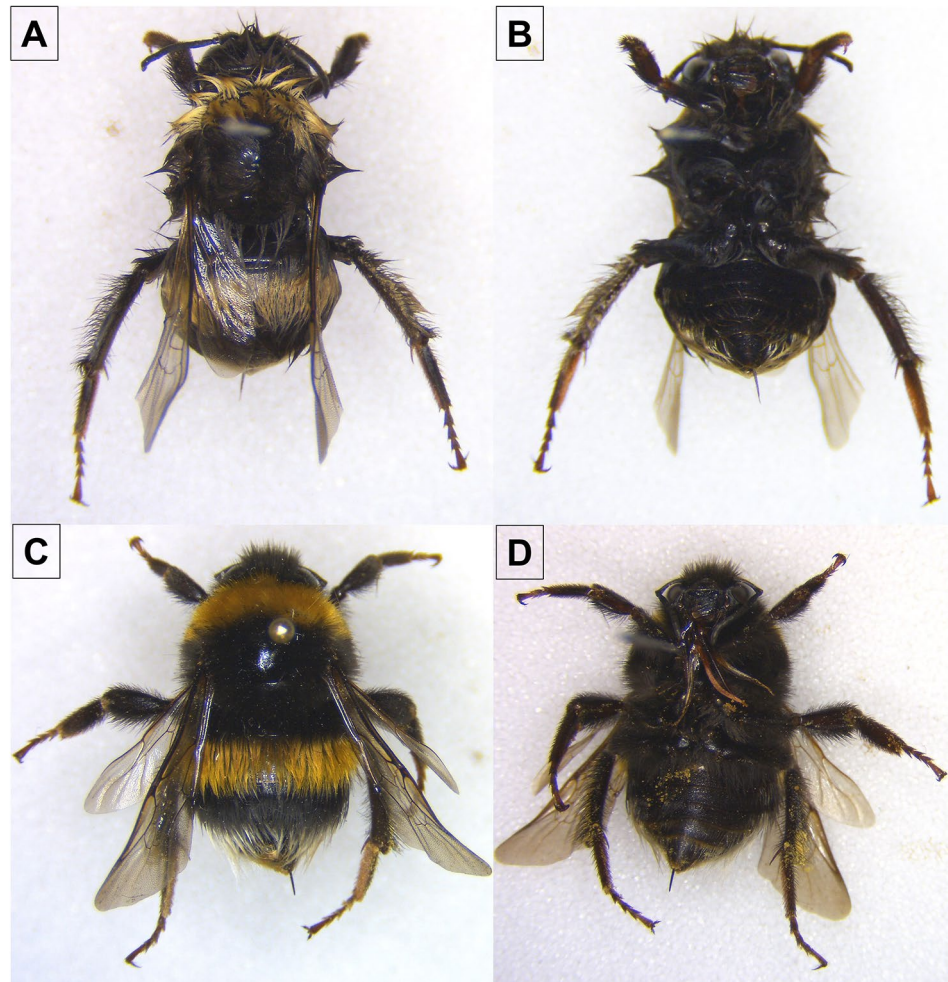


Figure 2. Dorsal and ventral comparison between the post-treatment in two *Bombus terrestris* individuals using the Gilbert et al.¹³ (A,B) and the here proposed (C,D) methods.

seems to affect DNA integrity and its. This degradation might be probably also linked to no DNA sequences obtained from all investigated *Ceratina* individuals.

The efficacy of this proposed method is also improved by the results obtained from the sequence analysis. Each obtained sequence matched (from 95 to 100% Identity) with *COI* sequences deposited. None of these sequences amplified a *Wolbachia* sp., a problem recently highlighted for the barcoding of bees²¹, which further highlights the robustness of our method.

Given the demand for the application of molecular taxonomy on museum specimens, our method can be effective. The museum collections preserved rare species and individuals collected in habitats that have changed through time, which could give not only important taxonomic and phylogenetic information but also ecological and evolutionary data. The next approach will be to test the method with museum specimens sampled more

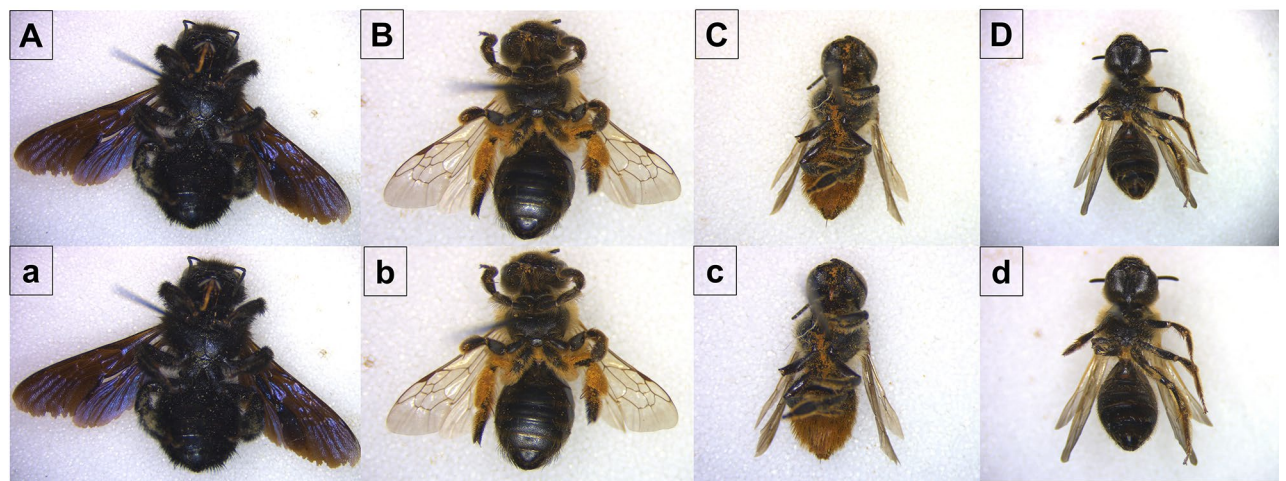


Figure 3. Ventral pictures of four bee individuals before (A–D) and after (a,b,c,d) the treatment using the swabs soaked with the digestion buffer. (A)/a *Xylocopa violacea* (L., 1758) (collected in Emilia-Romagna region in 2011), (B)/b *Andrena flavipes* (Panzer, 1799) (collected in Tuscany region in 1994), (C)/c *Megachile centuncularis* (L., 1758) (collected in Umbria region in 1996), (D)/d *Andrena lagopus* (Fabricius, 1775) (collected in Latium region in 1996).

than 30 years ago, to verify its feasibility for rare old materials. This method minimizes the risk of damaging the specimen, critical for the future of the field.

The proposed methods could increase the taxonomic information on bee individuals preserved in museums and historical entomological collections, as many of these materials are very rare and not yet investigated. Although we considered hairs as a possible source from which DNA was extracted, it cannot be ruled out that this method is also effective for even other arthropods. We have focused mainly on bees since that is our subject of study, but we hope that this method will be useful for the whole entomological research, implementing knowledge on species that are present in rare or even extinct. Due to the high heterogeneity of the insect class, it is impossible to define a single method for DNA extraction from preserved individuals in collections. This, therefore, makes it necessary to test its efficacy across taxa.

Methods

From the entomological collection of the Research Centre for Agriculture and Environment (CREA-AA), seventy-two dry bee specimens belonging to nine common and widely distributed species among 4 families, between 2021 to 1992, were selected for DNA analysis and sequencing (Table 1). All precautions have been taken to avoid environmental contamination, sterilizing the working tools and the worktop after each processed sample, and molecular works were performed in sterile conditions under a laminar flow hood. A specific digestion buffer was used for the analysis, modified from Gilbert et al.¹³, consisting of 5 mM CaCl₂, 2% sodium dodecyl sulphate (SDS), 65 mM dithiothreitol (DTT), 450 µg/ml proteinase K, 150 mM Tris buffer pH 8 and 100 mM NaCl.

The procedures were schematized in Fig. 4. A sterile microbiological swab, previously soaked in the digestion buffer for 5 min, was gently rubbed twenty times (in a total of 2 min) over the sternites of each investigated bee (Fig. 5). Due to the removal of locality and identification tags resulted very complicated, it was chosen to work on the sternites because these areas usually have few diagnostic characters related to hair. This procedure makes the proposed method even more specifically to avoid visually damaging the specimens.

Each swab was soaked in a 2 ml microtube, filled with 1 ml digestion buffer, and incubated for 18–22 h at 56 °C with gentle agitation. After the treatment, a sterile microbiological swab soaked with 100% EtOH was gently rubbed several times over the sternites to stop further digestion. Finally, the individuals were air-dried and replaced back in the collections.

Before DNA extraction, the swabs were removed from each 2 ml microtube, and DNA purification was performed using a phenol:chloroform extraction (Ultrapure™ Phenol:Chloroform:Isoamyl Alcohol, ThermoFisher Scientific, Waltham, MA, USA), following Gilbert et al.¹³. Briefly, 20 µg glycogen, 0.6 volumes 100% isopropanol and 0.1 volumes 3 M Sodium acetate (pH 5.2) were added, and the microtubes were immediately vortexed softly and centrifuged at room temperature at maximum speed (1400 g) for 30 min to collect DNA as a pellet. The supernatant was then removed, and the pellet was washed twice in 1.5 ml 85% ethanol, air-dried at 65 °C, and resuspended in 100 µl RNase-DNase-free water. The obtained DNAs were quantified using the spectrophotometer Infinite 200 PRO NanoQuant™ (TECAN Life Technologies, Männedorf, Switzerland) and placed at –20 °C until the analysis. For all of these processes, double-distilled RNase-DNase-free water was used as the negative control.

The extracted DNAs were analysed by PCR to amplify the *mtDNA* region. Primers amplified a 710-bp fragment within the highly conserved region coding for the *Cytochrome C oxidase subunit I (COI)* gene: LCO1490 (5'-GGTCAACAATCATAAAGATATTGG-3') and HC02198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3')²². The PCR was performed in 25 µl of volume using HotStarTaq Polymerase (Qiagen, Hilden, Germany) following manufacturers' instructions using 5 µl of DNA, forward and reverse probes (500 nM). The PCR assay

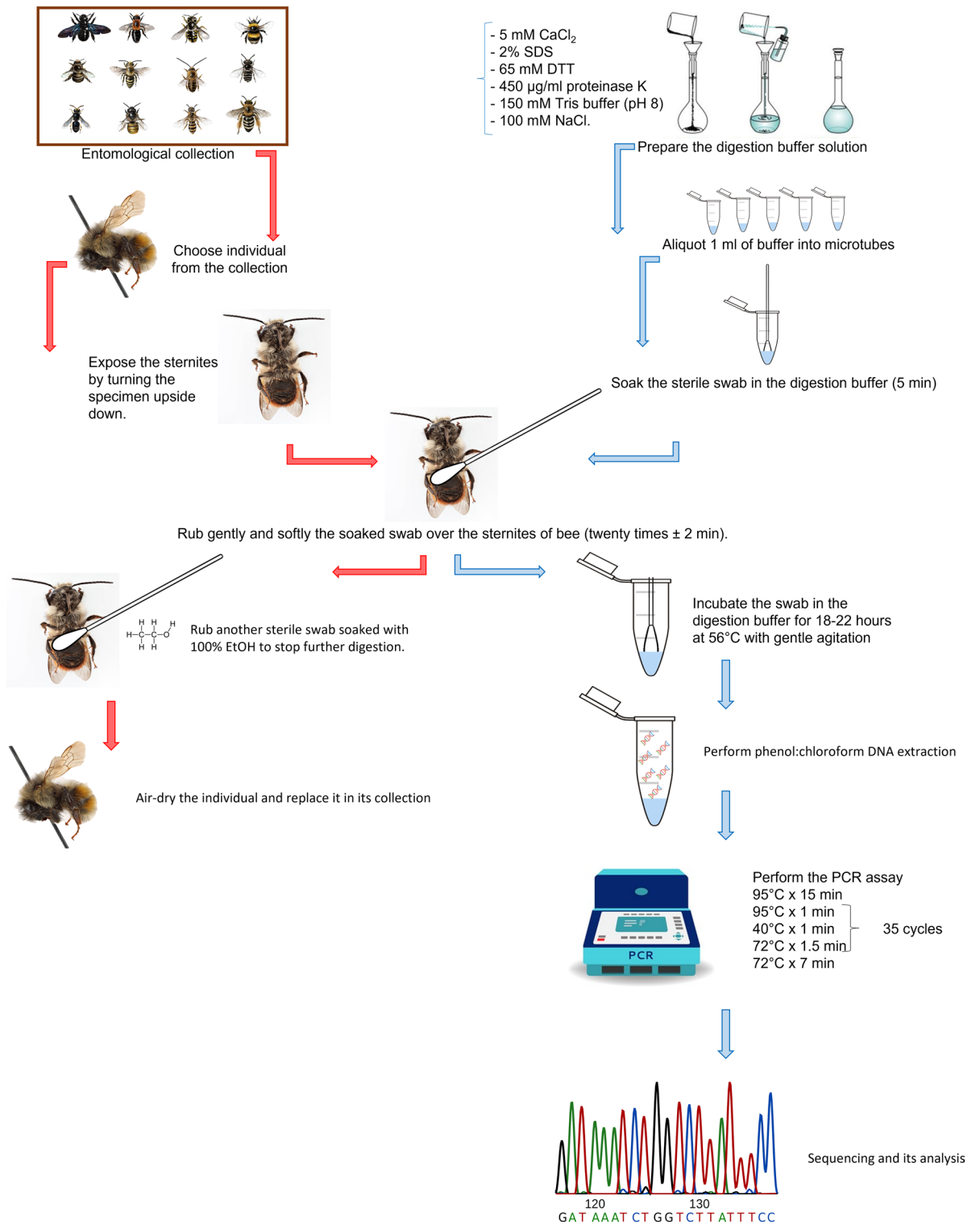


Figure 4. Schematic description of the experimental procedures.

was performed on Applied Biosystems® 2720 Thermal Cycler (ThermoFisher Scientific) and samples were amplified, after an initial activation at 95 °C for 15 min, through 35 cycles (1 min at 95 °C, 1 min at 40 °C, and 1.5 min at 72 °C), followed by a final extension at 72 °C for 7 min. All amplicons were visualized on a 1.5% agarose gel. The obtained amplicons were purified using ExoSAP-IT Express (ThermoFisher Scientific) and they were sequenced throughout the standard Sanger methodology (BMR Genomics, Padua, Italy). The obtained sequences were analysed using BioEdit²³ to create the consensus one aligning forward and reverse sequences and BLAST



Figure 5. Application of the proposed extraction method on the *Bombus terrestris* sternites.

(using megablast algorithm)²⁴ and compared to sequences deposited in the Barcode of Life Data Systems (BOLD Systems)²⁵.

Data availability

DNA sequences were deposited in GenBank (OL961135, OL966966, OL966999, OL967010, OL979169-OL979174, OL979211-OL979218, OL982531-OL982538, OL984023-OL984030; OL986022-OL986029).

Received: 9 February 2022; Accepted: 27 June 2022

Published online: 08 July 2022

References

- Rhodes, C. J. Pollinator decline—An ecological calamity in the making?. *Sci. Prog.* **101**, 121–160 (2018).
- Harrison, N. & Kidner, C. A. Next-generation sequencing and systematics: What can a billion base pairs of DNA sequence data do for you?. *Taxon* **60**, 1552–1566 (2011).
- Sperling, F. A. H. & Roe, A. D. Molecular dimensions of insect taxonomy. *Insect Biodivers. Sci. Soc.* <https://doi.org/10.1002/978144308211.CH16> (2009).
- Sharkey, M. J. *et al.* Minimalist revision and description of 403 new species in 11 subfamilies of Costa Rican braconid parasitoid wasps, including host records for 219 species. *Zookeys* **2021**, 1–665 (2021).
- Pauly, A., Noël, G., Sonet, G., Notton, D. G. & Boevé, J. L. Integrative taxonomy resuscitates two species in the *Lasioglossum villosulum* complex (Kirby, 1802) (Hymenoptera: Apoidea: Halictidae). *Eur. J. Taxon.* **2019**, 1–43 (2019).
- Pauly, A., Devalez, J., Sonet, G., Nagy, Z. T. & Boevé, J. L. DNA barcoding and male genital morphology reveal five new cryptic species in the West Palearctic bee *Seladonia smaragdula* (Vachal, 1895) (Hymenoptera: Apoidea: Halictidae). *Zootaxa* **4034**, 257–290 (2015).
- Roe, A., Dupuis, J. & Sperling, F. Molecular dimensions of insect taxonomy in the genomics era. *Insect Biodivers.* <https://doi.org/10.1002/9781118945568.CH16> (2017).
- Litman, J. R., Griswold, T. & Danforth, B. N. Phylogenetic systematics and a revised generic classification of anthidiine bees (Hymenoptera: Megachilidae). *Mol. Phylogenet. Evol.* **100**, 183–198 (2016).
- Dorchin, A., López-Urbe, M. M., Praz, C. J., Griswold, T. & Danforth, B. N. Phylogeny, new generic-level classification, and historical biogeography of the Eucera complex (Hymenoptera: Apidae). *Mol. Phylogenet. Evol.* **119**, 81–92 (2018).
- Suarez, A. V. & Tsutsui, N. D. The value of museum collections for research and society. *Bioscience* **54**, 66–74 (2004).
- Blaxter, M. L. The promise of a DNA taxonomy. *Philos. Trans. R. Soc. B Biol. Sci.* **359**, 669 (2004).
- Vogler, A. P., Desalle, R., Assmann, T., Knisley, C. B. & Schultz, T. D. Molecular population genetics of the endangered tiger beetle *Cicindela dorsalis* (Coleoptera: Cicindelidae). *Ann. Entomol. Soc. Am.* **86**, 142–152 (1993).
- Gilbert, M. T. P., Moore, W., Melchior, L. & Worebey, M. dna extraction from dry museum beetles without conferring external morphological damage. *PLoS ONE* **2**, e272 (2007).
- Phillips, A. J. & Simon, C. Simple, efficient, and nondestructive DNA extraction protocol for arthropods. *Ann. Entomol. Soc. Am.* **88**, 281–283 (1995).
- Flaminio, S., Nalinrachatakan, P., Warrit, N., Cilia, G. & Quaranta, M. *Trachusa vietnamensis*, a new species from Vietnam (Hymenoptera Apoidea Megachilidae). *Bull. Insectol.* **74**(74), 307–310 (2021).
- Rohland, N., Siedel, H. & Hofreiter, M. Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. *Biotechniques* **36**, 814–821 (2004).
- Lindahl, T. Instability and decay of the primary structure of DNA. *Nature* **362**(6422), 709–715 (1993).
- Nakahama, N., Isagi, Y. & Ito, M. Methods for retaining well-preserved DNA with dried specimens of insects. *Eur. J. Entomol.* **116**, 486–491. <https://doi.org/10.14411/eje.2019.050.html> (2019).
- Moreira, A. S., Horgan, F. G., Murray, T. E. & Kakouli-Duarte, T. Bumblebee (Hymenoptera: Apidae) sample storage for a posteriori molecular studies: Interactions between sample storage and DNA-extraction techniques. *Eur. J. Entomol.* <https://doi.org/10.14411/eje.2013.056.html> **110**, 419–425 (2013).
- Dillon, R. J. & Dillon, V. M. The gut bacteria of insects: Nonpathogenic interactions. *Annu. Rev. Entomol.* **49**, 71–92 (2004).
- Bleidorn, C. & Henze, K. A new primer pair for barcoding of bees (Hymenoptera: Anthophila) without amplifying the orthologous coxA gene of Wolbachia bacteria. *BMC Res. Notes* **14**(1), 1–6 (2021).
- Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **3**, 294–299 (1994).

23. Hall, T. A. A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98 (1999).
24. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
25. Ratnasingham, S. & Hebert, P. D. N. A DNA-based registry for all animal species: The Barcode Index Number (BIN) system. *PLoS ONE* **8**, e66213 (2013).

Acknowledgements

This research was funded by the project BeeNet (Italian National Fund under FEASR 2014-2020 from MIPAAF “Ministero delle politiche agricole alimentari e forestali”).

Author contributions

S.F. and G.C. wrote the main manuscript text, S.F. and M.Q. identified the specimens used for the barcoding, G.C. made all DNA-barcoding analyses, S.F. and G.C. prepared all pictures. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to S.F.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2022