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Development of a SNP-based tool for the identification and discrimination of *Melolontha melolontha* and *Melolontha hippocastani*

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Research Paper

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Abstract

The European (*Melolontha melolontha* L.) and Forest (*M. hippocastani* F.) cockchafer are widespread pests throughout Central Europe. Both species exhibit a 3–5-year life cycle and occur in temporally shifted populations, which have been monitored and documented for more than 100 years. Visual identification of adults and larvae belonging to these morphologically similar species requires expertise and, particularly in the case of larvae, is challenging and equivocal. The goal of the study was the development of an efficient and fast molecular genetic tool for the identification and discrimination of *M. melolontha* and *M. hippocastani*. We established a collection of both species from Switzerland, Austria and Northern Italy in 2016, 2017 and 2018. An approximately 1550 bp long fragment of the cytochrome c oxidase subunit 1 (CO1) mitochondrial gene was amplified and sequenced in 13 *M. melolontha* and 13 *M. hippocastani* beetles. Alignment of the new sequences with reference sequences (NCBI GenBank and BOLDSYSTEMS databases) and subsequent phylogenetic analysis revealed consistent clustering of the two species. After the identification of *M. melolontha* and *M. hippocastani* species-specific single nucleotide polymorphisms (SNPs) in the CO1 alignment, we developed an effective SNP tool based on the ABI PRISM® SNaPshot™ Multiplex Kit for the rapid and accurate species discrimination of adults and larvae.

Introduction

The genus *Melolontha* Fabricius, 1775 (Coleoptera: Scarabaeidae) includes approximately 60 morphologically described species distributed across the world (Li *et al.*, 2010; Sayers *et al.*, 2010; Löbl and Löbl, 2016). Amongst them, the Common cockchafer *Melolontha melolontha* Linnaeus, 1758 and the Forest cockchafer *M. hippocastani* Fabricius, 1801 are the prevalent *Melolontha* spp. in Central Europe (Keller and Zimmermann, 2005; Pozenel and Rot, 2007). A third *Melolontha* species, *M. pectoralis* Megerle, 1812 has been reported from various European countries, particularly in Eastern/South Eastern Europe (Löbl and Löbl, 2016). However, observations have been rare and its presence and distribution requires verification (Giannoulis *et al.*, 2011). *M. melolontha* and *M. hippocastani* are major pests, as the larvae feed on roots of several plant species including potato plants, grapes, fruit trees, herbs and grasses, and represent a major cause of economic loss in agriculture, forestry and horticulture (Strasser, 2004; Wagenhoff *et al.*, 2014; Sukovata *et al.*, 2015). Both species develop in soil by passing three larval instars, i.e., L1, L2 and L3, before pupation. Adults emerge in April–May and swarm along forest borders for 2–3 weeks. They feed on tree leaves, e.g., oak and beech. In severe cases, this can result in complete tree defoliation. After mating, females return to the fields from where they have emerged and deposit their eggs. *M. melolontha* generally completes its life cycle in 3 or 4 years, while the development of *M. hippocastani* usually lasts for 4 or 5 years (Jørgensen, 1960; Wagenhoff *et al.*, 2014). Populations of the Common and the Forest cockchafer have been observed in several European countries, such as Switzerland, Italy, Austria, Germany, the Czech Republic and France and have been monitored for several decades (Zweigelt, 1928; Kern and Gunthart, 1950; Richter, 1958; Muska, 1975; Ferron, 1977; Mattedi and Varner, 1996). Infested areas are typically inhabited by one temporally synchronized population of *M. melolontha* or *M. hippocastani*. However, there have been reports of the coexistence of temporally shifted populations of each of the two species in Germany and the Czech Republic (Ruther *et al.*, 2002; Švestka, 2010). Despite the considerable amount of historic and current knowledge on the spatial and temporal presence of the Common and Forest cockchafer in Europe, several ecological aspects related to the biology and life cycles of *M. melolontha* and *M. hippocastani*, and their interaction where both occur, remain unassessed. Furthermore, additional research is needed on the genetic

population structure of *M. melolontha* and *M. hippocastani* and on how it is affected by spatial separation and temporal isolation due to distinct swarming years.

Identification and discrimination of the larvae of *M. melolontha* and *M. hippocastani* based on morphological characters is difficult, and no effective dichotomous keys are available. In contrast, adults can be discriminated based on pygidium size and shape, which is longer and more slender in the Common as compared to the Forest cockchafer (Krell, 2004). Nevertheless, visual identification of *M. melolontha* and *M. hippocastani* adults in cases can be ambiguous and therefore requires expertise. Tools that allow for rapid and efficient species identification and discrimination of all stages (imaginal and preimaginal) of *M. melolontha* and *M. hippocastani*, particularly when dealing with large numbers of individuals, are relevant for an efficient assessment of species distribution and dispersal or for the development of specific and targeted control strategies. Giannoulis *et al.* (2011) have verified and confirmed the morphology-based species concept of *M. melolontha* and *M. hippocastani* studying mitochondrial cytochrome c oxidase subunit 1 (CO1) gene sequences, a standard taxon barcode used to investigate molecular evolutionary relationships in Metazoa (Lunt *et al.*, 1996; Hebert *et al.*, 2003; Dinsdale *et al.*, 2010). The CO1 gene is approximately 1600 base pairs long and includes the 710 base pair long Folmer region at the 5′ end, which has been addressed in numerous phylogenetic studies (Ståhls *et al.*, 2009; Kitahara *et al.*, 2010; Karimi *et al.*, 2012). Recently, a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach targeting the CO1 gene has been designed for the discrimination of *M. melolontha* and *M. hippocastani* (Tereba and Niemczyk, 2018). The method involves digestion of the PCR amplification product with the restriction enzyme SacI and subsequent analysis of fragment sizes. On the one hand, this approach allows for accurate *Melolontha* species identification, but on the other hand, it is based on one single polymorphic nucleotide at the restriction site.

The aim of the current study was the development of a new tool based on the characterization of single nucleotide polymorphism (SNP) in the entire CO1 gene allowing for fast, efficient and automated identification and discrimination of *M. melolontha* and *M. hippocastani* adults and larvae. We identified species-specific SNPs between *M. melolontha* and *M. hippocastani* specimens in a 1549 bp long PCR-amplified fragment of the CO1 gene. We developed a powerful, easy-to-use and rapid SNP-based tool for the identification and discrimination of adults and larvae of the Common and the Forest cockchafer.

Materials and methods

Adults and larvae of each *M. melolontha* and *M. hippocastani* were collected from four European countries and nine sites, i.e., Switzerland (five sites), Italy (two sites), France (one site) and Austria (one site). Specimens derived from samplings in 2016, 2017, 2018 or 2019 were stored at −80°C (table 1). Legs of individuals were frozen in liquid nitrogen and disrupted at 30 Hz for 30 s with the TissueLyser II (QIAGEN, Hilden, Germany). DNA was extracted with the Nucleospin® DNA Insect kit (Macherey & Nagel, Düren, Germany). Fragments of the CO1 gene were PCR-amplified from *M. melolontha* and *M. hippocastani* template DNA, respectively. PCR amplification was performed with forward primer LCO1490 (Folmer *et al.*, 1994) and reverse primer L2-N-3014 (Simon *et al.*, 1994) for *M. melolontha* adults or with forward primer N2-J-1006-Mel (Simon *et al.*, 1994) and reverse primer L2-N-3014 for *M. hippocastani* adults. PCR was performed in 20 µl containing 15 ng genomic DNA template, 0.6 mg ml^{-1} BSA, 0.2 mM dNTPs, 0.2 mM DMSO, 5x Phusion HF Buffer, 0.2 µM of forward and reverse primer, and 0.5 U Phusion Hot Start II DNA Polymerase (ThermoScientific, Waltham, MA, USA). Cycling conditions consisted of an initial denaturation of 30 s at 98°C followed by 38 cycles of 5 s at 98°C, 20 s at 57°C and 100 s at 72°C, with a final extension for 10 min at 72°C. Product quality was verified with 1%-agarose gel electrophoresis, and PCR products were purified with the Nucleospin® Gel and PCR clean-up kit (Macherey & Nagel). Purified PCR products of adult *Melolontha* spp. were sequenced with conserved and modified primers (Supplementary table S1; Folmer *et al.*, 1994; Simon *et al.*, 1994; Hajibabaei *et al.*, 2006) based on *Melolontha* spp. sequences obtained from the GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) and BOLDSYSTEMS databases (Ratnasingham and Hebert, 2007). Sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3500xL Genetic Analyser (Applied Biosystems) equipped with 50 cm capillaries and the POP-7 matrix. Sequences were assembled and manually edited using the software DNA baser® 4.7.0 (Heracle BioSoft, Mioveni, Romania). The new sequences were aligned with 40 *Melolontha* spp. sequences obtained from the BOLDSYSTEMS and GenBank databases using the software BioEdit® 7.0.9 (Ibis Biosciences, Carlsbad, CA, USA). Alignments were visually inspected and if necessary manually edited. Cluster analyses were performed using the maximum likelihood method with a General Time Reversible model plus a discrete γ distribution to model evolutionary rate differences implemented in MEGA 6.06 (Tamura *et al.*, 2013). The Kimura 2-parameter (K2P) model was used to calculate pairwise distances within *Melolontha* spp. (Kimura, 1980). *M. melolontha*-and *M. hippocastani*-specific SNPs were identified and primers were designed in conserved SNP flanking regions. SNP primers were tested on purified PCR products using the ABI PRISM® SNaPshot™ Multiplex Kit (Applied Biosystems) analysis on an ABI 3500xL (as described above). Electropherograms were analysed with GeneMarker® 2.4.0 (SoftGenetics, State College, PA, USA).

The final CO1-SNP assay protocol was defined as follows: (1) SNP-template amplification in 20 µl reaction volumes containing 15 ng genomic DNA template, 0.6 mg ml^{-1} BSA, 0.2 mM dNTPs, 1x GoTaq® Flexy Buffer, 2 mM $MgCl_2$, 0.2 µM forward N2-J-1006-Mel and reverse L2-N-3014 primers, and 1.25 U GoTaq® DNA Polymerase (Promega, Madison WI, USA). Cycling conditions consisted of an initial denaturation of 2 min at 95°C followed by 38 cycles of 30 s at 95°C, 1 min at 51°C and 100 s at 72°C, with a final extension at 72°C for 5 min. (2) SNP-template purification followed by multiplex SNP analysis including primers SNP1, SNP2 and SNP3 using kits and protocols as described above.

Data accessibility: sequences were deposited at GenBank database under the accession numbers MN699102–MN699127. Sequence alignments are available in the Supplementary Material to this article.

Results

Mitochondrial CO1 gene fragments of the approximate lengths of 1556 bp for *M. melolontha* and 2014 bp for *M. hippocastani* were amplified and sequenced from 13 *M. melolontha* and 13 *M. hippocastani* adult individuals (table 1). These 26 newly derived sequences

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Table 1. Origin of *M. melolontha* and *M. hippocastani* adults and larvae sampled for the present study.

Country	Site	Year	Coordinates WGS84	Individuals
Switzerland	Martina	2017	46°53′14″N 10°27′44″E	2 *M. melolontha* adults, 4 *M. hippocastani* adults
Switzerland	Matten	2017	46°41′10″N 7°51′42″E	2 *M. melolontha* adults
Switzerland	Strada	2017	46°52′01″N 10°26′05″E	13 *M. melolontha* adults, 12 *M. hippocastani* adults
Switzerland	Pignia	2019	46°36′50″N 9°26′27″E	4 *M. melolontha* larvae
Switzerland	Fuldera	2019	46°35′52″N 10°25′29″E	4 *M. hippocastani* larvae
Austria	Mils	2017	47°12′25″N 10°40′29″E	2 *M. melolontha* adults
France	Ingwiller	2019	48°54′27″N 7°29′17″E	7 *M. hippocastani* adults
Italy	Kastelruth	2016	46°34′55″N 11°33′54″E	2 *M. melolontha* adults
Italy	Glurns	2018	46°40′21″N 10°33′36″E	2 *M. melolontha* adults

were aligned with reference sequences of 26 *M. melolontha*, 12 *M. hippocastani* and two *M. pectoralis* individuals retrieved from either GenBank or BOLDSYSTEMS (Supplementary fig. S1). The alignment comprised 66 *Melolontha* spp. sequences, i.e., 22 full length and 44 partial sequences, and covered a 1549 bp long fragment of the CO1 gene. An internal fragment of 691 bp length (Supplementary fig. S2), which reached from position 724 to position 1414 of the 1549 bp alignment and represented the region covered by the two available *M. pectoralis* sequences, was selected to assess the relationship among *Melolontha* spp. derived sequences. The 691 bp alignment included 44 full sequences, i.e., 18 GenBank sequences, 25 new sequences (one of the 26 new sequences did not cover the region) and an *Aleochara puetzi* Assing, 1995 (Coleoptera: Staphylinidae) sequence (JQ990979.1) as outgroup. The 691 bp alignment revealed 97 parsimonious informative sites and was used to construct a Maximum likelihood dendrogram (fig. 1). The 26 *M. melolontha* and 15 *M. hippocastani* derived sequences formed two distinct clusters. *M. pectoralis* formed a sister cluster to the *M. melolontha* clade. The species grouping was supported with high (>70%) bootstrap values of 1000 iterations. The average pairwise distance within the *M. melolontha* and *M. hippocastani* clades was 0.4 and 0.1%, respectively. The average distance between the *M. melolontha* and *M. hippocastani* clades was 13.5%. The two *M. pectoralis* sequences differed at 58 positions and revealed a pairwise distance of 9.8%. The average distance was 7.8% between the *M. pectoralis* and *M. melolontha* clades and 16.2% between the *M. pectoralis* and *M. hippocastani* clades.

A total of 301 polymorphic sites (SNP) between *M. melolontha* and *M. hippocastani* were identified in the 1549 bp long alignment, of which 159 were species-specific. About half (76) of the *M. melolontha* and *M. hippocastani*-specific SNPs were located in the Folmer region. Different primers were designed in flanking regions adjacent to target nucleotides and quality tests were conducted to evaluate SNP primer efficacy. SNaPshot reactions were performed to test the primers individually in one *M. melolontha* and one *M. hippocastani* individual. The three SNP primers designed at positions 140 (SNP1), 770 (SNP2) and 1311 (SNP3) in the 1549 bp alignment (table 2; Supplementary Information fig. S1) revealed efficient extension with the complementary base and a suitable size to allow unequivocal scoring when analysed in triplex reactions. The product size determined by capillary electrophoresis differed from the expected size (primer size plus one base). SNP1, SNP2 and SNP3 products were five, nine and 13 base pairs longer in *M. melolontha* and five, seven and ten base pairs longer in *M. hippocastani*.

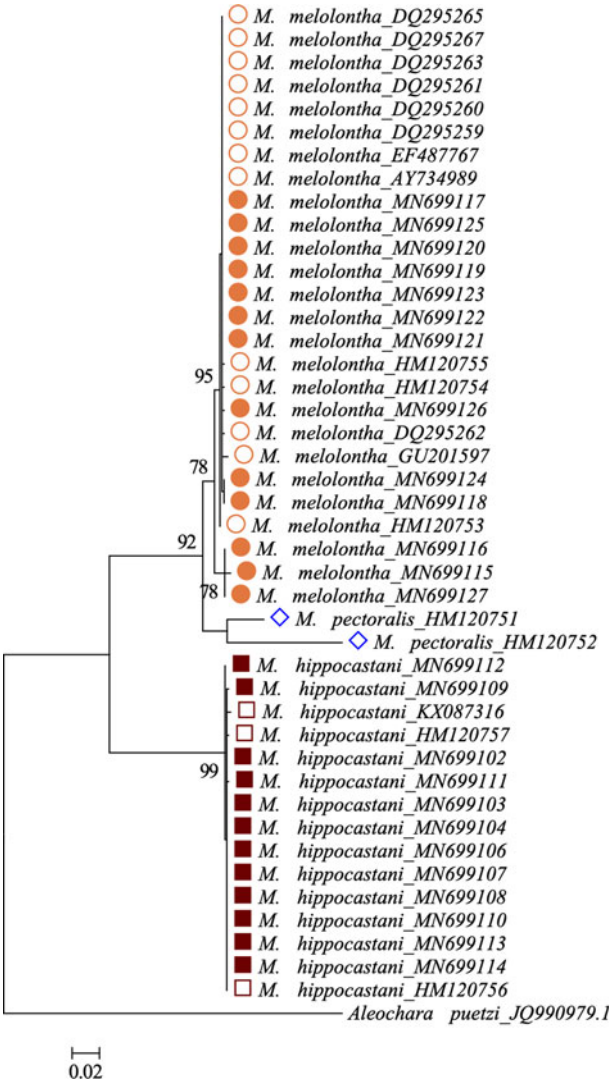

Figure 1. Phylogenetic tree based on partial CO1 sequences of 26 *M. melolontha* (orange), 15 *M. hippocastani* (brown) and two *M. pectoralis* (blue) adults. Filled symbols indicate sequences obtained in the frame of this study and open symbols indicate sequences retrieved from public databases. *Aleochara puetzi* JQ990979.1 was used as outgroup. Bootstrap values >70% of 1000 resamplings are shown. The scale represents a branch length as 0.02 substitutions per site.

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Table 2. Primers used in the triplex CO1-SNP assay and SNP detected in *M. melolontha* and *M. hippocastani*

Primer	Direction	Primer sequence (5′ to 3′)	Length	*M. melolontha*	*M. hippocastani*
SNP1	Reverse	ATAACAAAAGCRTGGGCTGTAACAATTACATTATA	35 bp	A	G
SNP2	Reverse	TATYCCTAAAGTGCCAAATGTTTCCTTTTT	30 bp	A	G
SNP3	Reverse	ATTTCAAGTTGTATAGGCATCAGG	24 bp	A	G

M. melolontha vs. *M. hippocastani* CO1-SNP assay was validated by applying the protocol to ten morphologically classified *M. melolontha* and ten *M. hippocastani* adults, which were collected in Strada (Switzerland), a site with simultaneous occurrence of both species in 2017. For all the 20 individuals, the CO1-SNP assay revealed the expected SNP profiles, which perfectly corresponded to the morphology-based species assignment. The CO1-SNP assay was further tested for its applicability to classify larvae of *M. melolontha* and *M. hippocastani*. The test included four *M. melolontha* larvae (Pignia, Switzerland) and four suspected *M. hippocastani* larvae collected from a population in a newly infested area in Switzerland (Fuldera). The developed CO1-SNP assay confirmed species identity of all four *M. melolontha* larvae and confirmed the suspected species affiliation of the four *M. hippocastani* larvae, i.e., the assay simultaneously detected the respective target SNPs in each larva.

Discussion

The use of morphological characters is the most common approach used for identifying and discriminating insect species. However, it requires thorough morphological and taxonomic expertise as differences between closely related species can be ambiguous. Furthermore, morphological identification is often restricted to the classification of adults and, for example, egg or larval stages cannot be identified. Correct larval identification is required to answer ecological questions regarding *M. melolontha* and *M. hippocastani* occurrence and opens up the potential for the establishment of targeted species-specific control strategies. Molecular genetic tools offer an efficient way to circumvent the above problems and allow for rapid and automated analysis of large numbers of samples, for instance, when analysing population genetic structure. In this study, we developed a reliable and efficient SNP-based tool for the discrimination and identification of the two common *Melolontha* species in Central Europe, i.e., *M. melolontha* and *M. hippocastani*. Importantly, the assay also allows species identification of the larvae, as shown by application of the SNP-based tool to eight second instar larvae. Species allocation of larvae is an important achievement as it bypasses the time required for the emergence of adults (3–4 years), which is typically necessary for species allocation of *Melolontha* individuals in newly infested regions.

Our molecular genetic analysis on intra- and inter-specific variations among the three Central European *Melolontha* spp., i.e., *M. melolontha*, *M. hippocastani* and *M pectoralis*, has confirmed the morphology-based species concept as well as the sequence-based results reported by Giannoulis *et al.* (2011). The latter study included a limited number of individuals, i.e., four *M. melolontha* and two *M. hippocastani* originating from France and two *M. pectoralis* originating from Greece. In this study, we confirmed previous results on samples representing a much wider range of European countries that suffer from *Melolontha* infestation. The phylogenetic analysis included 18 sequences from databases of individuals collected in Austria, Estonia, Finland, France, Germany, Iran, and Greece as well as 26 sequences from individuals collected in the frame of this study in Austria, France, Italy and Switzerland. As shown by Giannoulis *et al.* (2011), *M. melolontha* and *M. hippocastani* form distinct monophyletic clusters with a low average intraspecific genetic distance. Additional structuring was detected with *M. melolontha* individuals from Strada (MN699127) and Glurns (MN699116 and MN699115), which formed a well-supported (78% bootstrap support) subclade. The unequivocal discrimination of *M. melolontha* and *M. hippocastani* is based on a considerable amount of interspecific sequence polymorphisms. Whether the CO1 gene is suitable as a marker for the investigation of intraspecific variability and population structure awaits further analyses including larger sample numbers. Since only two sequences of *M. pectoralis* with a high average genetic distance are available in public databases, the reliability of the phylogenetic positioning and the classification of *M. pectoralis* is limited.

Identification of a genomic region that provides reliable species-specific signatures is a prerequisite for the development of a genetic tool for species identification. Our analyses of the CO1 sequence alignment resulted in the identification of 159 species-specific SNPs, confirming the suitability of the CO1 gene as a target for the development of such a tool. SNPs were located along the entire CO1 gene, including the Folmer region. Three of the designed and tested SNP flanking primers have been selected for the new tool as they enabled species identification by single base primer extension with fluorescently labelled ddNTPs using the ABI PRISM® SNaPshot™ Multiplex Kit. The SNP1 primer is positioned in the Folmer region, whereas the SNP2 and SNP3 primers are in the downstream region of the CO1 gene. The primers were designed to have different sizes, to allow simultaneous detection of the three SNP targets (i.e., three adenines in *M. melolontha* and three guanines in *M. hippocastani*) using capillary electrophoresis. As observed in other studies, the extension products were longer than expected, presumably due to the dyes used to label the ddNTPs, which affect fragment migration during electrophoresis (Tu *et al.*, 1998; Rotherham and Harbison, 2011).

The developed SNP-based tool as compared to CO1 gene sequencing targets and detects only a limited number of selected SNPs. However, the approach is fast and efficient in differentiating *M. melolontha* and *M. hippocastani* and thus preferable when simple species identification of large sample numbers is required. A similar approach was established by Tereba and Niemczyk (2018), who developed a PCR-RFLP method targeting a SNP site at position 1004 of the CO1 gene (Supplementary fig. S1) using the SacI restriction enzyme. *M. melolontha* is identified based on a successful restriction, whereas in *M. hippocastani* the restriction site is missing. The third position in a sequence codon typically suffers the highest mutation rate. In the order of Coleoptera, it is estimated to be 25.66×10^{-8} subs s^{-1} $year^{-1}$, corresponding to 25.66% per million years, whereas the

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substitution rate of the first and the second nucleotide position is assumed to be low, i.e., 0.079×10^{-8} subs s^{-1} $year^{-1}$ (Pons *et al.*, 2010). Indeed, sequence analyses revealed a point mutation at a third codon position of the restriction site in *M. melolontha* MN699115. Applying the PCR-RFLP typing method in this case would have resulted in a misidentification. Similarly, sequence analyses in *M. hippocastani* MN699111 also revealed a point mutation in the SacI restriction site, however in this case the mutation would remain undetected. The strength and big advantage of the SNP tool developed here is its ability to simultaneously reveal the identity of three distinct *M. melolontha* and *M. hippocastani* characteristic polymorphic sites, thus increasing the reliability and robustness of the test.

In this study, we developed an efficient SNP-based tool, which allows for accurate identification of *M. melolontha* and *M. hippocastani* adults and we showed that the SNP tool can be applied to larvae, enabling the identification of *M. melolontha* and *M. hippocastani* individuals at early developmental stages, which is ineffective based on morphology. The SNP tool can be employed for rapid and automated species identification/verification of large number of individuals (5 h for 200 individuals). It has thus great potential for use in ecological studies or monitoring programs as it enables accurate species classification of both adults and larvae of *M. melolontha* and *M. hippocastani*, and it might be helpful for the development of specific and targeted control strategies.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485320000784.

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