A quick PCR-based method for identification of *Melolontha melolontha* and *Melolontha hippocastani* (Coleoptera: Scarabaeidae)

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The common cockchafer (*Melolontha melolontha*) and the forest cockchafer (*Melolontha hippocastani*) are among the most destructive insect pests in many European countries. Larvae feed on the roots of numerous plant species, thus inflicting severe damage and heavy economic losses. The two species are often discussed together because they are difficult to distinguish during the larval stage. However, they differ slightly in ecology and development. The aim of this study was to develop a quick PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method for easily identifying the two *Melolontha* species through tissue samples or larvae, when reliable morphological identification is lacking. The strength of the method was tested on 43 *M. melolontha* and 37 *M. hippocastani* individuals. We demonstrate that the technique is rapid and inexpensive, with strong implications for the effective management of these insect pests.

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1. Introduction

Unique morphological features are often sufficient for species identification, but occasionally, these may be too few in number. Such is often the case for cryptic species, e.g. bats of *Myotis* spp. (*M. mystacinus* (Kuhl), *M. brandtii* (Eversmann), *M. alcathoe* von Helversen & Heller) (Boston *et al*. 2011), neotropical black flies (Hernández-Triana 2015), and *Schindleria* fishes (Kon *et al*. 2007). Species identification is essential to most biological research and requires a distinct approach from the identification of developmental stages like eggs (Freitas *et al*. 2014), larvae, or pupae (Chow *et al*. 2006). Two such pest species are the cockchafers *Melolontha melolontha* Linnaeus and *M. hippocastani* Fabricius (Scarabaeidae, Melolonthinae) in Europe.

The range of both the common cockchafer (*M. melolontha*) and the forest cockchafer (*M. hippocastani*) covers most of Europe, excluding the southernmost and northernmost territories (Sierpiński 1975). *Melolontha* spp. have a 3- to 5-year life cycle; in central Europe, the life cycle is typically 4 years (Švestka 2006, Sierpińska 2008). Adults feed on the leaves of broadleaf

Table 1. Sex, locations in two outbreak areas in Poland and date of collection for samples of two *Melolontha* species.

Samples	Species	Sex	Forest district	Sub-district	Sampling date
1–14	*M. melolontha*	Female	Smardzewice	Twarda	13–16 May 2015
15–20	*M. melolontha*	Male	Smardzewice	Twarda	13–16 May 2015
21–38	*M. hippocastani*	Female	Smardzewice	Twarda	13–16 May 2015
39–40	*M. hippocastani*	Male	Smardzewice	Twarda	13–16 May 2015
41–57	*M. hippocastani*	Male	Ostrowiec Św.	Bałtów	13–16 May 2015
58–75	*M. melolontha*	Male	Ostrowiec Św.	Bałtów	13–16 May 2015
76–80	*M. melolontha*	Female	Ostrowiec Św.	Bałtów	13–16 May 2015

trees, causing losses in agriculture, horticulture and forestry. However, cockchafer larvae (grubs) are more dangerous to biological-based production systems. In their first developmental stage (L_1), grubs feed on and damage herbaceous plant roots in the upper soil layer. Two- (L_2) and three-year-old (L_3) grubs feed on tree and shrub roots even more voraciously, making reforestation and afforestation difficult or impossible to implement. Recent mass outbreaks have established cockchafers to be the most harmful root-feeding pests in forestry, affecting nurseries, young plantations, and tree stands (Delb & Mattes 2001, Keller & Zimmermann 2005, Švestka 2006, Malinowski 2007, Giannoulis *et al.* 2011, Wagenhoff *et al.* 2014, Niemczyk 2015, Sukovata *et al*. 2015, Niemczyk *et al*. 2017).

Melolontha melolontha and *M. hippocastani* can only be clearly distinguished during the adult stage. The former is larger, with a long and slender pygidium, whereas the latter is smaller, with a short and knob-shaped pygidium. In contrast, accurate identification of these two species based only on morphological differences is extremely difficult during larval stages (lasting 3–4 years). As a result, *M. melolontha* and *M. hippocastani* are usually discussed together (Sukovata *et al*. 2015). Nonetheless, the two species show slight differences in developmental pace, habitat, and timing of the swarming period (Szujecki 1995). Some life-span, survival and weight differences also exist between the two insects when they are feeding on the same tree species (Woreta *et al*. 2016). Such differences, however, are difficult to apply in pest-control situations, where the goal is rapid identification. Furthermore, larval origin is as influential as the virulence of biological control agents (BCAs) in determining BCA efficacy (Kessler 2004). Therefore, improved identification of these two *Melolontha* species is potentially the first step in developing new, specific BCA that targets the appropriate pest while also accounting for unique soil requirements in a given region. Furthermore, distinguishing between the two insects is necessary for understanding their respective population structures, knowledge that is critical to both ecological studies and to plant-protection efforts in forestry and agriculture (e.g. decisions regarding population control).

Advancements in molecular techniques mean that DNA sequences can be used to provide additional diagnostic characters for species identification, beyond morphological features. In 2004, the Consortium for the Barcode of Life (CBOL) was formed as an international initiative to develop DNA barcoding as a global standard for identifying biological species. The CBOL database (http://www.barcodeoflife.org/content/about/barcoding-landscape) identifies different barcode sequences depending on the taxonomic group. For example, the cytochrome c oxidase subunit I gene is a standard for animals, the internal transcribed spacer region is that for fungi, whereas the rbcL and matK genes are for plants.

The aim of this study was to design a method for a quick and unequivocal identification of *M. melolontha* and *M. hippocastani* using simple polymorphisms in the cytochrome c oxidase subunit I (COI) molecular marker.

2. Materials and methods

Samples (Table 1) were collected from two of the most serious outbreak areas in Poland near Tomaszów Mazowiecki (51°28’27.97”N, 20°01’

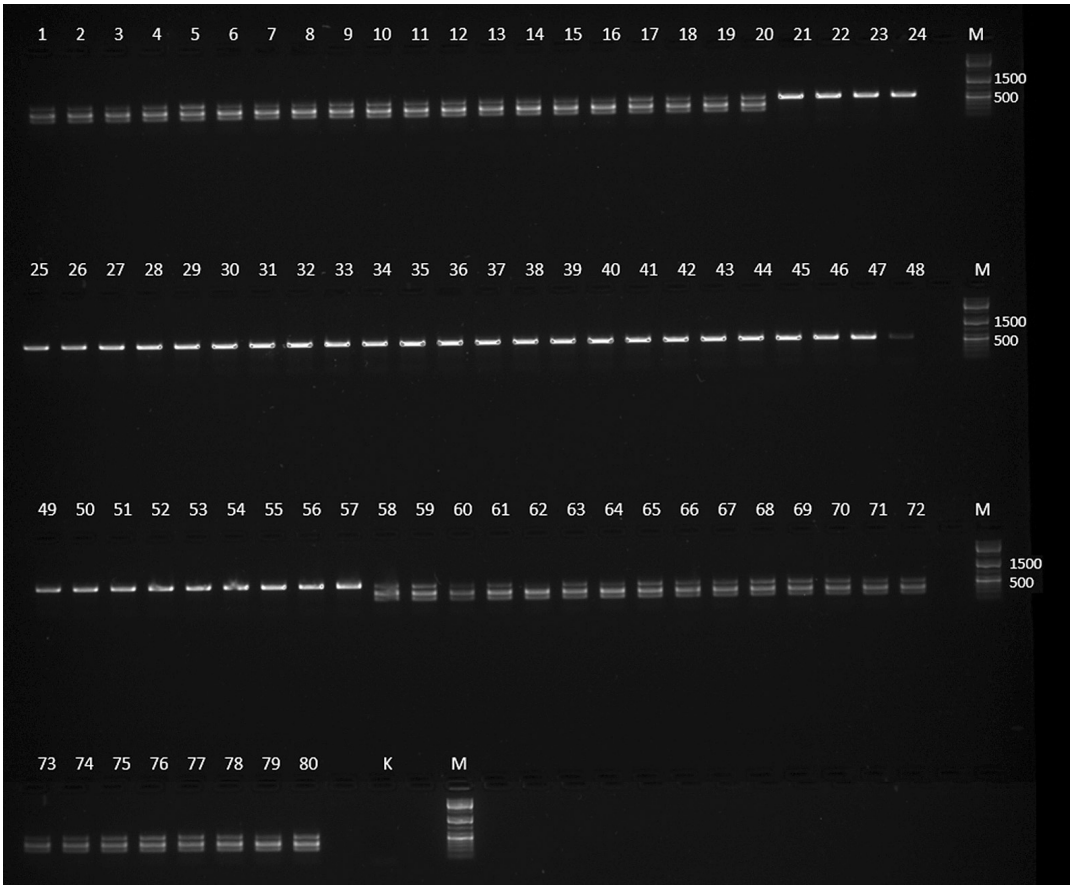

Fig. 1. Species-specific digestion of the cytochrome c oxidase subunit I (COI) fragment for *Melolontha melolontha* and *M. hippocastani* with SacI enzyme. For samples, see Table 1. "M" indicates size standards and "K" indicates the negative control for the PCR reaction.

39.66"E) and Ostrowiec Świętokrzyski (50°56' 00"N, 21°24'00"E). Adult insects were obtained from forested land (Smardzewice and Ostrowiec Świętokrzyski Forest Districts). The species and sex of adults were identified morphologically with a previously established key (Sierpiński 1975).

Genomic DNA was isolated from the leg muscle of 80 *Melolontha* specimens (43 *M. melolontha* and 37 *M. hippocastani*), using a NucleoSpin® Tissue Kit (Macherey-Nagel) in accordance with the manufacturer protocol. A single polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay was used for species identification.

We PCR-amplified fragments of the COI marker with two primers (MelCOIF, AAGGAAACATTTGGCACTTT and MelCOIR, GGGAAATTAGTGATCCGATAGA). Primers were designed in Primer3 ver. 4.0.0 (Koressaar & Remm 2007, Untergasser *et al*. 2012), based on *Melolontha* spp. COI sequences available in GenBank. Reaction volumes were 50 µL, including 15–35 ng of DNA, 1 µL per primer (10 µM concentration), 25 µL of REDTaq ReadyMix (ready-to-use mixture of Taq DNA polymerase, 99% pure deoxynucleotides, reaction buffer; Sigma-Aldrich, Germany), and 20 µL of PCR water. The thermocycling profile was as follows: 95 °C for 3 min, followed by 34 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 3 min. PCR products were digested with SacI restriction enzyme (Thermo Fisher Scientific) for 1 h at 37 °C and were inactivated at 65 °C for 20 min. Reactions were then performed in a total volume of 29 µL, consisting

of 16 µL PCR water, 10 µL PCR product, 2 µL of 10× buffer SacI, and 2 µL SacI restriction enzyme. After digestion, the products were visualised under a UV lamp on 1.5% agarose gels, treated with Nancy-520 (Sigma-Aldrich, Germany), and identified via band patterns.

3. Results

Specimens of 43 *M. melolontha* and 37 *M. hippocastani* were identified with both molecular and morphological methods, which fully agreed. For all 80 analysed samples, COI-based primers amplified 590 bp products. After SacI digestion of *M. melolontha* samples, we obtained two bands (or three if not fully digested), one 230 bp and the other 360 bp (Fig. 1). Restriction enzymes used in this experiment recognised a 6 bp sequence (GAGCTC) and cut at this site before the second C nucleotide. In contrast, SacI did not recognise any sites for *M. hippocastani*, resulting in a single 590 bp band.

4. Discussion

Species identification is essential for investigating intra-specific variation. The present study introduced a simple PCR-RFLP assay to identify *M. hippocastani* and *M. melolontha*. Our results indicated that the probability of misidentification based on a PCR-RFLP analysis is very low, after taking into account COI-marker variation for the number of analysed individuals. The method was also easy to use on the two *Melolontha* species, further indicating the diagnostic utility of PCR-RFLP markers. We thus provided a new and effective technique for resolving species-identification problems among the morphologically identical larvae of two major pest species. This also marks a breakthrough in the ability to separately investigate the distribution and ecology of *M. hippocastani* and *M. melolontha*.

Restriction enzyme polymorphisms provide a fast (up to 2 days), easy, and low-cost (cost of chemical reagents ~40 euros for 20 samples in this study) method for species identification, particularly applicable when dealing with organisms that cannot be distinguished based on morphology alone. In the case of *M. melolontha* and *M. hippocastani*, the morphologically similar grubs develop for approximately 3–4 years before they become morphologically distinct adults, so that a reliable and rapid species identification method would be useful in pest control efforts. Moreover, DNA polymorphisms can be used to identify other marginal species of *Melolontha*, once such analyses are optimised to be more robust and more molecular barcodes are incorporated. Currently, COI sequences are available for only *M. melolontha*, *M. hippocastani*, and *M. pectoralis* (Giannoulis *et al*. 2011). Within the scope of the present study, we chose not to include the latter species, because it has comparatively less negative economic impact throughout Europe. However, future studies should verify whether COI barcodes can successfully identify the latter species.

To our knowledge, this study is the first to develop a DNA-sequence-based method for identifying *Melolontha* species. We demonstrated that the COI region, a standard in phylogenetic analyses and invertebrate species identification (Folmer *et al*. 1994), is appropriate for distinguishing between *M. melolontha* and *M. hippocastani*. Our study follows the increasing trend of using DNA-based species-identification methods that do not require direct sequencing (e.g. cryptic mammals, Boston *et al*. 2011 and spiders, Raso *et al*. 2014). The decrease in cost and time requirements should prove promising for efforts to identify multiple species on a larger scale.

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