SHORT COMMUNICATION

Discriminating larvae of two syntopic *Cychramus* species (Coleoptera, Nitidulidae) by means of bar-HRM analysis

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Abstract

Molecular genetic methods are increasingly used to supplement or substitute classical morphology-based species identification. Here, we employ a COI mini-barcode coupled high-resolution melting analysis to quickly, cost-efficiently and reliably determine larvae of two closely related *Cychramus* (Coleoptera, Nitidulidae) species. Euclidean distance comparison (p < 0.01) and a Welch t-test of the melting point temperatures (p < 0.01) provide highly significant statistical evidence for species specific differences in melting and fluorescence curves, thus allowing the assignment of larvae to either of the two species. This protocol serves as a fast, low-cost and low-tech method to discriminate between pairs or groups of closely related species and can be adapted and applied to various ecological research questions.

Keywords Coleoptera · DNA-barcoding · High-resolution melting analysis · Larvae · Mini-barcodes · Sap beetles

Introduction

Many key questions asked today in basic and applied biological research require precise species identifications. Traditionally, species identification is based on morphological characters and depends on the organisms’ internal and/or external structures. However, taxonomic identification based only on morphology can be difficult to virtually impossible or very time consuming when two or more species are morphologically highly similar. Indeed, there is increasing evidence that the diversity of recognized morphospecies does by far not reflect the true species diversity, especially in inconspicuous and small taxa [1, 2]. In addition, certain life stages (i.e., eggs and larvae) or sexes are often morphologically indistinguishable among species [3], complicating inferences about species richness and ecological interactions.

In the last two decades, DNA sequence-based methods facilitated species determination in taxa where due to a shortage of reliable characters, morphological identification is difficult. Especially DNA-barcoding [4], which relies on DNA sequence variation of a short and standardized section of a specific gene or set of genes, has become a widely used tool among biologists. Indeed, this approach proved to be a powerful and invaluable method for discriminating a broad range of organisms [5]. Often even shorter fragments, so-called mini-barcodes, are sufficient for discriminating between closely related species and they are typically used for analyzing samples containing degraded DNA and in metabarcoding approaches to efficiently characterize entire communities [6, 7]. In addition, mini-barcodes can be combined with high resolution melting analysis (bar-HRM), which provides a time- and cost-effective way to discriminate DNA sequences with small, even single, nucleotide differences, thus avoiding the need of sequencing, which is the costliest step in standard DNA barcoding. The method is particularly suited for fast discrimination of a limited
number of species [8]. Briefly, following a real-time PCR, the products are denatured by increased temperature and the changes in fluorescence caused by the release of an intercalating dye from the DNA duplex are measured [9]. By comparing the melting curves of unknown samples, i.e. the change in intensity of the fluorescence signal with increasing temperature, with profiles of reliably identified samples, they can be assigned to known species [10, 11].

The sap beetle (Nitidulidae) genus Cychramus comprises six valid species, two of which, C. luteus and C. variegatus, are widely distributed across Eurasia and the only Cychramus species reported from Europe. Whereas the beetles are regular flower visitors feeding on pollen [12], with C. luteus even reported from bee hives [13], the larvae are found on various fleshy fungi, and are particularly common on representatives of the honey fungus species complex, Armillaria spp. [14–16]. Unlike the beetles, which are easy to identify, the larvae are almost indistinguishable based on morphological characteristics, especially at younger stages [17]. Due to a lack of reliable species identification, little is known about the larval presence and population dynamics of each of these two species, or the interaction among them. Because honey fungi are among the most important fungal pathogens of temperate and boreal forests, it is of substantial interest to gain better knowledge which of the mushroom-consuming species is prevalent under certain ecological conditions. To this end we developed a robust bar-HRM assay to rapidly identify larvae of C. luteus and C. variegatus that will facilitate studying ecological interactions between these two species, or the interaction among them. Because the larvae are almost indistinguishable based on morphological characteristics, especially at younger stages [17].

Materials and methods

Sampling, species determination and standard COI barcode generation

In total, 38 specimens (25 adult beetles, 13 larvae, Table 1) of the two closely related species C. luteus and C. variegatus were collected from two localities in Austria. Adult specimens were morphologically identified to species level. Standard-length DNA barcodes (658 bp) were generated for some of these specimens. Initial morphological identification of younger larval stages was omitted due to the scarcity of distinguishing characters. Total genomic DNA was extracted using the DNeasy blood and tissue kit (QIAGEN) following the manufacturer’s instructions. Polymerase chain reaction, enzymatic cleanup and cycle sequencing using C_LepFolF and C_LepFolR [18] followed [19] and [20]. Sequencing products were visualized on a 3130xl capillary sequencer (Applied Biosystems). Sequence editing and alignment was done in MEGA 6.06 [21].

Primer design for mini-barcodes and validation

Additional sequences were downloaded from GenBank (Table 1) to account for geographic genetic variation. A 153 bp fragment spanning from nucleotide position 51 to 204 of the standard barcode fragment containing sufficient nucleotide differences for species discrimination was selected for HRM analysis (Fig. 1). Primers Cyc-HRM-F 5′-TGAGAATCTTAATTCCGACTGAAAT and Cyc-HRM-R 5′-GGAACAGTCATTTCCAAATC were designed and their properties (annealing temperature, hairpins, etc.) checked with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Successful amplification and genetic species determination (including the larvae) by these mini-barcodes was confirmed. Protocols for PCR and cycle sequencing applied as mentioned above, only the PCR annealing temperature (49 °C) differed.

qPCR and high-resolution melting analysis of COI mini-barcodes

Quantitative real time PCR and subsequent HRM analyses were conducted in a Rotor-Gene 3000 thermal cycler (Corbett Research, Mortlake, New South Wales, Australia). PCR reactions using the Real Time 2× PCR Master Mix Eva-Green (A & A Biotechnology, Gdynia, Poland) and cycling conditions followed [8], only altering the annealing temperature to 49 °C. Optical measurements at 510 nm were recorded during each extension step. The final extension phase immediately initialized the heating process. Changes in fluorescence were detected during the increase of 0.1 °C increments per second between 60 and 95 °C. qPCR was repeated to obtain a technical replicate. The resulting fluorescence data was visualized using the Rotor-Gene 6.0.27 software.

Statistical analysis of melting and fluorescence curves

Statistical analyses were conducted with R version 3.6.3. For reproducibility a docker container was created with Rocker:Tidyverse image 3.6.3 [22, 23]. The R-code and raw relative fluorescence data is publicly available on GitHub and an automatically generated Docker image can be downloaded from Dockerhub. Raw data were normalized at 75 and 85 °C after visual examination of the relative fluorescence decline over time (Fig. 2a). The negative first derivative values (−d(RFU/dT)) from a geometric spline function were used for statistical analysis in the qpcR package. The threshold to identify the melting point (Tm) was set to 0.2, which resulted in a single peak area for all samples.
Distribution analysis was done visually with a Q-Q-plot. The $T_m$ from all samples grouped by taxa were compared with a two-sided Welch t-test using a 95% confidence interval and 10,000 bootstrap replicates. Euclidean distance comparison of melt curves followed [24]. p-values below 0.05 were considered significant.

Table 1 Information on specimens analyzed in the present study as well as sequences downloaded from online repositories are given

| Species       | Life stage | ID   | Locality       | Sampling site       | BOLD ID; Acc. No |
|---------------|------------|------|----------------|---------------------|------------------|
| C. luteus     | Larva      | Cyc1 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881657         |
|               | Larva      | Cyc2 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881658         |
|               | Larva      | Cyc3 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881659         |
|               | Larva      | Cyc4 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881660         |
|               | Larva      | Cyc5 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881661         |
|               | Larva      | Cyc6 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881662         |
|               | Larva      | Cyc7 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881663         |
|               | Larva      | Cyc8 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881664         |
|               | Beetle     | Cyc18| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT002-20; MT890466 |
|               | Beetle     | Cyc24| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT008-20; MT890467 |
|               | Beetle     | Cyc25| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT009-20; MT890468 |
|               | Beetle     | Cyc26| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT010-20; MT890469 |
|               | Beetle     | Cyc27| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT011-20; MT890470 |
|               | Beetle     | Cyc28| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT012-20; MT884449 |
|               | Beetle     | Cyc29| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT013-20; MT884448 |
|               | Beetle     | Cyc30| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT014-20; MT884447 |
|               | Beetle     | Cyc31| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT015-20; MT890471 |
|               | Beetle     | Cyc37| ST, Graz       | 47.0863 N; 15.4616 E| ANIT021-20; MT890472 |
|               | Beetle     | Cyc38| ST, Graz       | 47.0863 N; 15.4616 E| ANIT022-20; MT884446 |
|               | Beetle     | Cyc39| ST, Graz       | 47.0863 N; 15.4616 E| ANIT023-20; MT890473 |
|               | Beetle     | Cyc40| ST, Graz       | 47.0863 N; 15.4616 E| ANIT024-20; MT890474 |
|               | Beetle     | Cyc41| ST, Graz       | 47.0863 N; 15.4616 E| ANIT025-20; MT890475 |

Additional sequences

KJ962607; KJ965813; KJ966832; KJ962410; KJ964017; KJ962846; KMM448028; KM446407; KM451876; KM448866; KM448805; KM449494; KM449753; KM452505; KM445184; KM442734; KM446278; KU908905; KU910131; KU916564; KU915694; KU914876; KU910893; KM286278

| Species       | Life stage | ID   | Locality       | Sampling site       | BOLD ID; Acc. No |
|---------------|------------|------|----------------|---------------------|------------------|
| C. variegatus | Larva      | Cyc9 | UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881665         |
|               | Larva      | Cyc10| UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881666         |
|               | Larva      | Cyc14| UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881667         |
|               | Beetle     | Cyc15| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT001-20; MT884455 |
|               | Larva      | Cyc16| UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881668         |
|               | Larva      | Cyc17| UA, Gunskirchen| 48.1144 N; 13.9433 E| MT881669         |
|               | Beetle     | Cyc19| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT003-20; MT884454 |
|               | Beetle     | Cyc20| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT004-20; MT884451 |
|               | Beetle     | Cyc21| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT005-20; MT884450 |
|               | Beetle     | Cyc22| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT006-20; MT890476 |
|               | Beetle     | Cyc23| UA, Gunskirchen| 48.1144 N; 13.9433 E| ANIT007-20; MT890477 |
|               | Beetle     | Cyc32| ST, Graz       | 47.0863 N; 15.4616 E| ANIT016-20; MT884453 |
|               | Beetle     | Cyc33| ST, Graz       | 47.0863 N; 15.4616 E| ANIT017-20; MT884452 |
|               | Beetle     | Cyc34| ST, Graz       | 47.0863 N; 15.4616 E| ANIT018-20; MT890478 |
|               | Beetle     | Cyc35| ST, Graz       | 47.0863 N; 15.4616 E| ANIT019-20; MT890479 |
|               | Beetle     | Cyc36| ST, Graz       | 47.0863 N; 15.4616 E| ANIT020-20; MT890480 |

Additional sequences

KM286238; KJ965586

Acronyms UA and ST denote Upper Austria and Styria respectively
Results and discussion

The distinction between pairs or groups of sometimes strikingly similar species is often a key element when tackling biological, ecological or conservational research questions [24]. In the recent past, molecular biological methods have increasingly been used to aid in species assignment, albeit often requiring a substantial amount of infrastructure. Although standard genetic methods constantly aim for a reduction in hands-on time and financial efforts, few approaches actually decrease the necessary infrastructure. Here we present a fast, efficient and adaptable way to discriminate morphologically highly similar larvae of two closely related species of sap-feeding beetles of the family Nitidulidae, that will aid in investigating the population dynamics between these two sympatric species, but also the interactions of beetles, fungi and trees [25]. In the present study, we generated 10 new full-length DNA barcodes (MT884446-MT884455) and used them, together with previously published data, as a basis to create primers for a short mini-barcode fragment. High-resolution melting analysis of the mini-barcodes resulted in two clearly separated clusters of melting curves (see Fig. 2b). Subsequent statistical analyses of Euclidean distances (PERMANOVA, df = 1, pseudo-F = 29.6, p < 0.01, 10,000 permutations) and a two-sided Welch t-test (95% CI, df = 24.257, p < 0.01) of the melting point temperatures yielded significant differences in melting and fluorescence curves for C. luteus and C. variegatus (Fig. 2c), thus allowing for the assignment of the 13 larvae to either of the two species. The significant outcome of these tests indicates that shape, amplitude and melting peak do not just vary by chance [24]. The sensitivity of this method is known to account for single nucleotide differences [8]. The observed consistent differences in melting and fluorescence curves allow for the discrimination of species based on their melting profiles by eye. These results were corroborated by sequencing the short fragments and aligning them to the full-length barcodes. Thus, we conclude that HRM analyses of mini-barcode fragments present an adequate means to reliably differentiate morphologically similar specimens of these closely related species. Our workflow can be easily adapted for many applied and basic research questions whenever time and cost-efficient discrimination of a large number of samples of a limited number of species is necessary. Furthermore, our publicly available R-code can be used for any HRM study to provide statistical corroboration of visual results. Consequently, when short-fragment primers are established, only a qPCR machine and adequate software for visualization is required to facilitate high-sensitivity species discrimination.
Fig. 2 Relative fluorescence curve (a), identified melting point comparison (b) and melting rate curve (c). Species are indicated by blue (C. variegatus) and red (C. lutues) colors. Colored lines in (a) represent means, grey areas cover standard deviations. Colored dots in (b) mark the distribution of resulting melting points, black dots and error bars represent the means and 95% confidence intervals (bootstrap, BCa 10,000) respectively. (Color figure online)
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Author contributions CS, SK and LZ contributed to the study conception and design. Material preparation, data collection and analysis were performed by HH, ES, HO and LZ. The first draft of the manuscript was written by SK and LZ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Funding acquisition and resources were provided by CS and SK.

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Data availability DNA-barcoding data was stored on BOLD, BOLD-IDs and GenBank accession numbers are provided.

Code availability All code for the statistical analyses was deposited on GitHub (https://github.com/HannesOberreiter/melt_graz) and archived on zenodo (https://doi.org/10.5281/zenodo.3862848) and is publicly available on Dockerhub (https://hub.docker.com/r/hannesoberreiter/melt_graz).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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