

Bar-HRM for identification of cryptic earthworm species

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4	Bar-HRM for identification of cryptic earthworm species
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- 18 Abstract
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20 Accurate species identification is crucial in ecological research on sentinel species such as earthworms. Here, we developed a reliable and cost-efficient method for the rapid 21 22 identification of taxa within the Allolobophora chlorotica complex of cryptic earthworm 23 species. We combined high resolution melting (HRM) analysis with DNA barcoding (Bar-24 HRM) in a three-step approach: (i) selection of a mini-barcode in the 16S mitochondrial gene, 25 (ii) test of the method on a panel of 16 reference individuals and (iii) identification at the species level of 24 unknown specimens. Thus, we proved the efficacy of Bar-HRM to 26 27 discriminate between cryptic sister (or sibling) earthworm species and we provided a 28 standardized method that can be easily adapted to other taxa.

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32 Key Words

33 Allolobophora chlorotica; complex of species; high resolution melting analysis;
34 mitochondrial lineages; species identification

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Accurate species identification is a fundamental issue of current biological and 38 39 ecological researches (e.g. Elphick, 2008). Until quite recently, earthworm species 40 identification was almost only conducted on morphological characters (Bartlett et al., 2010). 41 Nowadays, molecular techniques are more frequently used and multiple studies revealed 42 cryptic diversity (i.e. two or more distinct species that were classified as a single one due to 43 morphological similarity, Bickford et al., 2007) even within long known earthworm 44 morphospecies (e.g. King et al., 2008; James et al., 2010; Novo et al., 2010; Taheri et al., 45 2018). Biologically relevant differences were identified between cryptic species, highlighting 46 that correct identification of earthworm specimens is crucial for further study of these 47 ecologically important species (review in Marchan et al., 2018).

In the last decade, the DNA barcoding approach (Hebert et al., 2003) using the 48 mitochondrial gene coding for cytochrome c oxidase subunit I (COI) has become the most 49 50 used molecular taxonomy tool in earthworms (e.g. Decaens et al., 2016; Porco et al., 2018). In 51 addition to allowing the detection of cryptic or overlooked diversity cases in earthworms, this 52 approach is a way to recover specific level data for juvenile specimens and to process 53 numerous specimens without the intervention of a taxonomist (Porco et al., 2018). While this 54 approach holds many advantages, it can turn out to be relatively expensive when applied to large sample sizes and requires an access to a Sanger-sequencing or a high-throughput next-55 56 generation sequencing (NGS) platform.

57 Recently, DNA-based identification of genetic variants was made possible without any 58 sequencing step. The high resolution melting (HRM) analysis is a highly sensitive method 59 allowing to discriminate DNA sequences differing of only one substitution or one base pair 60 indel (Wittwer, 2009). Following a real-time PCR, DNA fragments (from 80 to 250 bp long) 61 are denatured with increasing temperature and the resulting changes in fluorescence caused by 62 the release of an intercalating dye from the DNA duplex are monitored. Combining HRM

analysis to DNA barcoding (Bar-HRM) is thus a way to rapidly distinguish genetically
differentiated groups based on the thermal denaturation curves of amplified mini-barcodes
(Behrens-Chapuis et al., 2018; Chen et al., 2019). Thereby, Bar-HRM has appeared to be an
effective, simple, cheap (in long term and large scale investigation) and time-saving tool for
species identification (Fernandes et al., 2017; Fidler et al., 2017; Sun et al., 2017;
Osathanunkul et al., 2018).

The goal of the present study was to propose a standardized method of species 69 70 identification using Bar-HRM, from the evaluation of mini-barcode discriminating power to 71 the species assignation of unknown individuals, and to test its usefulness to distinguish cryptic 72 earthworm species using the Allolobophora chlorotica aggregate as a model. Two colour 73 morphs were described in the Allolobophora chlorotica aggregate, a green morph 74 representing a single taxon although composed of two divergent mitochondrial lineages (L2 75 and L3), and a pink colour morph, composed of at least two taxa (L1 and L4, Dupont et al., 76 2016). The two colour morphs are known to have contrasting ecological preferences linked to 77 soil moisture (Satchell, 1967; Lowe and Butt, 2007) and to present postzygotic reproductive 78 isolating mechanisms (Lowe and Butt, 2008). Confirmation of species status for the L1, 79 L2/L3 and L4 lineages was obtained with microsatellite markers by Dupont et al. (2016) who revealed that hybridization was rare among these taxa. The status of three other lineages (i.e. 80 81 L5, L6 and L7) remains however unclear (King et al., 2008; Dupont et al., 2011).

Here, we focused on the relatively well-defined L1, L2/L3 and L4 taxa. The development of the bar-HRM method was led in three main steps. In a first time, we tested whether the set of EwD/EwE primers developed by Bienert et al. (2012) for earthworms and allowing to amplify a 111 bp mini-barcode region in the mitochondrial 16S rRNA gene was adequate to discriminate these taxa. In order to estimate the distribution of the theoretical melting temperatures (i.e. temperature at which 50% of DNA double-strands are dissociated)

of this mini-barcode among the L1, L2/L3 and L4 taxa, we used 41 published A. chlorotica 88 agg. 16S sequences (Genbank Accession Numbers AM774359-93, KJ912500-505, 89 90 JN869755-756). To compute the expected melting temperatures (Tm_{THEO}) from DNA 91 sequences of the EwD/EwE region, we implemented the model described in Khandelwal & 92 Bhyravabhotla (2010). Results showed significantly differentiated means between taxa (L1 =93 78.4 ± 0.30 , L2/L3 = 79.7 ± 0.47 and L4 = 80.6 ± 0.38) but slightly overlapping Tm_{THEO} 94 ranges (Fig. 1). Because HRM takes into account the shape of the whole curve, in addition to 95 the expected melting temperature, we assumed that it should still be possible to correctly 96 identify specimens even from different DNA fragments displaying close melting 97 temperatures. Interestingly, despite the divergence between L2 and L3 haplotypes in a 98 phylogenetic tree built using a longer fragment of the 16S gene (Supplementary material, Fig S1), the Tm_{THEO} of these lineages, belonging to a single species, were not distinguishable 99 100 using the EwD/EwE mini-barcode.

101 In a second time, we composed a panel of 16 reference individuals belonging to each 102 of the three taxa (4 L1, 8 L2/L3 and 4 L4). These reference individuals were selected based on 103 their already published COI sequences (Genbank Accession Number -AN- in supplementary 104 material Table S1) in order to capture both intra and inter-lineage diversity and a 445 bp 105 fragment of the 16S gene was subsequently sequenced using EwA/EwF primers (Bienert et 106 al., 2012; AN in Table S1). The HRM analysis of these reference panel individuals was 107 carried out in triplicates using MeltDoctor HRM Master Mix (Applied Biosystems) according 108 to the manufacturer protocol in 10 µL reaction volume and using EwD/EwE primers. The 109 fluorescence data was normalized by the use of the exponential background removal method 110 described in Palais and Wittwer (2009) in order to obtain the curves describing the decrease of 111 double-stranded DNA fractions in response to the temperature. We observed a clear separation of the melting curves of the three taxa (Fig. 2a). The experimental melting
temperatures (Tm_{OBS}) ranges, i.e. between the lower and the higher Tm_{OBS}, did not overlap.

114 Finally, species identification was tested on 24 unknown specimens sampled in 115 Parisian parks and morphologically identified as members of the A. chlorotica aggregate. 116 Because the samples were stored in ethanol before DNA extraction (using the DNeasy ® 117 Blood & Tissue Kit, Qiagen, according to manufacturer protocol), and have therefore lost 118 their pigmentation, their colour morph could not be determined. The HRM analyses of the 119 unknown samples and the reference panel samples were conducted at the same time. The 120 distances between each unknown sample melting curve and every reference panel curves were 121 computed as the mean integral of the absolute difference between the two curves considered 122 (Palais and Wittwer, 2009). The unknown specimens could thus be assigned to one of the taxa 123 based on the lowest distance between their melting curve and the one of the reference samples 124 (Fig. 2b). Then, the identification of the 24 specimens was controlled by the sequencing of 125 their 16S gene using EwA/EwF primers (Table S1). Among them, 11 different haplotypes 126 were identified (Table S1) using the Dna SP V 5.10.01 software (Librado and Rozas, 2009). 127 HRM results matched with the 16S sequencing results for 10 of these haplotypes (i.e. 22 128 individuals). An erroneous identification comes from a haplotype (present in 2 individuals, 129 Table S1) assigned to the L4 taxa using the bar-HRM method while it clustered with L2 haplotypes in a phylogenetic tree (Supplementary material, Fig S1). This 16S haplotype 130 131 sequence was indeed expected to display a melting temperature closer to the L2/L3 group 132 than to the L4 group (Fig. 3). So, there is a discrepancy, in this particular case, between the 133 expected and observed melting temperatures and this haplotype appears to be a significant 134 outlier (Bonferroni test). Although this 16S haplotype clustered with the L2 lineage, it is 135 nevertheless noteworthy that the phylogenetic tree revealed a sequence quite divergent (Supplementary material, Fig S1), a result that could partly explain the discrepancy. In order 136

to increase the specific identification accuracy and therefore correctly assign the identified outliers, we propose to use a multi-locus bar-HRM method. For instance, a mini-barcode designed in the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene allowed to discriminate between L1/L2 and L3/L4 groups of lineages (Primers designed for this study: ND1F374 – TRGCTGGATGAAGHTCAAA and ND1R519 – GCAAGYCARGCATGRAAA; 163 bp amplicon, $T_a = 52^{\circ}C$, Fig. S2 and S3).

143 To conclude, we demonstrated the effectiveness of HRM for distinguishing cryptic 144 species of earthworms. We developed a simple and rapid bar-HRM method allowing to 145 successfully identify most of the tested specimens belonging to the A. chlorotica agg. We 146 believe that this Bar-HRM method could be easily adapted to other earthworm species 147 following the three-step procedure described here. It could be used for accurate species 148 identification, which is an essential step in many fields of ecology. For instance, rearing of earthworms may be necessary for laboratory experiments in ecotoxicology or soil restoration 149 150 (Lowe and Butt, 2005). To ensure a successful rearing, it is necessary to mix specimens from 151 the same species (i.e. in order to avoid reproductive isolation). The bar-HRM method may be 152 particularly useful to rapidly confirm morphological species distinction. This method is 153 indeed much faster than sequencing because PCR and HRM steps are performed in the same 154 reaction tube without any other post-PCR manipulations. For species confirmation, melting 155 profiles of samples can be compared to those of reference species in a database. The 156 prerequisite is thus to have a good knowledge of the taxonomical status of the target taxa, as it 157 is now the case for several complexes of earthworm species (e.g. A. chlorotica, Dupont et al., 158 2011; Pontoscolex corethrurus, Taheri et al. 2018).

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- 251 252

254 Figure legends

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Figure 1: Expected melting temperatures (Tm_{THEO}) of the 16S EwD/EwE fragment according
to the three *Allolobophora chlorotica* agg. taxa, computed from published sequences.

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Figure 2: Melting curves profiles. a) panel of 16 reference individuals (L1 yellow, L2/L3 blue, L4 orange) and b) panel of 16 reference individuals (dotted line) and 24 assigned unknown specimens (solid line) with non-matching control individuals in purple. HRM analysis of each individual was carried out in triplicates.

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Figure 3: Relationship between expected melting temperatures (computed) and experimental melting temperatures (measured by HRM) for all the individuals (reference panel individuals and assigned individuals) with the assignment to the three species indicated by colours (L1 yellow, L2/L3 blue, L4 orange). The 2 non-matching control individuals sharing the same haplotype are indicated in purple.

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