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1 **Short communication**

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4 **Bar-HRM for identification of cryptic earthworm species**

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17

18 **Abstract**

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20 Accurate species identification is crucial in ecological research on sentinel species
21 such as earthworms. Here, we developed a reliable and cost-efficient method for the rapid
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
26 species level of 24 unknown specimens. Thus, we proved the efficacy of Bar-HRM to
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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38 Accurate species identification is a fundamental issue of current biological and
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).

48 In the last decade, the DNA barcoding approach (Hebert et al., 2003) using the
49 mitochondrial gene coding for cytochrome c oxidase subunit I (COI) has become the most
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
55 large sample sizes and requires an access to a Sanger-sequencing or a high-throughput next-
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

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

69 The goal of the present study was to propose a standardized method of species
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
80 revealed that hybridization was rare among these taxa. The status of three other lineages (i.e.
81 L5, L6 and L7) remains however unclear (King et al., 2008; Dupont et al., 2011).

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

88 of this mini-barcode among the L1, L2/L3 and L4 taxa, we used 41 published *A. chlorotica*
89 agg. 16S sequences (Genbank Accession Numbers AM774359-93, KJ912500-505,
90 JN869755-756). To compute the expected melting temperatures (T_{mTHEO}) 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 T_{mTHEO}
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
99 S1), the T_{mTHEO} of these lineages, belonging to a single species, were not distinguishable
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 μ L 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

112 separation of the melting curves of the three taxa (Fig. 2a). The experimental melting
113 temperatures (T_{mOBS}) ranges, i.e. between the lower and the higher T_{mOBS} , 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
130 haplotypes in a phylogenetic tree (Supplementary material, Fig S1). This 16S haplotype
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
136 (Supplementary material, Fig S1), a result that could partly explain the discrepancy. In order

137 to increase the specific identification accuracy and therefore correctly assign the identified
138 outliers, we propose to use a multi-locus bar-HRM method. For instance, a mini-barcode
139 designed in the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene allowed to
140 discriminate between L1/L2 and L3/L4 groups of lineages (Primers designed for this study:
141 ND1F374 – TRGCTGGATGAAGHTCAAA and ND1R519 –
142 GCAAGYCARGCATGRAAA; 163 bp amplicon, $T_a = 52^\circ\text{C}$, 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
149 earthworms may be necessary for laboratory experiments in ecotoxicology or soil restoration
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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254 **Figure legends**

255

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

258

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

263

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

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