



**HAL**  
open science

## Bar-HRM for identification of cryptic earthworm species

Gaspard Baudrin, Virginie Roy, Agnès Gigon, Lise Dupont

► **To cite this version:**

Gaspard Baudrin, Virginie Roy, Agnès Gigon, Lise Dupont. Bar-HRM for identification of cryptic earthworm species. *Pedobiologia*, 2020, 80, pp.150634 -. 10.1016/j.pedobi.2020.150634 . hal-03489572

**HAL Id: hal-03489572**

**<https://hal.science/hal-03489572>**

Submitted on 20 May 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Short communication**

2

3

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

5

6

7 Gaspard Baudrin<sup>1,2,3</sup>, Virginie Roy<sup>1,2,3</sup>, Agnès Gigon<sup>1,2,3</sup> & Lise Dupont<sup>1,2,3\*</sup>

8

9

10 <sup>1</sup> *Univ Paris-Est Creteil, CNRS, INRAE, IRD, IEES-Paris, F-94010 Creteil France*

11 <sup>2</sup> *Sorbonne Université, IEES-Paris, F-75005 Paris, France*

12 <sup>3</sup> *Université de Paris, IEES-Paris, F-75013 Paris, France*

13

14

15

16 \* Corresponding author: [lise.dupont@u-pec.fr](mailto:lise.dupont@u-pec.fr)

17

18 **Abstract**

19

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.

29

30

31

32 **Key Words**

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

35

36

37

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  $78.4 \pm 0.30$ , L2/L3 =  $79.7 \pm 0.47$  and L4 =  $80.6 \pm 0.38$ ) 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  $\mu$ 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).

159

160



161 **Acknowledgements**

162

163 This work was partly funded by the French National Research Agency (ANR) as a part of the  
164 project Ecoville No. ANR-14-CE22-0021. The authors are grateful to H. Audusseau for her  
165 help in sampling earthworms in Parisian parks and to the PRAMMICS platform of the OSU  
166 Efluve for making qPCR thermocycler available.

167

168 **References**

- 169 Bartlett, M.D., Briones, M.J.I., Neilson, R., Schmidt, O., Spurgeon, D., Creamer, R.E., 2010. A  
 170 critical review of current methods in earthworm ecology: From individuals to  
 171 populations. *European Journal of Soil Biology* 46, 67-73.
- 172 Behrens-Chapuis, S., Malewski, T., Suchecka, E., Geiger, M.F., Herder, F., Bogdanowicz, W.,  
 173 2018. Discriminating European cyprinid specimens by barcode high-resolution  
 174 melting analysis (Bar-HRM)-A cost efficient and faster way for specimen assignment?  
 175 *Fisheries Research* 204, 61-73.
- 176 Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K., Das,  
 177 I., 2007. Cryptic species as a window on diversity and conservation. *Trends in*  
 178 *Ecology & Evolution* 22, 148-155.
- 179 Bienert, F., De Danieli, S., Miquel, C., Coissac, E., Poillot, C., Brun, J.J., Taberlet, P., 2012.  
 180 Tracking earthworm communities from soil DNA. *Molecular Ecology* 21, 2017-2030.
- 181 Chen, Y.Q., Wang, B., Wang, P., Huang, B.S., Liu, H.G., Xiong, C., Wu, M.L., Wang, N.L.,  
 182 Liu, D., Hu, Z.G., Chen, S.L., 2019. Rapid identification of common medicinal snakes  
 183 and their adulterants using the Bar-HRM analysis method. *Mitochondrial DNA Part A*  
 184 30, 367-374.
- 185 Decaens, T., Porco, D., James, S.W., Brown, G.G., Chassany, V., Dubs, F., Dupont, L.,  
 186 Lapied, E., Rougerie, R., Rossi, J.-P., Roy, V., 2016. DNA barcoding reveals diversity  
 187 patterns of earthworm communities in remote tropical forests of French Guiana. *Soil*  
 188 *Biology & Biochemistry* 92, 171-183.
- 189 Dupont, L., Lazrek, F., Porco, D., King, R.A., Rougerie, R., Symondson, W.O.C., Livet, A.,  
 190 Richard, B., Decaens, T., Butt, K.R., Mathieu, J., 2011. New insight into the genetic  
 191 structure of the *Allolobophora chlorotica* aggregate in Europe using microsatellite and  
 192 mitochondrial data. *Pedobiologia* 54, 217-224.
- 193 Dupont, L., Porco, D., Symondson, W.O.C., Roy, V., 2016. Hybridization relics complicate  
 194 barcode-based identification of species in earthworms. *Molecular Ecology Resources*  
 195 16, 883-894.
- 196 Elphick, C.S., 2008. How you count counts: the importance of methods research in applied  
 197 ecology. *Journal of Applied Ecology* 45, 1313-1320.
- 198 Fernandes, T.J.R., Costa, J., Oliveira, M., Mafra, I., 2017. DNA barcoding coupled to HRM  
 199 analysis as a new and simple tool for the authentication of Gadidae fish species.  
 200 *Food Chemistry* 230, 49-57.
- 201 Fidler, G., Kocsube, S., Leiter, E., Biro, S., Paholcsek, M., 2017. DNA Barcoding coupled with  
 202 High Resolution Melting analysis enables rapid and accurate distinction of *Aspergillus*  
 203 species. *Medical Mycology* 55, 642-659.
- 204 Hebert, P.D.N., Cywinska, A., Ball, S.L., Dewaard, J.R., 2003. Biological identifications  
 205 through DNA barcodes. *Proceedings of the Royal Society of London Series B:*  
 206 *Biological Sciences* 270, 313-321.
- 207 James, S.W., Porco, D., Decaens, T., Richard, B., Rougerie, R., Erseus, C., 2010. DNA  
 208 Barcoding reveals cryptic diversity in *Lumbricus terrestris* L. 1758 (Clitellata):  
 209 resurrection of *L. herculeus* (Savigny, 1826). *Plos One* 5, e15629.
- 210 Khandelwal, G., Bhyravabhotla, J., 2010. A phenomenological model for predicting melting  
 211 temperatures of DNA sequences. *Plos One* 5, e12433.
- 212 King, R.A., Tibble, A.L., Symondson, O.C., 2008. Opening a can of worms: unprecedented  
 213 sympatric cryptic diversity within British lumbricid earthworms. *Molecular Ecology* 17,  
 214 4694-4698.
- 215 Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA  
 216 polymorphism data. *Bioinformatics* 25, 1451-1452.
- 217 Lowe, C.N., Butt, K.R., 2005. Culture techniques for soil dwelling earthworms: A review.  
 218 *Pedobiologia* 49, 401-413.
- 219 Lowe, C.N., Butt, K.R., 2007. Life-cycle traits of the dimorphic earthworm species  
 220 *Allolobophora chlorotica* (Savigny, 1826) under controlled laboratory conditions.  
 221 *Biology and Fertility of Soils* 43, 495-499.

222 Lowe, C.N., Butt, K.R., 2008. *Allolobophora chlorotica* (Savigny, 1826): Evidence for  
223 classification as two separate species. *Pedobiologia* 52, 81-84.

224 Marchan, D.F., Cosin, D.J.D., Novo, M., 2018. Why are we blind to cryptic species? Lessons  
225 from the eyeless. *European Journal of Soil Biology* 86, 49-51.

226 Novo, M., Almodovar, A., Fernandez, R., Trigo, D., Cosin, D.J.D., 2010. Cryptic speciation of  
227 hormogastrid earthworms revealed by mitochondrial and nuclear data. *Molecular*  
228 *Phylogenetics and Evolution* 56, 507-512.

229 Osathanunkul, M., Osathanunkul, K., Wongwanakul, S., Osathanunkul, R., Madesis, P., 2018.  
230 Multiuse of Bar-HRM for *Ophiocordyceps sinensis* identification and authentication.  
231 *Scientific Reports* 8

232 Palais, R., Wittwer, C.T., 2009. Mathematical algorithms for high-resolution DNA melting  
233 analysis. In: Johnson, M.L., Brand, L. (eds) *Methods in Enzymology: Computer*  
234 *Methods*, Vol 454, Pt A, pp. 323-343

235 Porco, D., Chang, C.-H., Dupont, L., James, S., Richard, B., Decaëns, T., 2018. A reference  
236 library of DNA barcodes for the earthworms from Upper Normandy: Biodiversity  
237 assessment, new records, potential cases of cryptic diversity and ongoing speciation.  
238 *Applied Soil Ecology* 124, 362-371.

239 Satchell, J.E., 1967. Colour dimorphism in *Allolobophora chlorotica* Sav. (Lumbricidae).  
240 *Journal of Animal Ecology* 36, 623-630.

241 Sun, W., Yan, S., Li, J.J., Xiong, C., Shi, Y.H., Wu, L., Xiang, L., Deng, B., Ma, W., Chen,  
242 S.L., 2017. Study of commercially available *Lobelia chinensis* products using Bar-  
243 HRM technology. *Frontiers in Plant Science* 8

244 Taheri, S., James, S., Roy, V., Decaens, T., Williams, B.W., Anderson, F., Rougerie, R.,  
245 Chang, C.H., Brown, G., Cunha, L., Stanton, D.W.G., Da Silva, E., Chen, J.H.,  
246 Lemmon, A.R., Lemmon, E.M., Bartz, M., Baretta, D., Barois, I., Lapied, E., Coulis,  
247 M., Dupont, L., 2018. Complex taxonomy of the 'brush tail' peregrine earthworm  
248 *Pontoscolex corethrurus*. *Molecular Phylogenetics and Evolution* 124, 60-70.

249 Wittwer, C.T., 2009. High-Resolution DNA Melting analysis: advancements and limitations.  
250 *Human Mutation* 30, 857-859.

251

252

253

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.

269

270

271

272







