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1 **Identifying the last bloodmeal of questing sheep**
2 **tick nymphs (*Ixodes ricinus* L.) using high**
3 **resolution melting analysis.**

4

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21 Note: Supplementary data associated with this article

22

23 **ABSTRACT**

24 The sheep tick, *Ixodes ricinus* L., is an important hematophagous
25 vector of zoonotic disease of both veterinary and public health
26 importance in Europe. Risk models for tick-borne diseases can be
27 improved by identifying the main hosts of this species in any given
28 area. However, this generalist tick stays on a host for only a few days
29 a year over its life cycle, making the study of its feeding ecology
30 difficult. In contrast, ticks can easily be collected from vegetation
31 when they are questing. Molecular methods have proved to be a
32 reliable alternative to field observation, but most current methods
33 have low sensitivity and/or low identification success (i.e. hosts are
34 only identified to taxonomic levels higher than species). In this study
35 we use Real-time PCR coupled with High Resolution Melting
36 Analysis (HRMA) to identify the source of the last bloodmeal in
37 questing tick nymphs. Twenty of the most important tick hosts were
38 grouped taxonomically and six group-specific primer sets, targeting
39 short mitochondrial DNA regions, were designed *de-novo*. Firstly,
40 we show that these primers successfully amplify target host DNA
41 (from host tissue or engorged ticks), and that HRMA can be used to
42 reliably identify hosts to species (or genera in the case of *Sorex* and
43 *Apodemus*). Secondly, the new protocol was tested on field-collected
44 questing nymphs. Bloodmeal source was identified in 65.4 % of 52
45 individuals. In 83.3 % of these, the host was identified to species or
46 genera using HRMA alone. Moreover, the primer sets designed here
47 can unequivocally identify mixed bloodmeals. The combination of
48 sensitivity and identification success together with the closed-tube
49 and single step approach that minimizes contamination, make Real-

50 time HRMA a good alternative to current methods for bloodmeal

51 identification.

52 *Index Keywords:*

53 Bloodmeal

54 Host identification

55 Host-parasite interactions

56 Vector ecology

57 Hematophagy

58 HRMA

59

60 **1. Introduction**

61

62 Understanding the feeding biology of arthropod vectors is
63 essential for defining the relative importance of various hosts in
64 maintaining and transmitting zoonotic pathogens, facilitating the
65 improvement of disease risk models used in veterinary and public
66 health strategies (Kent, 2009; Gómez-Díaz and Figuerola, 2010). The
67 sheep tick, *Ixodes ricinus*, considered the "most important multi-
68 potent vector in Europe" (Randolph, 2009), obtains its bloodmeal
69 from a wide range of wild and domestic vertebrate species including
70 companion animals and livestock, many of which are competent
71 reservoir hosts of pathogenic organisms (Gray, 2001). However,
72 direct field observations of feeding *I. ricinus* on hosts are difficult
73 and labour intensive, and may provide inaccurate estimates of host
74 choice in epidemiological cycles, especially because ticks only spend
75 a few days per life stage on hosts (Kirstein and Gray, 1996). In the
76 last decade, cheap, rapid molecular methods for amplifying degraded
77 DNA have become widely available and are frequently applied to the
78 bloodmeal analysis of hematophagous arthropods, including ticks
79 (reviewed in Mukabana et al., 2002; Alcaide et al., 2009; Kent, 2009;
80 Gariepy et al., 2012; Thiemann et al., 2012). However, molecular
81 identification of the source of a questing tick bloodmeal poses
82 particular technical challenges, because questing ticks may have had
83 their last bloodmeal in their previous life stage, up to one year before
84 collection (Randolph et al., 2002). Consequently: i) host DNA is
85 compromised by digestive and hemolytic processes so that only few
86 degraded copies of DNA are likely to be present in the endosomes of

87 the tick midgut (Sonenshine, 1991; Kirstein and Gray, 1996; Kent,
88 2009; Sojka et al., 2013); ii) a high concentration of heme molecules
89 tend to inhibit DNA amplification; iii) environmental or human DNA
90 contamination may confound the DNA bloodmeal signature, as is
91 typical of any molecular study using low quality/quantity DNA
92 (Taberlet et al., 1999; Humair et al., 2007). Nevertheless, published
93 studies on laboratory fed and captive-reared ticks have shown that
94 host DNA can be detected up to 10 months after the bloodmeal
95 (Kirstein and Gray, 1996; Pichon et al., 2003).
96 The above limitations are reflected in the results obtained with
97 Reverse Line Blotting Hybridization (RLBH), which has been widely
98 applied on large numbers of questing ticks (Kent, 2009; Allan *et al.*,
99 2010; Estrada-Peña et al., 2013 and references therein). Reported
100 sensitivity is low and varies considerably; in addition, the ability to
101 discriminate and identify the source of the bloodmeal (to taxonomic
102 group, genus and, rarely, species level) also varies depending on the
103 molecular markers and set of probes used (Pichon et al., 2005;
104 Pichon et al., 2006; Morán Cadenas et al., 2007). Recently, a
105 restriction fragment length polymorphism (RLFP) protocol, targeting
106 *12S* rDNA amplified fragments has been developed (Wodecka et al.,
107 2014). RLFP was first proposed by Kirstein and Gray in1996,
108 although they targeted the *cytb* gene. Wodecka et al. (2014) claim
109 that the RFLP pattern of the targeted 12S rDNA fragment is available
110 for about 60 host species. This method has been applied to a large
111 sample set and appears to provide a higher mean sensitivity than
112 reported for RLBH. Alternatives to the DNA-based methods have
113 been tested, such as proteome profiling and stable isotope analysis

114 (Gomez-Díaz and Figeruola, 2010; Laskay et al., 2012; Estrada-Peña
115 et al., 2013 and references therein); however, none have been widely
116 adopted.

117 We decided to test the applicability of High Resolution Melting
118 Analysis (HRMA) to bloodmeal identification in questing ticks, since
119 it is a simple, rapid, post-PCR method that does not require
120 expensive DNA sequencing, and has already proven useful for a wide
121 variety of applications (Wittwer et al., 2003; Reed et al., 2007; Lin et
122 al., 2008; Erali and Wittwer, 2010), including species identification
123 (Winder et al., 2011; Kang and Sim, 2013). HRMA is known to be a
124 sensitive and reliable method even when DNA is of low quantity and
125 quality (Do et al., 2008); for example, it has been applied to
126 bloodmeal analysis of the Chagas disease vectors up to 30 days after
127 feeding (Peña et al., 2012). Our aim was to optimize a simple, rapid
128 and reliable protocol for identifying questing tick bloodmeal sources
129 to species with a higher sensitivity than previously published
130 methods, at the same time minimizing amplification of contaminant
131 DNA.
132

133 **2. Materials and methods**

134

135 *2.1. Target species*

136 Twenty tick hosts, including livestock and companion animals,
137 were selected based on their importance as maintenance hosts, and
138 their epidemiological relevance (reservoir competence for the most
139 common tick borne zoonotic pathogens). All of these hosts occur in
140 the European Alps, where our ticks were collected (Gray, 1998;

141 Morán Cadenas et al., 2007; De la Fuente et al., 2008; Bown et al.,
142 2011; Marsot et al., 2012; Wodecka et al., 2014). In addition, the
143 availability of control samples and GenBank sequences were also
144 taken into account. After selection, these hosts were then grouped
145 taxonomically (i.e. into: Order Passeriformes; Superfamily
146 Muroidea; Families: Soricidae, Cervidae, Canidae; Subfamily
147 Caprinae; Table 1). Please note that the use of these taxonomic
148 names hereafter refers only to the amplification of the chosen host
149 species and does not imply potential amplification of all species in
150 these groups). *Homo sapiens* was not included as a target species,
151 because humans have not been considered among the main tick hosts
152 in previous studies (e.g. Humair et al., 2007; Pichon et al., 2003;
153 Morán Cadenas et al., 2007; Wodecka et al., 2014).
154

155 *2.2. Marker choice and primer design*

156 Since the majority of field-collected questing *I. ricinus* nymphs had
157 fed as larvae many months before collection (Randolph et al., 2002),
158 host DNA is likely to be of very low quantity and quality, as
159 mentioned in the Introduction. Consequently, only mitochondrial
160 DNA amplicons of less than 200 bp were considered likely to
161 amplify consistently enough to be reliable markers (Kirstein and
162 Gray, 1996).
163 In order to select the most appropriate primers for each taxonomic
164 host group, we used Clustal X v. 2.0 to align mtDNA sequences of
165 relevant host species (mainly retrieved from GenBank but also
166 generated by us, see accession numbers: **KJ676686** *Turdus merula*;
167 **KJ676687** *Turdus philomelos*; **KJ676688** *Erithacus rubecula*), as

168 well as non-target species (Supplementary file 1). A *Homo sapiens*
169 reference sequence was also included since we wanted to avoid the
170 amplification of contaminant human DNA. This is not easily
171 achieved when working with extremely low quality/quantity DNA
172 using highly sensitive methods even if both field sampling and
173 subsequent handling of ticks are carried out using all possible
174 precautions to avoid contamination (Pääbo et al., 2004; see also
175 below). Alignments were checked visually to identify DNA regions
176 that would optimize the discriminating power of HRMA in low
177 quantity/quality host DNA; i.e. highly conserved intraspecific
178 mtDNA regions of about 200 bp with well-defined interspecific
179 variation (at least two single nucleotide polymorphisms, SNPs). Non-
180 degenerate group-specific primers were selected in order to avoid
181 contaminant human DNA and amplification of non-target species, to
182 have melting temperatures (T_m) of about 60 °C and a low probability
183 of dimer formation as predicted by the Oligo Analysis Tool
184 (www.operon.com/tools/oligo-analysis-tool.aspx). Identified mtDNA
185 regions were tested for their species identification potential with
186 uMELT™ (https://www.dna.utah.edu/umelt/um.php; Dwight et al.,
187 2011) using standard parameters and the thermodynamic parameter
188 set of Unified-SantaLucia (SantaLucia, 1998). Finally, each sequence
189 was blasted (BLASTn; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to test
190 if the selected mtDNA fragment allowed unequivocal and correct
191 species identification.
192
193 *2.3. HRMA optimization*

194 Tissue samples from host species and/or engorged ticks
195 collected from the host while feeding were available from previous or
196 ongoing projects at the Fondazione Edmund Mach (Italy) and stored
197 in 70 % alcohol at -80 °C. DNA was extracted using Qiagen Dneasy
198 Blood and Tissue kit (Qiagen, Valencia, CA, USA). These control
199 samples (see Table 1 for details) were used i) to test that the primer
200 pairs amplified the target fragments correctly and reliably using
201 conventional PCR; ii) to validate the species-discriminating power of
202 HRMA and; iii) as positive samples in Real-time HRMA of questing
203 ticks with unknown bloodmeal sources. In addition to control
204 samples of the target species for each host-group, control samples of
205 some non-target species were included in the conventional PCR of
206 Muroidea (*Capreolus capreolus*, *Canis lupus familiaris*, *Sorex*
207 *antinorii*), Soricidae (*C. capreolus*, *C. l. familiaris*, *Myodes*
208 *glareolus*), Passeriformes (*C. capreolus*, *Apodemus flavicollis*) and
209 Caprinae (*C. capreolus*, *A. flavicollis).* Moreover, each group-
210 specific primer set was tested using conventional PCR on three
211 human DNA templates extracted from a partially engorged nymph
212 collected while feeding, whole human blood, and human hair. The
213 PCR included a negative control of the extraction and positive
214 controls for each primer set (see Supplementary file 2 for details).
215 Conventional PCR was performed at a final volume of 20 μL,
216 containing 0.5 μM of each primer, 0.25 mM of each dNTP, 1x
217 HotMaster *Taq* Buffer, 1.25 U HotMaster Taq (5-Prime), and 1 μL of
218 template DNA. The thermal cycling consisted of initiation at 94 °C
219 for 2 min; 40 cycles with denaturation at 94 °C for 30 s, annealing
220 with T_a (°C) of the group-specific primer set (Table 1) for 30 s,

221 elongation at 65 °C for 1 min; and termination at 65 °C for 10 min,
222 and was performed in a Veriti® Thermal Cycler (Applied Biosystems,
223 Foster City, CA, USA). Capillary electrophoresis of PCR products
224 was performed on a QIAxcel system (Qiagen, Valencia, CA, USA);
225 the DNA High Resolution Cartridge, QX 15 bp-3 Kb size marker and
226 the OM500 method were used for the run. Results were checked
227 using the software QIAxcel ScreenGel 1.0.2.0. At least one PCR
228 product per group-specific primer set was purified with Exo-SAP-
229 IT™ (GE Healthcare, Little Chalfont, England).Both forward and
230 reverse strands were sequenced on an ABI 3130 XL using Big Dye
231 Terminator v3.1 (Applied Biosystems, Foster City, CA, USA).
232 Sequencher v. 5. 1 was used for the electropherogram check and
233 consensus sequence creation. A BLASTn search was carried out to
234 verify the amplicon identity.
235 Real-time PCR coupled with HRMA was conducted on an
236 ECO™ Real-Time PCR machine (Illumina®, San Diego, USA) twice
237 for each sample at a final volume of 15 µL, containing 0.3 µM of
238 each primer, 1x SsoFast™ EvaGreen® Supermix (Bio-Rad,
239 Hercules, CA), and 3 µL of genomic DNA. Thermal cycling
240 conditions included initiation at 95 °C for 5 min; 50 cycles of
241 denaturation at 95 °C for 15 s and annealing and elongation with T_a
242 (°C) of the group-specific primer set (Table 1) for 15 s; directly
243 followed by HRMA carried out at 95 °C for 15 s, 55 °C for 15 s, then
244 by an increase of temperature from 55 °C to 95 °C, and 95 °C for 15
245 s. Fluorescence data was collected every 0.1 °C. HRMA was
246 performed using ECO™ v. 4.0 (Illumina®, San Diego, USA). Raw
247 fluorescence plots were normalized by setting pre- and post-melting

248 regions to 100% and 0%, respectively. Both normalized and
249 derivative graphs were analysed for melting temperatures (T_m °C) and
250 melting profile shapes. HRMA of control samples was used to define
251 the minimum and maximum T_m for each host species. Note that the
252 observed T_m was generally 2-6 °C lower than that predicted by
253 uMELT™ (Table 1).
254
255 *2.4. Testing the HRMA protocol*
256 In order to test the potential of our HRMA protocol to identify
257 the bloodmeal of questing ticks, *I. ricinus* nymphs were collected
258 from the vegetation in four localities in the Province of Trento
259 (Condino - CO: 45°53’03”N, 10°36’06”E, 450 m a.s.l.; Transacqua -
260 TR: 46°09’48”N, 11°49’56”E, 850 m a.s.l.; Cadine - CA:
261 46°05'48”E, 11°04'26”E, 550 m a.s.l.; Pietramurata - PI: 46°00'52”N,
262 10°55'26”E, 680 m a.s.l.) between April and June 2012 using
263 conventional blanket-dragging (Sonenshine, 1993) and sterile
264 forceps. Nymphs were frozen individually at -80 °C until DNA
265 extraction. According to Randolph et al. (2002), these spring-
266 collected nymphs are presumed to have consumed their first
267 bloodmeal as larvae during the previous spring/summer,
268 consequently bloodmeal will be nine to 14 months old. We decided
269 to optimize our technique for nymphs, because this stage of the tick
270 life cycle is considered the most important for the transmission of
271 tick-borne diseases to humans (Rizzoli et al., 2011). Prior to
272 processing, each tick was confirmed morphologically as *I. ricinus*
273 (Cringoli et al., 2005) using a dissecting microscope and washed
274 twice in DNA-free distilled water to rehydrate them and to eliminate

275 possible surface contaminants. Total DNA was extracted from 52
276 individuals using the QiaAmp® Investigator Kit (Purification of Total
277 DNA from Nail Clipping and Hair Protocol; Qiagen, Valencia, CA,
278 USA). Minor modifications to the pre-purification protocol were
279 implemented: briefly, each nymph was placed in a sterile vial
280 containing 230 μL ATL Buffer (Qiagen, Valencia, CA, USA) and cut
281 into small pieces with a sterile scalpel; 40 μL Proteinase-K 10
282 mM/mL (Sigma-Aldrich, Saint Louis, USA) and 30 μL DTT 1 M
283 (Sigma-Aldrich, Saint Louis, USA) were added to the solution.
284 Overnight digestion at 56 °C was performed in an incubator on a
285 rotary tube mixer. Total DNA was eluted in 60 μL ATE buffer. To
286 avoid contamination with environmental DNA, all DNA extraction
287 procedures were carried out under a laminar flow hood (UV-
288 sterilized); to check for cross-contamination, a negative control was
289 included in each DNA extraction procedure. DNA was stored at -20
290 °C until use. Real-time amplification and HRMA were performed as
291 previously described, testing each tick twice for each of the group-
292 specific primer sets, with minor modifications (i.e. 55 cycles of
293 amplification was needed for the Muroidea and Soricidae primers).
294 For each Real-time HRMA reaction, one positive control for each
295 target species and one negative control were included. Normalized
296 and derivative HRMA plots were obtained using ECO™ v. 4.0
297 (Illumina®, San Diego, USA). Amplicons from questing ticks were
298 assigned to species or genera by visually matching their melting
299 patterns (T_m, melting curve shape, number of melting peaks) to those
300 of control samples (see also Results for examples). Since the identity
301 of the hosts was unknown, this assignment to host species was

302 effectively ‘blind’. To verify the accuracy of this classification
303 procedure, all amplified samples were sequenced, both with reverse
304 and forward primers. Amplicons with T_m and melting curve profiles
305 divergent from those of control samples were also sequenced. Using
306 Sequencher v. 5.1 (Gene Codes Corporation, Ann Arbor, USA),
307 consensus sequences were created, visually checked and then aligned
308 in the corresponding group-specific alignment; in addition, a
309 BLASTn search was carried out.
310
311 **3. Results**
312
313 Six group-specific primers, targeting the 20 chosen host
314 species, were selected to allow identification of tick bloodmeal
315 sources using HRMA (Table 1). For each of these primer pairs,
316 conventional PCR resulted in amplification of the expected mtDNA
317 target for all the control samples from both tissue and engorged ticks,
318 as confirmed by BLASTn searches. None of the PCR negative
319 controls or the human DNA samples were amplified. Primer dimers
320 or short aspecific multiple amplicons were occasionally visible in the
321 QIAxcel images (reported in Supplementary file 2), as is typical of
322 PCR reactions for which appropriate template is lacking. Since
323 amplification of non-target species included in the testing of
324 Muroidea, Soricidae, Passeriformes or Caprinae primers did not
325 occur during conventional PCR (data not shown), only DNA control
326 samples from the target species were included in the testing of Real-
327 time HRMA of each host group.

328 Results obtained from Real-time HRMA on control samples
329 provide the proof-of-principle confirmation that HRMA can be used
330 to reliably identify target host species. The normalized and derivative
331 melting plots in Figure 1 and the T_m in Table 1 show that the
332 Muroidea species can be easily identified from bloodmeals except
333 those of the genus *Apodemus* (Fig. 1A, B). Similarly, for Soricidae,
334 Crocidura species are easily distinguishable but not those of the
335 genus *Sorex* (Fig. 1C, D; Table 1). In the case of Passeriformes, *T.*
336 *merula* is easily identified, but since only 0.1 – 0.4 °C separates the
337 melting peaks of *T. philomelos* and *E. rubecula* (Fig. 1E, F; Table 1),
338 amplicons falling within the ranges of these two species had to be
339 sequenced to confirm host identification. Some variability in melting
340 profiles was recorded in the Canidae, but *C. l. familiaris* was always
341 easily distinguishable from *Vulpes vulpes* (Fig. 1G, H). The melting
342 profile of Caprinae target species is characterized by multiple peaks,
343 but *Capra hircus* can be distinguished from *Rupicapra rupicapra* by
344 their non-overlapping first peaks (Table 1; Fig. 1I, J). There was also
345 variability in melting temperature between different control samples
346 of *C. capreolus*, but in any case, all *C. capreolus* samples were easily
347 distinguished from those of *Cervus elaphus* (Fig. 1K, L).
348 In order to test the HRMA protocol on questing ticks, 52
349 randomly chosen, field-collected questing *I. ricinus* nymphs were
350 analyzed using the above method separately for each of the six
351 group-specific primers. In most cases, identification of the bloodmeal
352 was straightforward: i.e., the T_m and melting profile of questing tick
353 (i.e. unknown) samples were clearly within the range of control
354 samples (see example of Cervidae in Fig. 2). Very occasionally,

355 amplicons gave T_m and/or melting curve profiles clearly divergent
356 from those of control samples (see example in Fig. 3A). These
357 amplicons were removed from the HRMA melting curve graphs to
358 make examining the remaining curves easier (see example in Fig.
359 3B). The aspecific amplicons were then sequenced to verify their
360 identity. A BLASTn search confirmed that these sequences were
361 mainly derived from tick DNA or simply short primer dimer
362 amplifications (Supplementary file 3). However, while testing the
363 Caprinae primers on questing ticks, by sequencing three unusual
364 amplicons, we confirmed that *Bos taurus* was also amplified (in
365 samples 2_CO, 3_CO and 5_CO), and has an HRMA profile similar
366 to but clearly distinguishable from those obtained for the tested
367 Caprinae species, with double melting peaks and a T_m of 80.8-81.0
368 and 83.7-84.1 °C, respectively (Fig. 4).
369 Using our primers and the described HRMA protocol,
370 bloodmeals were successfully identified in 34 out of 52 questing
371 nymphs analyzed (i.e. sensitivity was 65.4 %; Table 2). However, as
372 a result of mixed bloodmeals (see below) an additional eight
373 amplicons were generated. Sequencing confirmed that HRMA
374 allowed the correct identification of 35 out of 42 (83.3 %) bloodmeal
375 sources to species level (including *Bos taurus*), and five to genus
376 level (*Sorex* sp. and *Apodemus* sp.; samples 14_CA and 13_PI: *S.*
377 *antinorii*; samples 12_CO, 1_PI and 5_PI: *A. flavicollis*;
378 Supplementary data 3). Of the remaining two amplicons, sample
379 12_TR had an aspecific melting profile, within the target species
380 range (see Supplementary data 3)and was confirmed by sequencing
381 as *Crocidura leucodon* (). The last amplicon, 5_PI, was identified by

382 HRMA as *M. glareolus*, whereas the BLASTn search of the
383 sequenced amplicon suggested *A. flavicollis* (98% probability;
384 Supplementary data 3). Alignment of sample 5_PI and the *A.*
385 *flavicollis* control sample sequences revealed a 3 bp deletion (GTG)
386 in sample 5_PI that caused a variation in melting temperature to
387 match that of *M. glareolus.*
388 Identification of more than one host from the same tick (i.e.
389 amplification by more than one host-group primer set), occurred in
390 eight out of 34 (23.5 %) nymphs. *B. taurus* and *C. l. familiaris* were
391 both found in three nymphs; for the other five nymphs, the pairs of
392 hosts included: *Apodemus* sp. and *Crocidura suaveolens*; *Apodemus*
393 sp. and *C. elaphus*; *C. leucodon* and *C. elaphus*; *C. leucodon* and *V.*
394 *vulpes*; and *C. l. familiaris* and *C. elaphus.*
395 As reported in Table 2, amplicons from at least one questing
396 tick tested positive for each of the targeted Canidae and Cervidae
397 hosts; for Soricidae, *Sorex araneus* and *Crocidura russula* were not
398 found in any questing ticks, nor were *Mus musculus domesticus* and
399 *M. glareolus* from Muroidea host group. No nymphs were positive
400 for Caprinae or Passeriformes.
401
402 **4. Discussion**
403
404 This paper reports new primer sets and the necessary protocols
405 for the application of HRMA to the investigation of tick feeding
406 ecology, and in particular, the role of different host species in the
407 epidemiological cycles of tick-borne diseases. By using Real-time
408 amplification and HRMA, this method allows the simultaneous

409 screening and identification of bloodmeal sources in questing ticks
410 for 17 of the most important European vertebrate tick host species (8
411 wild ungulates, rodents, shrews and birds, as well as the domestic
412 dog, livestock species - goat, sheep and cattle -, and the commensal
413 house mouse), and two genera (*Apodemus* and *Sorex*).
414 Our results confirm the power of HRMA to identify the host
415 species or genus from both control DNA samples and questing
416 nymphs. Importantly, HRMA is a non-destructive post-PCR method,
417 meaning that amplification products from questing ticks can be
418 sequenced to confirm species identity in certain cases (e.g. to
419 distinguish *T. philomelos* and *E. rubecula*) and to reach species-level
420 identification for cases in which HMRA can only clarify the genus
421 (e.g. *Sorex* sp. and *Apodemus* sp.). In order to avoid unnecessary
422 expense, we would recommend post-HRMA sequencing of only
423 those amplicons with unusual melting profiles that fall within the T_m
424 range of target species (e.g. sample 12_TR; Supplementary data 3),
425 or that have similar shape to target species but are outside the
426 reported T_m range. These are worth sequencing as they may extend
427 the T_m range of that species (by identifying intraspecific sequence
428 variation), or even the list of target species for a particular primer set
429 (as in the case reported here of *B. taurus*). However, as shown here,
430 profiles that fall well beyond the range of target species or have a
431 very unusual shape are unlikely to yield host DNA, but are more
432 likely to be contaminant DNA or primer dimers.
433 In this study, we were able to identify bloodmeals in 65.4 % of
434 questing nymphs. This sensitivity is higher than mean sensitivities
435 published thus far for RLBH or PRC-RFLP for *I. ricinus* nymphs

436 (24.5 %: Estrada-Pena et al., 2005; 49.4 %: Pichon et al., 2005; 33 %:
437 Pichon et al., 2006; 38.2 %: Humair et al., 2007; 40.6 %: Morán
438 Cadenas et al., 2007) and is similar to that of Wodecka et al., (2014;
439 62.8 %). Our HRMA protocol also proved to have high identification
440 success, correctly assigning host DNA to species or genera using
441 HRMA alone in 40/42 (95.2 %) amplicons obtained from questing
442 nymphs; of these, 35/42 (83.3%) were identified to species. This
443 compares favourably to the 72 % and 62.3 % identification success to
444 genera or species reported in Morán Cadenas et al. (2007) and in
445 Humair et al. (2007), respectively, while all other above-mentioned
446 authors were only able to identify host DNA to group, family or
447 occasionally genus level. Unfortunately, direct comparison of all
448 three currently available methods, by testing the same nymphs with
449 both RLBH, PCR-RFLP and HRMA, is not possible at this time
450 because of the limited quantity of eluted DNA available from each
451 questing nymph. Only application of this new HRMA protocol to
452 large collections of questing ticks will confirm its place among
453 bloodmeal identification methods.
454 The lack of host identification in 18 out of 52 questing ticks,
455 may be a result of the time since the last bloodmeal, which we
456 estimated as nine to 14 months (Kirstein and Gray, 1996; Randolph
457 et al., 2002), or specific individual developmental dynamics, heavily
458 affected by site and climatic conditions (Morán Cadenas et al., 2007).
459 However, it may be that some nymphs fed as larvae on species not
460 included in our primer design process (e.g. *Podarcis muralis*,
461 *Erinaceus europapaeus*, *Lepus europapaeus*, *Sciurus vulgaris*, *Sus scrofa,*
462 etc.), which are currently considered minor hosts in our study area.

463 For this reason, additional primer sets are currently being designed to
464 expand host coverage.
465 HRMA led to host misidentification in only one individual, as
466 a result of sequence variation at the intraspecific level, not
467 predictable during amplicon selection and primer design. These
468 errors derive from the relative scarcity of available GenBank mtDNA
469 sequences for some of the selected host-species (e.g. *Apodemus* sp.)
470 that are not currently the object of intensive genetic study, despite
471 their importance in zoonotic disease cycles.
472 DNA from multiple hosts was detected in 23.5 % of tested
473 nymphs. Although this result is based on a relatively small number of
474 samples, it is comparable to that obtained in 2007 by Morán Cadenas
475 et al. (19.5 %), although the efficacy of PCR-RLBH and PCR-RFLP
476 protocols to detect mixed bloodmeals has not been thoroughly
477 investigated to our knowledge (see Humair et al., 2007; Wodecka et
478 al., 2014). In addition, the presence of DNA from more than one host
479 may increase the complexity of host identification with the above
480 methods as well as those using direct sequencing (Alcaide et al.,
481 2009; Kent, 2009). However, our HRMA approach allows
482 unambiguous detection of multiple hosts, at least when these are
483 species belonging to different host groups. Further investigations are
484 needed to determine whether mixed bloodmeals of species within the
485 same host group are being overlooked (Albonico et al., 2013;
486 McCarthy et al., 2013). As noted by Morán Cadenas et al. (2007),
487 further testing should be also done to confirm whether the multiple
488 host DNA is a result of voluntary drop off and secondary questing by

489 the tick (true mixed bloodmeals), involuntary interrupted feeding, or
490 unsuccessful attachment.

491 The fact that no nymphs were found to have fed as larvae on
492 targeted species of Passeriformes and Caprinae should not be taken
493 as an indication of the suitability of the primer set, but simply
494 regarded as a result of the small sample size; in fact, ongoing
495 bloodmeal screening using this protocol provide amplification of *T.*
496 *merula*, *T. philomelos*, *E. rubecula* and *R. rupicapra* DNA from
497 questing nymphs (Collini *et al.* in prep).

498 We are currently attempting to improve the time- and cost-
499 effectiveness of the HRMA method described here by introducing
500 automated DNA extraction and Real-time HRMA reaction set-up.
501 However, although six different amplifications must be carried out
502 on each tick, the single-step Real-time HRMA design described here,
503 enabling reaction processing, screening and genotyping on the same
504 instrument, still make it simple and fast compared to other methods.
505 In addition, as is, the method is extremely useful for unambiguous
506 identification of multiple host DNA. Our protocol also reduces errors
507 common in multi-step molecular protocols and avoids amplification
508 of both environmental and human contaminating DNA, a recurring
509 problem in low quantity/quality DNA studies. Moreover, the lack of
510 the sequencing step in all but the most dubious cases lowers the cost
511 of analysis. In addition, Real-time HRMA reagents have costs
512 comparable to those for conventional PCR, and are usually provided
513 as a *supermix*, further reducing errors (Reed et al., 2007). In future,
514 the development of a multiplex assay with unlabeled probes (Reed et

515 al., 2007; Seipp et al., 2008) would further increase the time- and
516 cost-saving properties of the devised method.
517 Although the technique described here is optimized for
518 nymphs, our protocol should also be easily applicable to adult
519 questing ticks, given the larger bloodmeal of the nymphal stage
520 compared to the larval one. This protocol could also be applied to
521 other species of ticks, and other hematophagous insects in general, if
522 this were convenient, by expanding primer sets if necessary.
523
524 **5. Conclusion**

525 In this paper we demonstrate that Real-Time HRMA is a reliable
526 method for bloodmeal analysis in questing ticks. Six newly designed
527 host group specific primers target 21of the most important *I. ricinus*
528 hosts, and HRMA allows species level identification for 17 of them
529 (plus the genera *Apodemus* and *Sorex*). The application of this new
530 tool to questing ticks demonstrated its capacity to retrieve host DNA
531 from the remnant larval bloodmeal in 65.4 % of individuals, while
532 83.3% of these positive samples could be identified to species by
533 HRMA alone. These are notable improvements on the sensitivity and
534 identification success of most currently widely-used methods. In
535 addition, the protocol described here demonstrated good
536 contamination control, and is relatively rapid and simple.
537
538 **Acknowledgements**

539
540 We thank Fondazione Edmund Mach, the Università degli Studi di
541 Milano and the European Union grant FP7-261504 EDENext for

542 funding. This article is catalogued by the EDENext Steering
543 Committee as EDENext 000 (http://www.edenext.eu). We thank the
544 technical staff of the Department of Biodiversity and Molecular
545 Ecology of the Fondazione E. Mach for field and laboratory
546 assistance and advice and two anonymous Reviewers for helping to
547 improve the manuscript.

548

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704

Table 1

Target species within each host-group, features of host-group specific primers and amplicons and related HRMA parameters. All species within each host group can be distinguished from one another using the HRMA method described here unless otherwise indicated.

Targets and amplification parameters			**Amplicon features**					**HRMA**			
Host – group	**mtDNA Target Primer (5' - 3')**	**T_a (°C)**	**Target species**	**Size (bp)**	**GC %**	**T_m sim (°C) T_m1**	**T_m2**	**Control samples[a]**	**T_m obs (°C) T_m1**	**T_m2**	**HRMp[b] Fig. 1**
Muroidea	*d-loop*	60	*A. flavicollis*	175	45	86.4	-	2E	81.4 [c]	-	A - B
	F_TCTGGTTCTTACTTCAGGGC		*A. sylvaticus*	175	44	86.1	-	2T	81.3 [c]	-	
	R_TTCATGCCTTGACGGCTATG		*M. glareolus*	176	44	85.9	-	2E	81.0-81.2	-	
			M. m. domesticus	175	43	85.5	-	2T	80.7-80.9	-	
Soricidae	*d-loop*	62	*S. minutus*	137	44	84.2	-	1T	80.3-80.5[d-e]	-	C - D
	F_TCAGCCCATGCCGACACAT		*S. antinorii*	137	43	83.9	-	1T – 1E	80.1-80.4 [d]	-	
	R_GCCCCCATAGAGAATAAGCC		*S. araneus*	137	42	83.4	-	n.a.			
			C. leucodon	136	45	84.7	-	1T – 1E	82.1-82.3	-	
			C. suaveolens	137	47	86.0	-	1T – 2E	81.4-81.5	-	
			C. russula	136	46	85.0	-	n.a.			
Passeriformes	*12S*	60	*T. merula*	155	52	90.2	-	1T – 3E	80.4-80.6	-	E - F
	F_ATCCACGATATTACCTGACCATT		*T. philomelos*	155	50	90.0	-	1T – 2E	83.9-84.0	-	
	R_TACCCCATTGCTTCCATTCC		*E. rubecula*	156	50	89.6	-	1T – 3E	83.5-83.8	-	
Canidae	*d-loop*	61	*C. l. familiaris*	147	44	82.7	85.9	4E	80.3-81.3[f]		G - H
	F_CCGCAACGGCACTAACTCTA		*V. vulpes*	146	49	84.5	88.6	1T	81.3	83.5-83.6[e]	
	R_CCATTGACTGAATAGCACCTTG										
Caprinae	*12S*	57	*C. hircus*	158	37	80.1	86.3	1T	76.5	80.5	I - J
	F_TAAATCTCGTGCCAGCCA		*R. rupicapra*	158	37	79.6	86.6	2E	75.3-75.7	80.3-80.6	
	R_GTAGGGTTACTTTCGTCAT		*O. aries*	158	36	78.3	88.0	3E	74.5-75.0	81.7-82.0	
Cervidae	*d-loop*	60	*C. capreolus*	168	45	84.5	88.6	1T – 2E	80.5-80.9	82.5-82.9	K - L
	F_CGATGGACTAATGACTAATCAG		*C. elaphus*	169	47	85.2	88.9	1T – 2E	80.7-80.8	83.6-83.7	
	R_TTATGGGGATGCTCAAGATG										

1 T_a, annealing temperature; T_m sim., simulated melting temperature from uMELT^{TM} (Dwight *et al.* 2011); T_m1, melting temperature peak 1; T_m2, melting temperature peak 2 (if present); T_m obs, observed melting temperature expressed as *minimum-maximum* range; n.a., not available.

6 [a]Number of control DNA samples used in HRMA testing; T, extracted from host tissue; E, extracted from engorged ticks (each tick was collected from a different individual of the host species).

10 [b]HRM profiles

11 [c]The melting temperature, as well as the melting profiles, for *A. sylvaticus* and *A. flavicollis d-loop* amplicons are fully overlapping, so discrimination of these two species is not possible using these primers.

15 [d]The melting temperature for *S. antinorii* and *S. minutus d-loop* amplicons are not sufficiently different from each other to be diagnostic for species identification.

18 [e]Reported range refers to the variation observed between the two replicates of the same single control sample.

20 [f]Wide range of T_m is related to mutations in the sequence of the used control samples (see Fig. 1G).

22

Table 2

Results of host identification with HRMA using field-collected questing nymphs.

Host DNA identification		Site[a]				Total
Host group	**Host genera/species**	**CO**	**TR**	**CA**	**PI**	
Muroidea	*Apodemus sp.*	1			2	3
	M. glareolus					0
	M. m. domesticus					0
Soricidae	*Sorex sp.*			1	1	2
	S. araneus					0
	C. leucodon		2	1	4	7
	C. suaveolens	1	1	1	2	5
	C. russula					0
Passeriformes	*T. merula*					0
	T. philomelos					0
	E. rubecula					0
Canidae	*C. l. familiaris*	6	3		1	10
	V. vulpes		2			2
'Caprinae'	*O. aries*					0
	R. rupicapra					0
	C. hircus					0
	B. taurus	3				3
Cervidae	*C. capreolus*	1		1		2
	C. elaphus	1	2	1	4	8
n ticks with identified bloodmeal/*n* ticks tested		9/13	8/12	5/14	12/13	34/52
% ticks with identified bloodmeal		69.2	66.7	35.7	92.3	65.4
n mixed bloodmeals		4	2	0	2	8
n different host species/genera identified		6	5	5	6	9

[a]CO, Condino; TR, Transacqua; CA, Cadine; PI, Pietramurata.

56 **Figure Legend**

57

58 **Fig. 1.**

59 HRMA of positive control samples of the species listed in the
60 legends using group-specific primer sets. (A, B) Muroidea, (C, D)
61 Soricidae, (E, F) Passeriformes, (G, H) Canidae, (I, J) Caprinae, (K,
62 L) Cervidae; Figs. 1A, C, E, G, I and K are normalized melting plots,
63 while Figs. 1B, D, F, H, J and L are derivative melting plots. Yellow
64 bars delineate pre- and post- melting normalization regions. For
65 Canidae (I, J), alignment of the sequences obtained from the two
66 most differentiated *C. l. familiaris* HRMA profiles (*a*, *b*) showed that
67 sample *a* had one transversion (T->C) compared to sample *b* causing
68 a + 0.5 °C shift in T_m. In Fig.1K, L melting profiles from *C.*
69 *capreolus* tissue (*) are notably different from melting profiles from
70 engorged ticks (#), possibly related to a difference in DNA
71 concentration.

72

73 **Fig. 2.**

74 Normalized melting plot of the Cervidae primer set showing the
75 melting profile obtained from control samples and questing nymphs
76 that provided amplification. Note that the melting curves of unknown
77 samples are very similar to those of positive samples despite a slight
78 deviation of T_m, ensuring correct species identification of unknown
79 (i.e. questing tick) bloodmeals.

80 **Fig. 3.**

81 Analysis of HRMA data by means of melting plots: an example using
82 Canidae HRMA on questing nymphs. (A) Normalized melting graph

83 of all amplified samples: note that the HRMA curves obtained for
84 amplicons from questing ticks are very similar to (1) or clearly
85 different from (2) control sample curves (in this case: *C. l . familiaris*
86 and *V. vulpes*); (B) normalized melting graph reporting only those
87 samples with melting profiles matching those of control samples.
88 **Fig. 4.**
89 HRM plots for Caprinae. (A) Normalized; (B) derivative. Grey lines
90 indicate unexpected HRMA profiles obtained from two replicates of
91 samples 2_CO, 3_CO and 5_CO, later identified by sequencing as
92 *Bos taurus*. Note that these curves are similar in shape to those of
93 other hosts, and very different from the unusual curves in Fig. 3A.
94
95 **Supplementary File 1**
96 Partial host group alignments with primers sequences.
97 **Supplementary File 2**
98 QIAxcel capillary electrophoresis images of conventional PCR of a
99 representative subset of DNA control samples obtained from host
100 tissue or from engorged ticks directly collected from the host, and
101 from human DNA, for each pair of group-specific primers. *
102 sequenced PCR products. Size markers can be seen at 15 bp and
103 3000 bp. Bands appearing at about 40 bp are primer dimers. These
104 bands disappeared when we increased the quantity of DNA template
105 in the Real-time reaction. **(A) Muroidea**: *M. m. domesticus*: lane 1,
106 tissue; *M. glareolus*: lanes 2-3, tissues; lanes 4-5, engorged *I. ricinus*
107 larvae; *A. sylvaticus*: lane 6, 1:100 diluted tissue; lane 7, tissue; *A.*
108 *flavicollis*: lane 8, tissue; lanes 9-10, engorged *I. ricinus* larvae; **(B)**
109 **Soricidae**: *S. antinorii*: lane 1, tissue; lane 2, engorged *I. ricinus*

110 larva; *S. minutus*: lane 3, tissue; *C. leucodon*: lane 4, tissue; lane 5,
111 engorged *I. ricinus* larva; *C. suaveolens*: lane 6, tissue; lanes 7-8,
112 engorged *I. ricinus* larvae; **(C) Passeriformes**: *T. merula*: lane 1,
113 tissue; lane 2, engorged *I. ricinus* nymphs; *T. philomelos*: lane 3,
114 tissue; lane 4, engorged *I. ricinus* larva; *E. rubecula*: lane 5, tissue;
115 lane 6, engorged *I. ricinus* larva; **(D) Canidae**: *C. l. familiaris*: lanes
116 1-3, engorged *I. ricinus* females; *V. vulpes*: lane 4, tissue; **(E)**
117 **Caprinae**: *R. rupicapra*: lanes 1-2, tissues; lanes 3-5, engorged *I.*
118 *ricinus* females; *C. hircus*: lanes 6-8, tissues; *O. aries*: lanes 9-10,
119 engorged *I. ricinus* females; **(F) Cervidae**: *C. capreolus*: lanes 1-3,
120 tissues; *C. elaphus*: lanes 4-6, tissues. K-pcr: PCR negative control.
121 **(G) Conventional PCR on human DNA for each group-specific**
122 **primer set:** lane 1, human DNA extracted from partially engorged *I.*
123 *ricinus* nymph collected while feeding; lane 2, whole human blood;
124 lane 3, human hair; lane 4, negative extraction control; lane 6,
125 negative PCR control. Lane 5, target host species DNA control
126 sample, as follows: a. *M. m. domesticus* (tissue); b. *C. suaveolens*
127 (tissue); c. *T. merula* (engorged *I. ricinus* nymph); d. *V. vulpes*
128 (tissue); e. *C. hircus* (tissue); f. *C. capreolus* (engorged *I. ricinus*
129 female).
130
131 **Supplementary File 3**
132 Table reporting identification results by HRMA and sequence
133 BLASTn.

Table1

Table 1

Target species within each host-group, features of host-group specific primers and amplicons and related HRMA parameters. All species within each host group can be distinguished from one another using the HRMA method described here unless otherwise indicated.

Targets and amplification parameters			**Amplicon features**					**HRMA**			
Host – group	**mtDNA Target Primer (5' - 3')**	T_a **(°C)**	**Target species**	**Size (bp)**	**GC %**	T_m **sim (°C)** T_m1	T_m2	**Control samples**[a]	T_m **obs (°C)** T_m1	T_m2	**HRMp**[b] **Fig. 1**
Muroidea	*d-loop*	60	*A. flavicollis*	175	45	86.4	-	2E	81.4[c]	-	A - B
	F_TCTGGTTCTTACTTCAGGGC		*A. sylvaticus*	175	44	86.1	-	2T	81.3[c]	-	
	R_TTCATGCCTTGACGGCTATG		*M. glareolus*	176	44	85.9	-	2E	81.0-81.2	-	
			M. m. domesticus	175	43	85.5	-	2T	80.7-80.9	-	
Soricidae	*d-loop*	62	*S. minutus*	137	44	84.2	-	1T	80.3-80.5[d-e]	-	C - D
	F_TCAGCCCATGCCGACACAT		*S. antinorii*	137	43	83.9	-	1T – 1E	80.1-80.4[d]	-	
	R_GCCCCCATAGAGAATAAGCC		*S. araneus*	137	42	83.4	-	n.a.			
			C. leucodon	136	45	84.7	-	1T – 1E	82.1-82.3	-	
			C. suaveolens	137	47	86.0	-	1T – 2E	81.4-81.5	-	
			C. russula	136	46	85.0	-	n.a.			
Passeriformes	*12S*	60	*T. merula*	155	52	90.2	-	1T – 3E	80.4-80.6	-	E - F
	F_ATCCACGATATTACCTGACCATT		*T. philomelos*	155	50	90.0	-	1T – 2E	83.9-84.0	-	
	R_TACCCCATTGCTTCCATTCC		*E. rubecula*	156	50	89.6	-	1T – 3E	83.5-83.8	-	
Canidae	*d-loop*	61	*C. l. familiaris*	147	44	82.7	85.9	4E	80.3-81.3[f]		G - H
	F_CCGCAACGGCACTAACTCTA		*V. vulpes*	146	49	84.5	88.6	1T	81.3	83.5-83.6[e]	
	R_CCATTGACTGAATAGCACCTTG										
Caprinae	*12S*	57	*C. hircus*	158	37	80.1	86.3	1T	76.5	80.5	I - J
	F_TAAATCTCGTGCCAGCCA		*R. rupicapra*	158	37	79.6	86.6	2E	75.3-75.7	80.3-80.6	
	R_GTAGGGTTACTTTCGTCAT		*O. aries*	158	36	78.3	88.0	3E	74.5-75.0	81.7-82.0	
Cervidae	*d-loop*	60	*C. capreolus*	168	45	84.5	88.6	1T – 2E	80.5-80.9	82.5-82.9	K - L
	F_CGATGGACTAATGACTAATCAG		*C. elaphus*	169	47	85.2	88.9	1T – 2E	80.7-80.8	83.6-83.7	
	R_TTATGGGGATGCTCAAGATG										

T_a, annealing temperature; T_m sim., simulated melting temperature from uMELT™ (Dwight *et al.* 2011); T_m1, melting temperature peak 1; T_m2, melting temperature peak 2 (if present); T_m obs, observed melting temperature expressed as *minimum-maximum* range; n.a., not available.

[a]Number of control DNA samples used in HRMA testing; T, extracted from host tissue; E, extracted from engorged ticks from different individuals.

[b]HRM profiles

[c]The melting temperature, as well as the melting profiles, for *A. sylvaticus* and *A. flavicollis d-loop* amplicons are fully overlapping, so discrimination of these two species is not possible using these primers.

[d]The melting temperature for *S. antinorii* and *S. minutus d-loop* amplicons are not sufficiently different from each other to be diagnostic for species identification.

[e]Reported range refers to the variation observed between the two replicates of the same single control sample.

[f]Wide range of T_m is related to mutations in the sequence of the used control samples (see Fig. 1G).

Table2

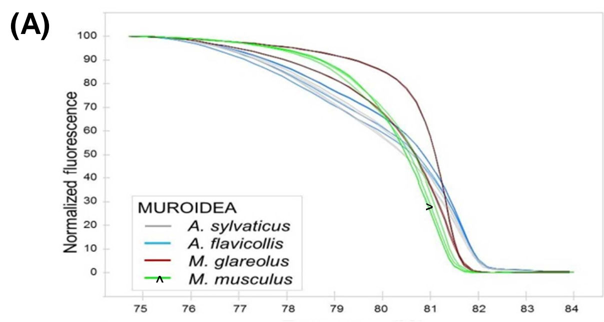
Table 2

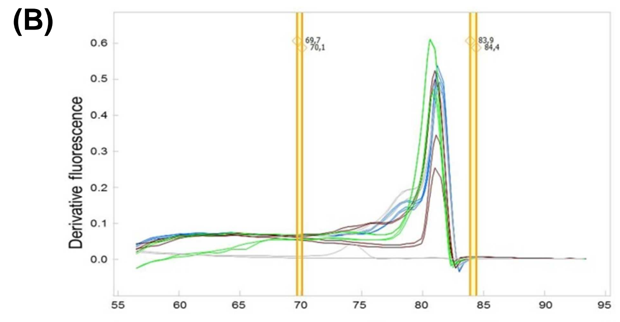
Results of host identification with HRMA using field-collected questing nymphs.

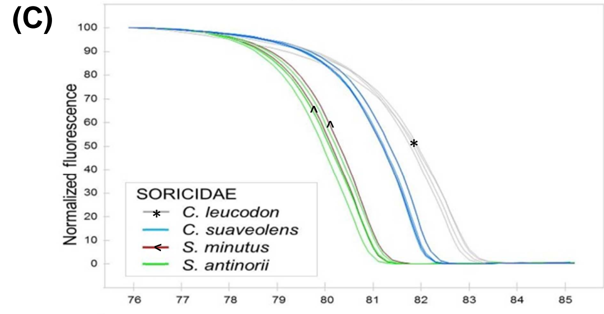
Host DNA identification		**Site**[a]				**Total**
Host group	**Host genera/species**	**CO**	**TR**	**CA**	**PI**	
Muroidea	*Apodemus sp.*	1			2	3
	M. glareolus					0
	M. m. domesticus					0
Soricidae	*Sorex sp.*			1	1	2
	S. araneus					0
	C. leucodon		2	1	4	7
	C. suaveolens	1	1	1	2	5
	C. russula					0
Passeriformes	*T. merula*					0
	T. philomelos					0
	E. rubecula					0
Canidae	*C. l. familiaris*	6	3		1	10
	V. vulpes		2			2
'Caprinae'	*O. aries*					0
	R. rupicapra					0
	C. hircus					0
	B. taurus	3				3
Cervidae	*C. capreolus*	1		1		2
	C. elaphus	1	2	1	4	8
n ticks with identified bloodmeal/*n* ticks tested		9/13	8/12	5/14	12/13	34/52
% ticks with identified bloodmeal		69.2	66.7	35.7	92.3	65.4
n mixed bloodmeals		4	2	0	2	8
n different host species/genera identified		6	5	5	6	9

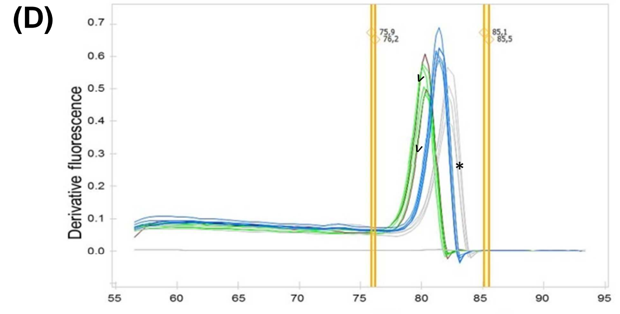
[a]CO, Condino; TR, Transacqua; CA, Cadine; PI, Pietramurata.

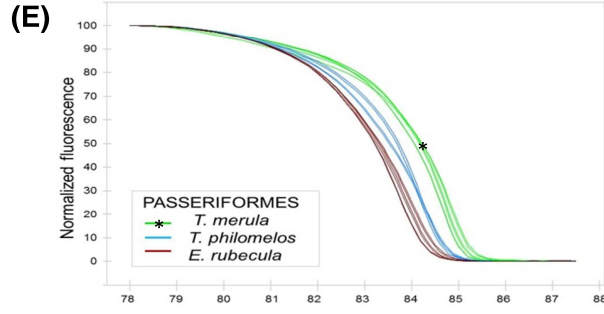
Figure_colour for online publications

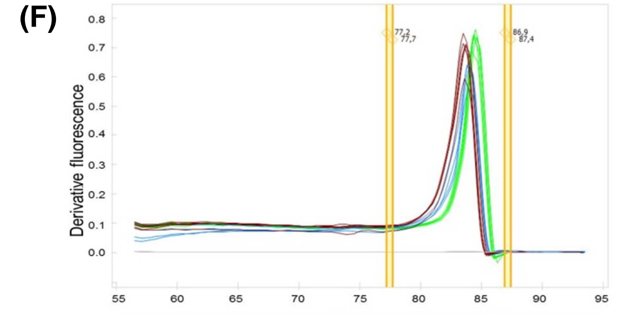


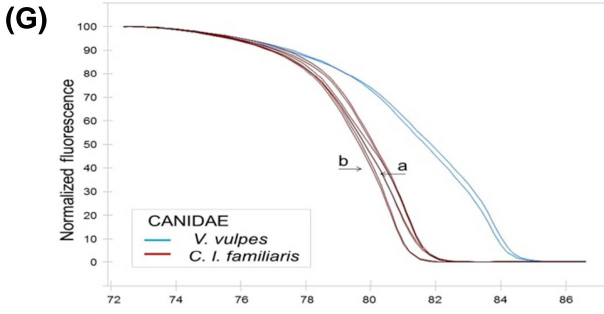


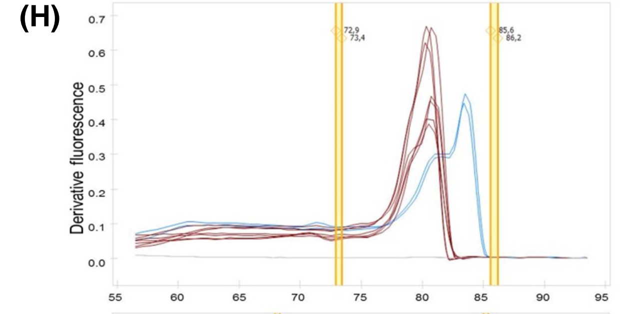


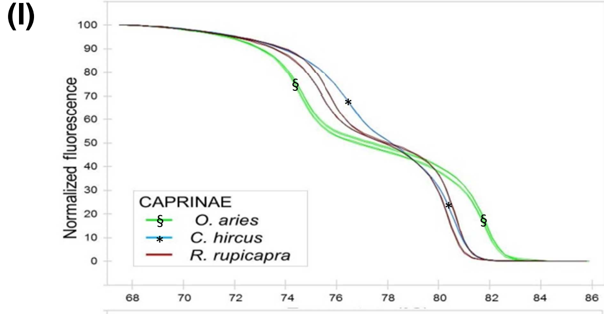


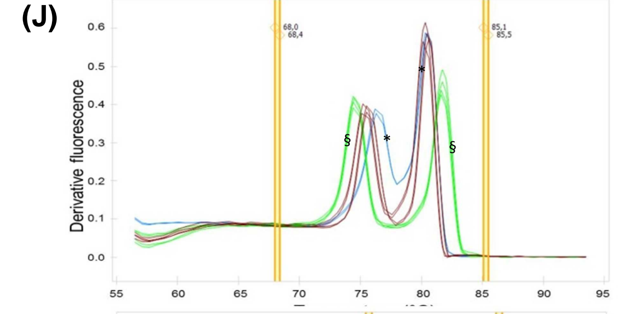


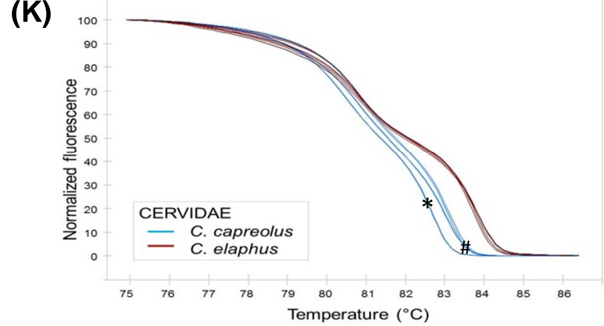


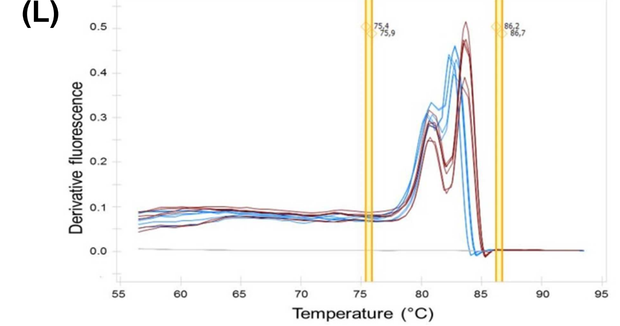












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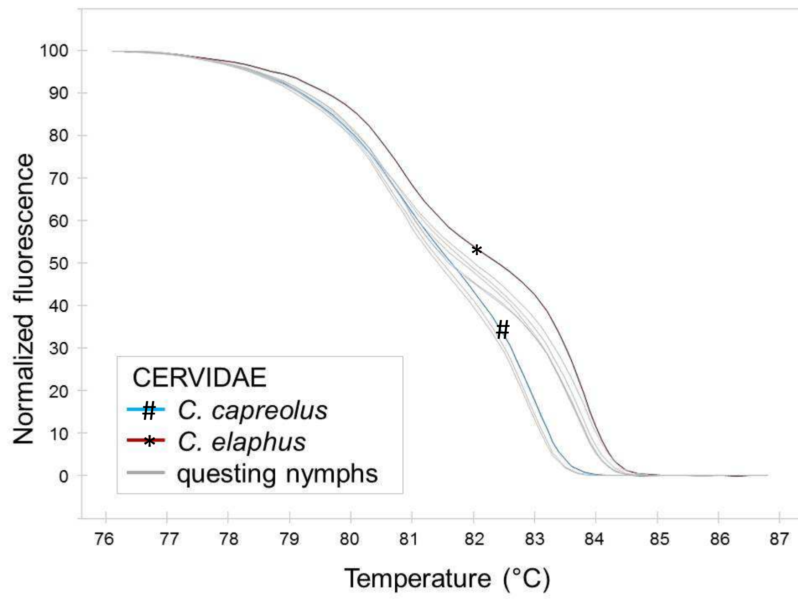

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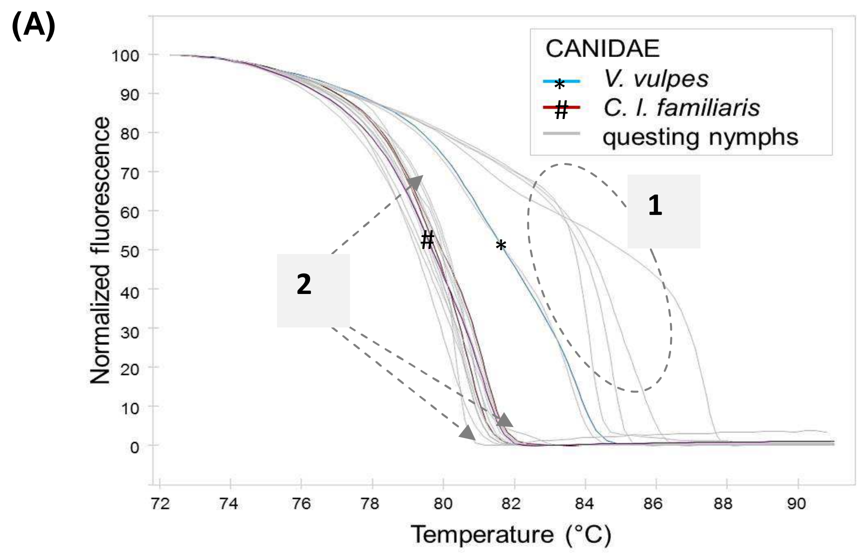

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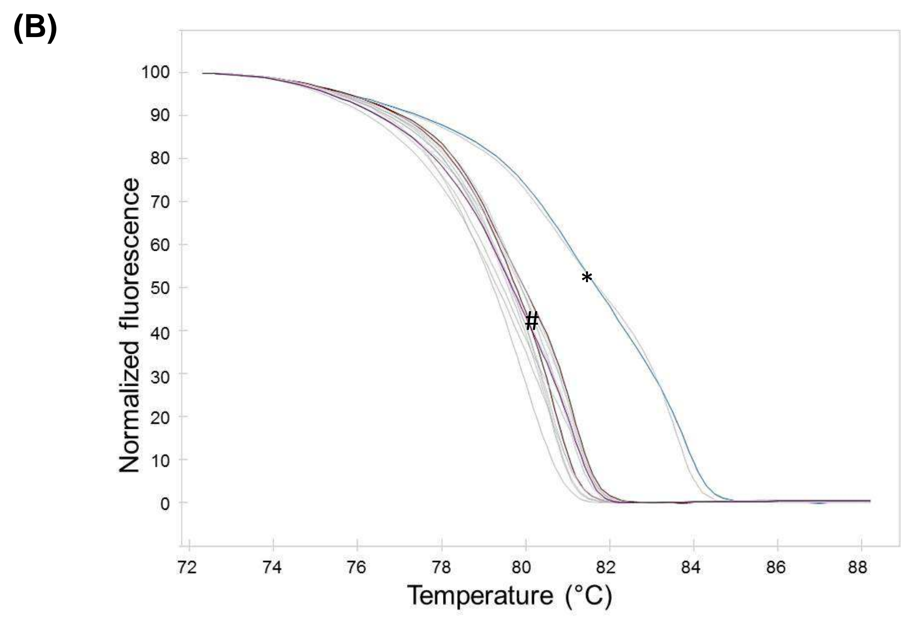

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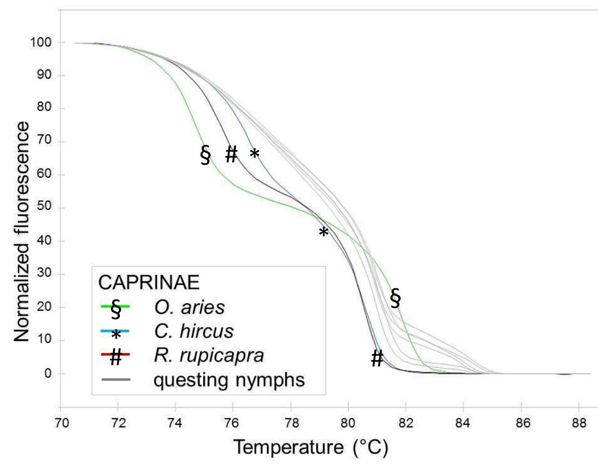

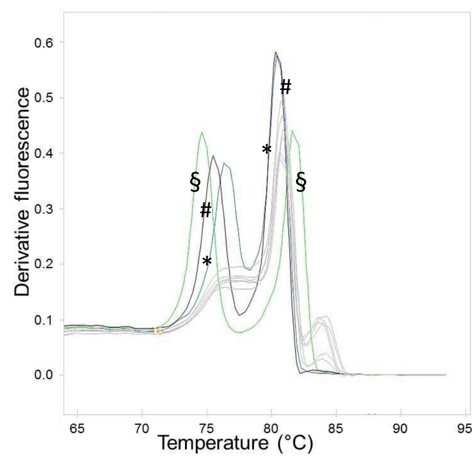

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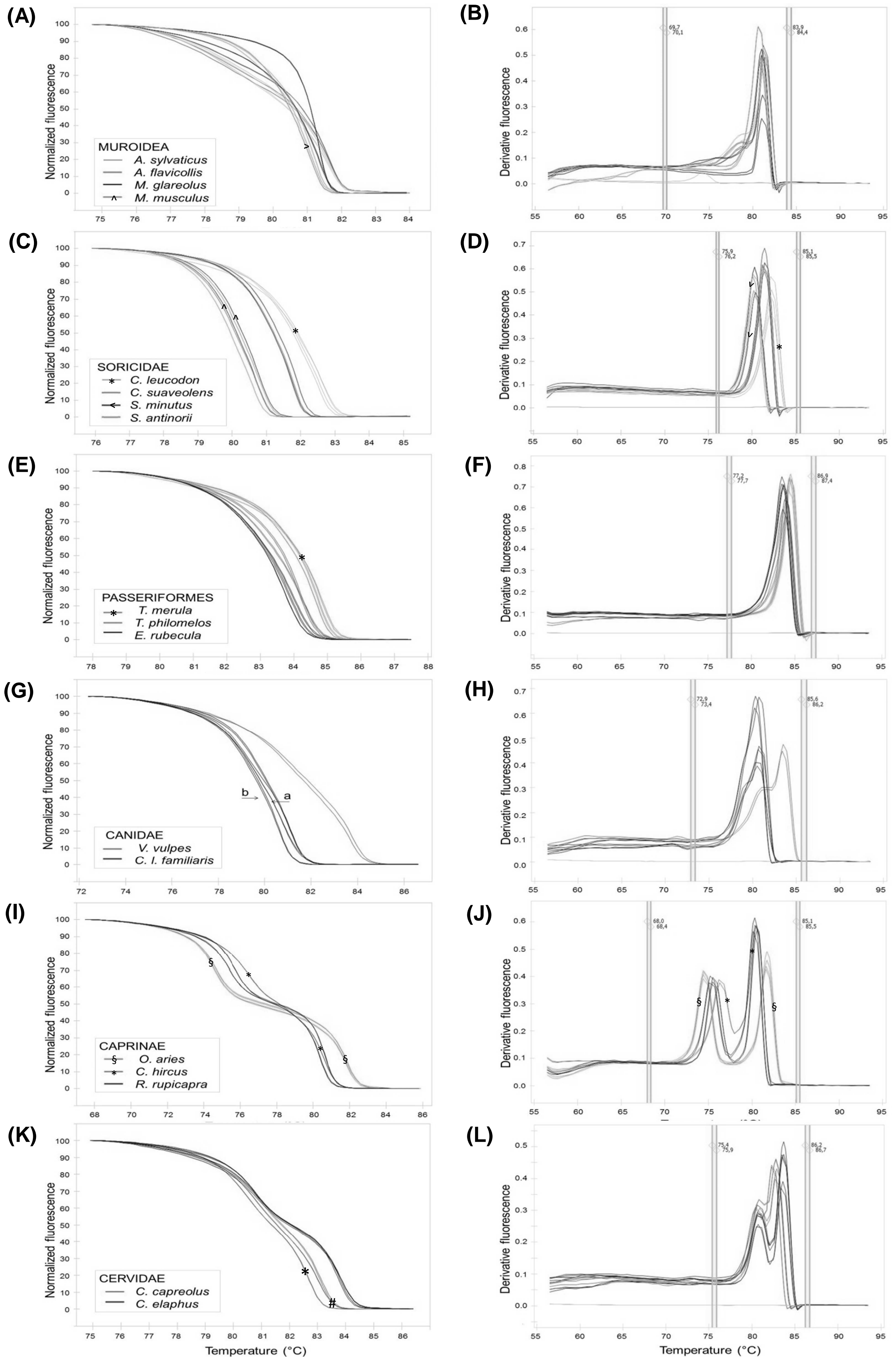

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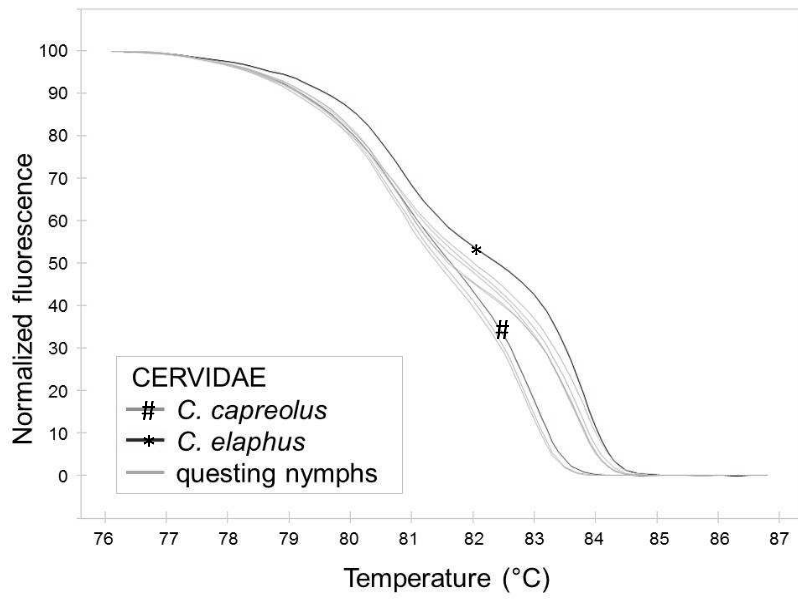

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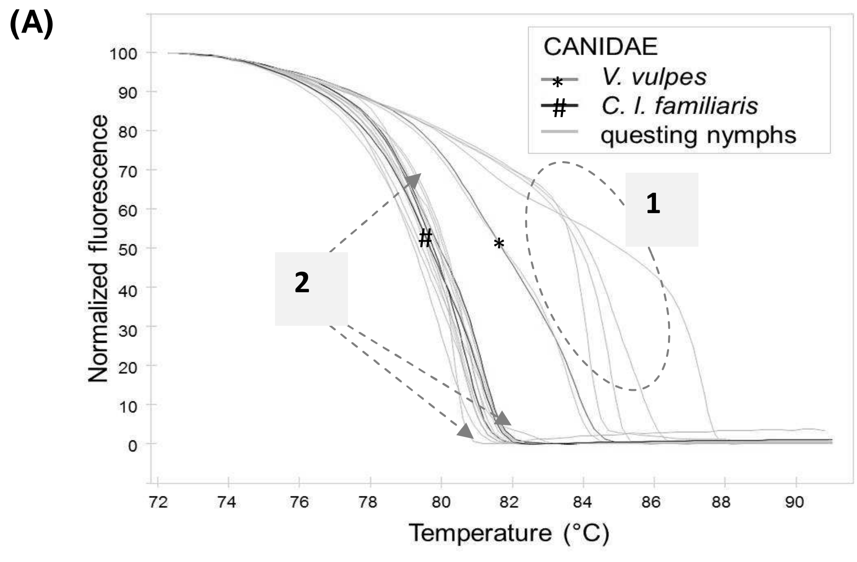

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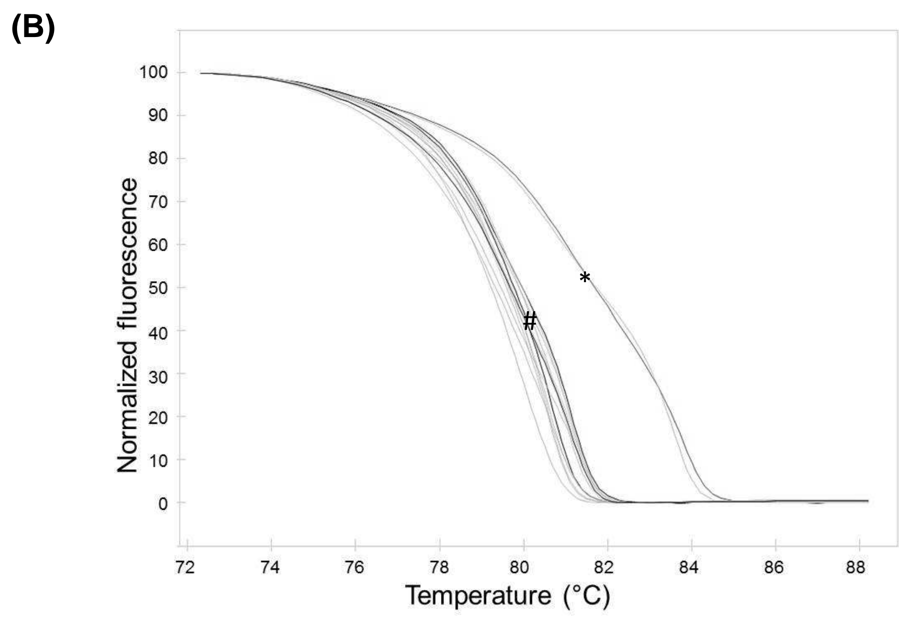

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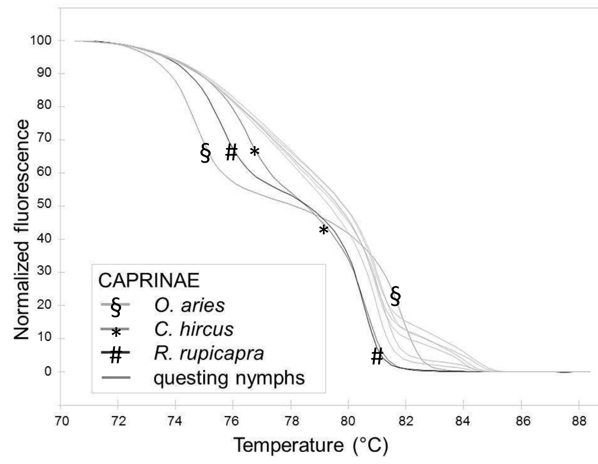

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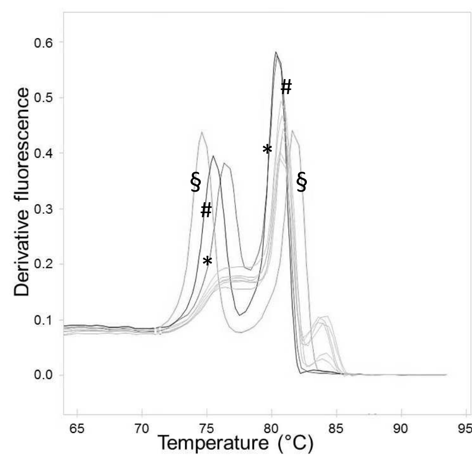

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