Int J Legal Med (2010) 124:277–285
DOI 10.1007/s00414-009-0396-x

TECHNICAL NOTE

Identification of forensically important blowfly species (Diptera: Calliphoridae) by high-resolution melting PCR analysis

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Received: 13 March 2009 / Accepted: 18 November 2009 / Published online: 16 January 2010

Abstract We describe here the successful coupling of real-time polymerase chain reaction (PCR) and high-resolution melting (HRM) analysis to rapidly identify 15 forensically important species of blowfly from the family Calliphoridae (Diptera), which occur in Poland. Two short regions (119 and 70 base pairs, respectively) of cytochrome oxidase gene subunit I with sufficient sequence diversity were selected. In the case of lacking taxa (e.g., reference species) these amplicons can be synthesized using sequences deposited in gene banks. The technique utilizes low template DNA concentration and is highly reproducible. The melting profile was not altered up to a 10,000-fold difference in DNA template concentration (ranging from 5 pg to 50 ng). The several HRM runs performed on different specimens from Poland belonging to the same species and on different days resulted in only minor variations in the amplification curves and in melting temperatures of the peaks. Intraspecific variation in a larger scale was tested using synthesized oligonucleotides from cosmopolitan *Lucilia illustris* originating from Poland, France, Great Britain, India, and USA. As HRM PCR analysis is sensitive to even single base changes, all geographic variants of this species were identified. This technique is also cost-effective and simple, and it may even be used by non-geneticists. A working protocol was ultimately constructed for the purpose of rapid and accurate species identification in most countries in Europe regardless of which stage or which part of a blowfly was collected.

Keywords Forensic entomology · Blowflies · Species identification · COI · Real-time PCR · High-resolution melting

Introduction

One of the challenges facing modern biology is to develop accurate and reliable methods for the rapid identification of species in numerous fields of study, such as taxonomy, epidemiology, forensics, archeology, and ecology. Molecular identification is also central to the diagnosis, treatment, and control of infections caused by different pathogens. In recent years, a variety of DNA-based approaches have been developed to identify species in many taxonomic groups [7, 43, 63].

Forensic entomology is a scientific discipline that focuses on the presence of insects and other arthropods in criminal and general legal investigations. Identifying species found in association with a corpse is one of the first steps a forensic entomologist performs in seeking to estimate the post-mortem interval (PMI) [61]. Among these species, a leading role is played by the blowflies from the family Calliphoridae, which occur in abundant numbers and are among the first to colonize dead bodies. Blowflies feed on decaying organic matter and thus by themselves may show how much time has passed between a death and the time of discovery of the corpse [37]. The speed and effectiveness with which maggots operate was even known to the famous naturalist Carolus Linnaeus [31, p. 990], who noted that "Tres Muscae consumunt cadaver Equi, aeque

Electronic supplementary material The online version of this article (doi:10.1007/s00414-009-0396-x) contains supplementary material, which is available to authorized users.

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cito ac Leo" ("Three flies will consume the carcass of a horse as quickly as a lion", in the sense of them producing large masses of maggots). Nevertheless, their use in PMI estimation has been hampered by difficulties associated with correct species identification at all developmental stages (eggs, larvae, pupae, adults), particularly when only fragments of insect evidence are available for analysis. Immature stages of most species of blowflies are very similar, and even taxonomists have serious difficulty with species identification based solely on morphological characters. This similarity of many species of blowfly leaves a DNA-based identification approach to be more advantageous than morphology. The genetical methods which have already been used in identification of calliphorids include RFLP-PCR [1, 53] and DNA sequencing [e.g., 4, 35, 56, 59, 62, 64]. The latest method is currently the most frequent approach used in molecular identification of different taxa at the species level. However, current sequencing technology produces error rates in the order of 1.5% for a 550 base-pair (bp) read (ABI 3700 analyzer specifications) in a single run. It is also a time-consuming procedure.

Real-time polymerase chain reaction (PCR) offers the significant advantage over conventional PCR-based identification systems of allowing work to be done without post-PCR handling, thereby reducing the risk of carryover contamination in the laboratory. It also offers increased sensitivity, by permitting discrimination of spurious PCR amplifications from non-target DNA. This is also a relatively fast genotyping technique, with some platforms affording high throughput automation. The recent developments in rapid genotyping and mutation scanning include high-resolution melting (HRM) PCR [18]. The HRM PCR is a close-tube and post-PCR method enabling the quick analysis of genetic variation in PCR amplicons. HRM PCR involves precise monitoring of changes in fluorescence caused by the release of an intercalating DNA dye from a DNA duplex as it is denatured by increasing temperature. This technique does not require fluorescently labeled probes or separation steps and has already been used in zygosity testing, epigenetics, and single nucleotide polymorphism (SNP) typing/point mutation detection [28, 33, 67]. The HRM approach has also been applied successfully in the identification of different microorganisms, such as viruses [30], bacteria [5, 12, 14, 25, 44, 60], and protozoa [40]. It has also been used in the rapid identification of grapevine and olive cultivars [32], cooked poisonous mushrooms [34], some tuna fish [6], and humans [13]. Hybridization probes allow genotyping and species identification but are expensive and require multichannel real-time PCR instrumentation.

So far, 68 species of blowfly (Calliphoridae) have been recorded in Poland [8, 9, 57, 58], and of these, 16 may be of relevance to forensic entomology [e.g., 3, 15, 21, 26, 36, 55]. In the work we described here, we tested the efficacy of HRM real-time PCR for the rapid identification of the forensically important species of calliphorid which occur in Poland.

Materials and methods

Specimens and DNA extraction

Currently, 16 blowfly species of forensic importance are known from Poland: *Calliphora loewi* Enderlein, 1903; *Calliphora stelviana* Brauer et Bergenstamm, 1891; *Calliphora subalpina* (Ringdahl, 1931); *Calliphora uralensis* Villeneuve, 1922; *Calliphora vicina* Robineau-Desvoidy, 1830; *Calliphora vomitoria* (Linnaeus, 1758); *Cynomya mortuorum* (Linnaeus, 1761); *Lucilia ampullacea* Villeneuve, 1922; *Lucilia caesar* (Linnaeus, 1758); *L. illustris* (Meigen, 1826); *Lucilia richardsi* Collin, 1926; *Lucilia sericata* (Meigen, 1826); *Lucilia silvarum* (Meigen, 1826); *Phormia regina* (Meigen, 1826); *Protophormia terraenovae* (Robineau-Desvoidy, 1830) [8]; and the recently discovered *Chrysomya albiceps* (Wiedemann, 1819) [58].

Genomic DNA was extracted from the thorax of three specimens of each species using a GenElute Mammalian Genomic DNA Purification Kit (Sigma-Aldrich, Milwaukee, WI), following the manufacturer's instructions. DNA was eluted in 100 μl H_2O and quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The level of degradation was estimated by DNA electrophoresis in 1% agarose gel. Samples of DNA were deposited at the National Plant, Fungi and Animal DNA Bank in Poland (www.bankdna.pl).

All specimens were caught in Poland (see www.bankdna.pl). Unfortunately, the genetic material originating from Polish samples of *C. stelviana* and *L. ampullacea*, despite several attempts, was too degraded to be used in our analyses. In addition, *C. albiceps* was found in Poland only recently [58], and at the time of our work, no specimen belonging to this species was available for our study. To overcome these problems, we synthesized missing oligonucleotides using resources deposited in GenBank, although originating from other countries (Supplementary Table 1). Finally, the only species lacking in our analyses was *C. stelviana* (no DNA sequence of interest deposited in GenBank). The species is quite rare and limited in its distribution to montane–boreal regions of Europe, Asia, and North America [16, 42, 49, 50]. To look at geographic variability, we also synthesized oligonucleotides for *L. illustris* originating from Great Britain, France, India, and USA (Supplementary Table 1). All synthesized oligonucleotides were purchased from Sigma-Aldrich at the price of about Euro 45 for a 119 bp DNA fragment.

DNA sequencing

The cytochrome oxidase gene subunit I (COI) was selected as a target in designing PCR amplicons. Several studies have shown that sequence diversity in a ~650 bp region near the 5′ end of this gene provides strong species-level resolution in several groups of animals [22, 45], including calliphorids [21]. The corresponding sequences from calliphorid species were aligned with Clustal X [29]. Two regions with sufficient sequence diversity flanked by conserved regions were selected (Supplementary Table 1).

Amplicon I spans from 904 to 1,022 bp (119 bp) and amplicon II from 1,438–1,507 bp (70 bp; numbering according to *C. albiceps* from Egypt, sequence AF083657, which served as a reference species). Primers to COI were designed by Primer3 software http://frodo.wi.mit.edu/primer3 [52]. Two pairs of primers were designed. The first pair forward 5′-TCGAGCTTACTTTACTTCAGC TACA-3′ and reverse 5′-GCTCATAAAGTAGCAGGG GAAT-3′ for amplicon I and the second pair (forward 5′-TAGCAACTCTTTATGGAACTCAA-3′ and reverse 5′-GCTCATAAAGTAGCAGGGGAAT-3′) for amplicon II. Specificity of amplified products was confirmed by DNA sequencing. Excess dNTPs and unincorporated primers were removed from the PCR product using the Clean-Up Purification Kit (A & A Biotechnology, Gdynia, Poland). DNA was eluted in 40 µl H_2O, and sequencing reactions consisted of 30 cycles of 96°C for 20 s, 50°C for 20 s, and 60°C for 4 min. Each 20-µl reaction mix was prepared using 3 µl GenomeLab DTCS Start Mix (Beckman Coulter, Fullerton, CA), 2 µl of 1.6 µM primer, 10 µl of PCR product, and H_2O to a total 20 µl reaction volume. For the purification of sequencing products 2 µl 3 M sodium acetate pH 4.8, 2 µl 0.1 M EDTA, 1 µl glycogen (Beckman Coulter, Fullerton, CA), and 60 µl frozen 96% ethanol were added to the reaction mix. Samples were centrifuged for 15 min at 12,000×*g* at 4°C in a centrifuge 5804R (Eppendorf AG, Hamburg Germany). The supernatant was carefully removed, and the DNA pellet washed with 150 µl of frozen 70% ethanol. Samples were centrifuged 5 min at 12,000×*g* at 4°C; the supernatant was removed, and samples dried at room temperature for 5 min. Dried samples were dissolved in 36 µl of sample loading solution. Automated sequencing and sequence data analysis was conducted with CEQ 8000 Beckman Coulter DNA sequencer (Beckman Coulter).

High-resolution melting PCR

Amplification of target sequences was carried out on a Rotor-Gene 6500 thermal cycler (Corbett Research, Mor-

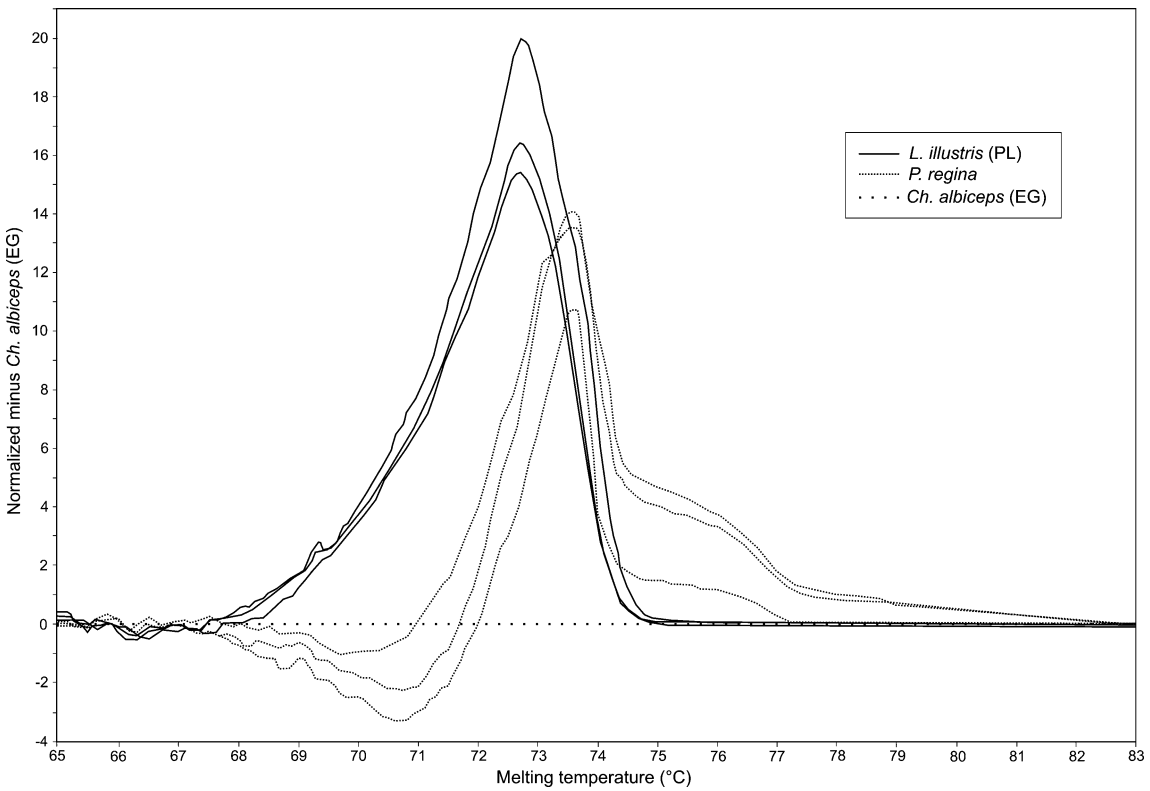

Fig. 1 Independence of melting temperature from the DNA template origin of specimens belonging to the same species and occurring in Poland. DNA templates obtained from three specimens of each analyzed species were used to show reproducibility of different melting curves. *C. albiceps* (EG) used as a reference

tlake, NSW, Australia). This instrument has the thermal uniformity ±0.01°C and the resolution ±0.02°C. PCR primers were purchased from IBB Oligo (Warsaw, Poland). Other PCR reagents were purchased from Quantace (London, UK). Each PCR reaction mix was prepared using 10 μl 2× SensiMix HRM, 4 μl of 10 μM each primer, 4 μl DNA extract, and 2 μl H_2O to total 20 μl volume. PCR cycling and HRM analysis were performed on the Rotor-Gene 6500 (Corbett Research). The thermal program consisted of an initial denaturation step of 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Optical measurements in the green channel (excitation at 470 nm and detection at 510 nm) were recorded during the extension step. After 40 PCR cycles had been completed, melting curve data were generated by increasing the temperature from 60°C to 95°C at 0.1°C per second and recording fluorescence. HRM curve analysis was performed using the software Rotor-Gene 1.7.27, and the HRM algorithm was provided.

Results

The designed primer sets produced amplicons of the expected size when analyzed by agarose gel electrophoresis (data not shown). Specificity of amplified sequences was confirmed by amplicon sequencing. Obtained DNA sequences were identical or very similar to sequences deposited in GenBank, indicating that the selected pair of primers amplified the mitochondrial COI gene region and not nuclear pseudogene(s) sequences.

Reproducibility and intraspecific consistency of melting profiles were tested by running three replicates on DNA template isolated from three different specimens of each species. The crossing points for the real-time PCR ranged from 18.7 to 26.6 cycles in different species when 50 ng of DNA template per reaction was used. These differences may result from the different quality of DNA isolated from specimens used in our research, or from incomplete identity of primer to template sequences, or be due to the presence of inhibitors. Nevertheless, these differences in amplification efficiency do not affect the HRM difference in melting profiles (Fig. 1). The several HRM runs performed on different days resulted in minor variations in the amplification curve and in melting temperatures of the peaks but were consistent with those presented above.

Sensitivity of the analysis was demonstrated for *C. vicina* by running PCR and melting curve analysis on a serial dilution of DNA. The crossing point ranged (mean±SE) from 19.1±0.43 to 32.4±0.58 cycles for the DNA

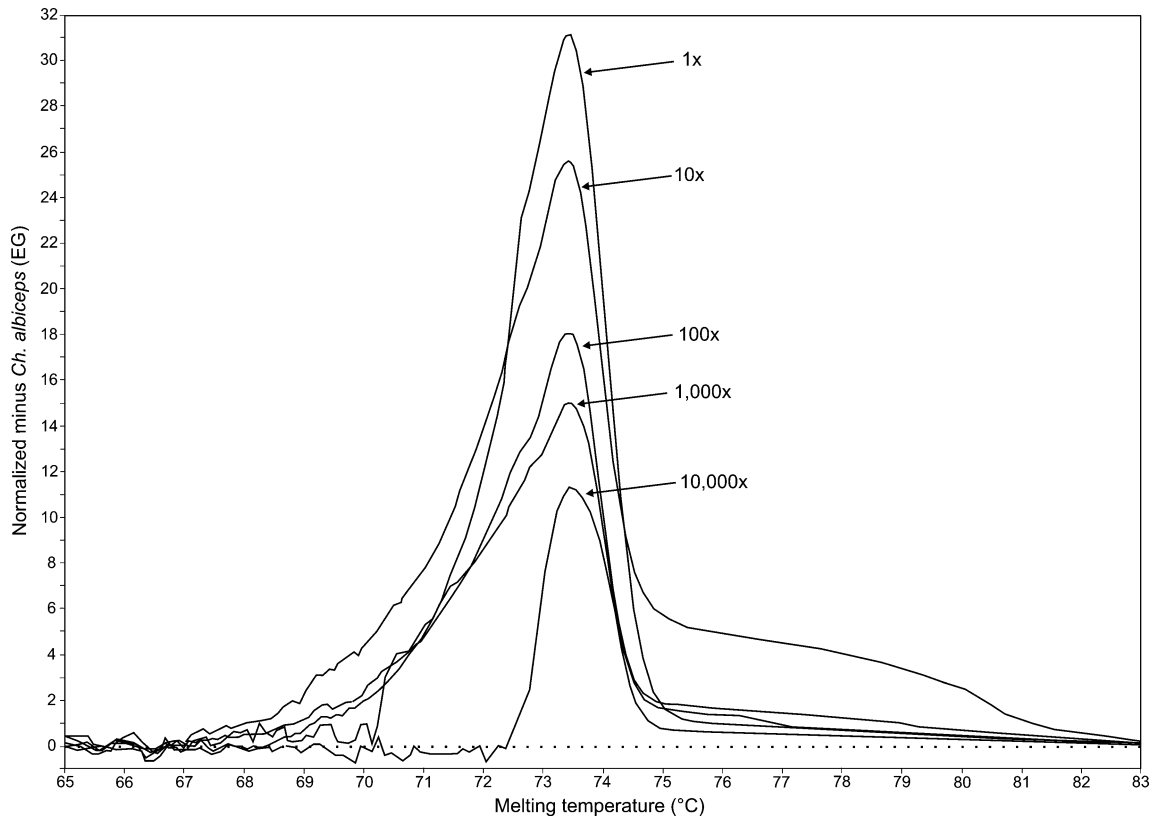

Fig. 2 Independence of melting temperature from the DNA template amounts used in the PCR reaction. Serial dilution *C. vicina* DNA (amplicon I, original concentration 50 ng) was applied for real-time PCR and following HRM analysis. *C. albiceps* (EG) used as a reference

template, when it was present at amounts ranging from 50 ng to 5 pg. The difference melting profile was not altered up to a 10,000-fold difference in template concentration. The relative insensibility of the melting profile to template concentration permits using practically non-measured amounts of DNA for the PCR reaction (Fig. 2).

Difference melting profiles were distinguishable among species from the peak position and the melting temperature (Fig. 3). When *C. albiceps* (EG) was selected as a reference, most of the analyzed species were separated into two multispecies groups (Fig. 4): group A with *L. caesar* and *C. uralensis*, and group B with *C. loewi*, *C. subalpina*, *C. vicina*, *C. vomitoria*, *C. mortuorum*, *L. richardsi*, *L. sericata*, *P. regina*, and *P. terraenovae*. Group B also contained geographical variants of *L. illustris* from Great Britain, USA, and India (not shown in Fig. 3, but see Fig. 4). Other taxa had clearly different melting profiles allowing their quick identification. *L. caesar* and *C. uralensis* from group A have six and nine nucleotide substitutions, respectively, and produced the positive peak at about 73.0–73.1°C. The ten species from group B have from six to 15 nucleotide substitutions and had the positive peak at about 73.5°C. Other taxa had either positive or negative melting peaks (or both, as in *L. silvarum*; see Fig. 3) and contained from seven (*L. illustris* from Poland) to nine (*L. illustris* from France and *L. silvarum*) nucleotide substitutions in the analyzed amplicon (Supplementary Table 1).

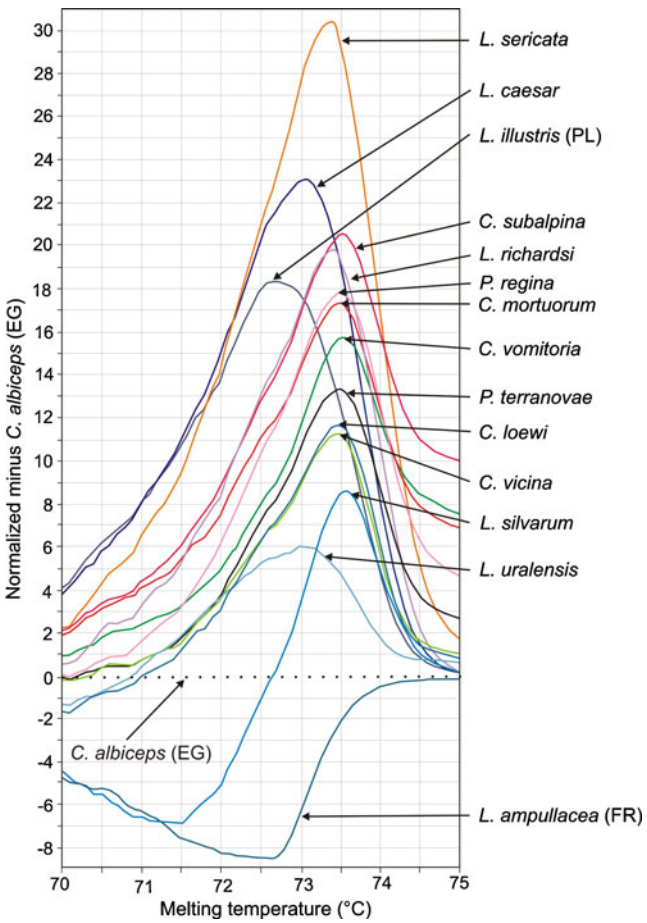

Fig. 3 The HRM difference plot of 15 species of calliphorid (amplicon I). To decrease graph complexity only one representative of each species is shown. *C. albiceps* (EG) used as a reference

We used two strategies in identifying the species of Calliphoridae within each melting group. In the first approach, we used the possibility offered by Rotor-Gene for a change of reference species after PCR and HRM analysis. Changing reference species from *C. albiceps* (EG) to *L. illustris* (US) allowed separating group B into four subgroups, among which three subgroups had clearly visible and well-defined melting peaks: B1 with *L. illustris* from India, *L. richardsi*, and *L. sericata*, B2 with *C. subalpina*, *C. vomitoria*, and *C. mortuorum*, and B3 with *L. illustris* from Great Britain, and *C. albiceps* from Egypt. The fourth subgroup (B4) contained three taxa: *C. loewi*, *C. vicina*, and *P. terranovae*. They had low, hardly defined peaks, and melting curves positioned close (±3 units in the y-axis) to the reference line. Further species discrimination was possible by performing a PCR for amplicon II and by changing the reference species. The work flow for identification of these calliphorids is as presented in Fig. 4.

Discussion

One of the approaches taken in species identification is direct DNA sequencing followed by comparison of an obtained sequence with a reference sequence. Ideally, the structure of the DNA region to be analyzed has to consist of a variable sequence with low intraspecific and high interspecific variability flanked by conserved regions. COI has all these features and has been chosen as a standard barcode sequence for the identification of different animal species [e.g., 22, 45]. COI was also used successfully in the identification of calliphorid flies [1, 4, 19–21, 41, 62, 64]. Nevertheless, the development of techniques independent of DNA sequencing is necessitated by errors occurring during DNA sequencing, as well as by the presence of nuclear mtDNA pseudogenes.

The high-resolution melting is a novel, close-tube, and post-PCR method enabling the analysis of genetic variation in PCR amplicons. Because different genetic sequences melt at slightly different rates, they can be viewed, compared, and detected using melting curves and measuring the subtle differences in melting temperature (T_m). Depending on the type of SNP, the difference in T_m between the two homozygous alleles can vary from 0.1°C

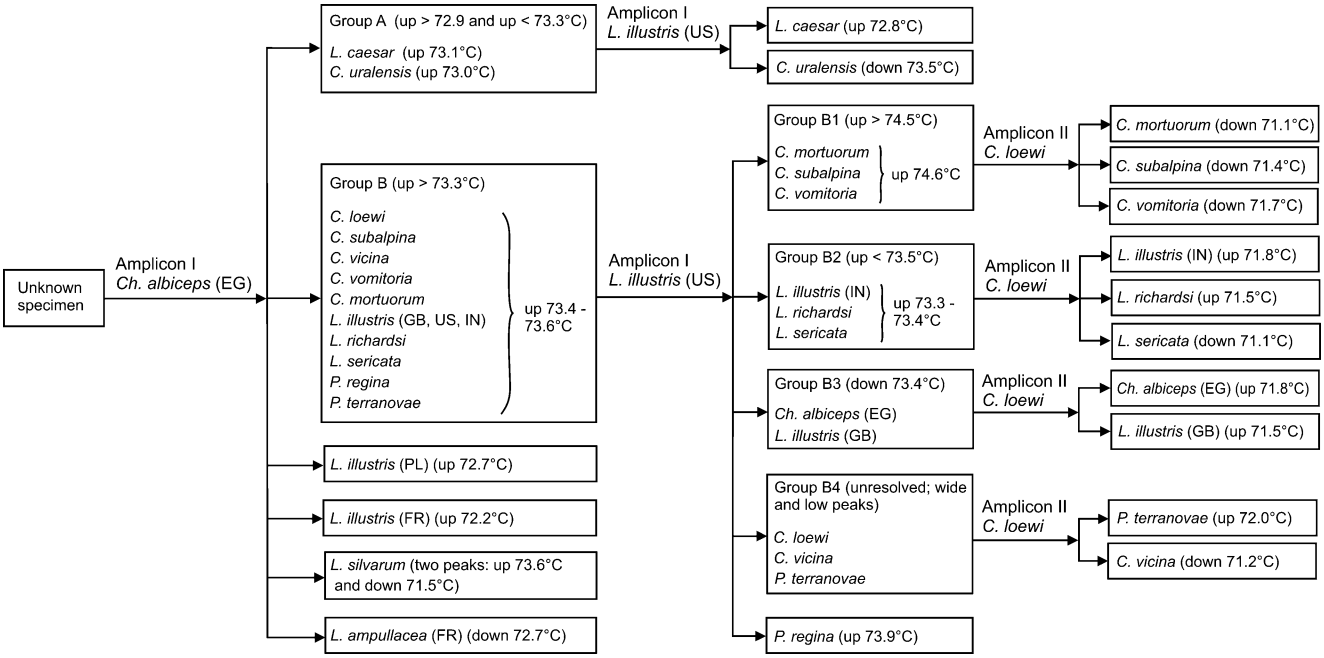

Fig. 4 The work flow for identification of necrophagous calliphorids, including samples of different origin of cosmopolitan *L. illustris*. "Up" and "down" refer either to above or below the reference line

up to 1.5°C. Evaluation of HRM analysis for mutation scanning performed by National Genetics Reference Lab (Wessex), Salisbury, and Regional Genetics Service and CRUK Mutation Detection Facility, Leeds, on 11 amplicons and all possible homo- and heteroduplex types showed 100% sensitivity and 95.3% specificity (www.ngrl.org.uk/wessex/downloads/pdf/HWCMGS06.pdf).

HRM analysis has already been used for the identification of different microorganisms [e.g., 5, 11, 12, 25, 30], fungi [34], fish [6], and humans [13]. According to Cheng et al. [5], melting temperature (T_m)-based differentiation is possible down to the genus or species level. Within a group of 25 bacterial species frequently encountered in positive blood cultures, nine taxa presented unique T_ms, whereas the remaining 16 species could be classified into four distinct melting groups. In the same study, species making up melting groups were discriminated successfully by a second real-time PCR targeting a different region of the 16S rRNA gene. Robinson et al. [48] applied HRM analysis to distinguish between seven species of *Naegleria* (Heterolobosea: Protista) and to differentiate them from the closely related *Willaertia magna*, using a single amplicon (~330–400 bp) representing ITS-1, 5.8S, and ITS-2 of nuclear rDNA. The same approach was used to the identification of grapevine and olive varieties [32] and for classifying strains of *Mycoplasma synoviae* [25]. The HRM analysis of the two hypervariable regions HVI and HVII of mtDNA has also been suggested to function as a rapid and inexpensive pre-screening method for forensic samples of humans prior to DNA sequencing [13].

We performed HRM analysis with a view to identifying the forensically significant calliphorids occurring in Poland. The entire analysis was performed within 2 h—from the start of DNA amplification to the final result (or within 3 h of the beginning of DNA isolation). This represents an order-of-magnitude increase in speed and decrease in time when set against other analytical methods typically used in species identification. Another significant advantage of HRM for molecular taxonomy was found to be the relatively limited degree to which melting temperature depends on template concentration (in our study no shift of melting temperature in probes from 5 pg to 50 ng of DNA template). Some publications also reported a marked dependence of HRM on salt and buffer conditions in the PCR reaction [17, 54]. While our analyses involved individual DNA extractions in which these parameters could not be identical, no differences between HRM profiles from different individual samples were observed. This is in agreement with the HRM reproducibility research performed by Hermann et al. [23].

Forensic entomology takes into account all aspects capable of influencing the development of carrion-feeding insects and the rate of cadaver decomposition (i.e., temperature, relative air humidity, insolation, illumination, precipitation, etc.). Further important factors taken into account when establishing dates of death are the geographical location and habitat type in which a body is found [e.g., 46, 47], its positioning in respect to the sun, the cause of death, and the place and means of the corpse's discovery (summarized in Kaczorowska and Draber-Mońko [26]). For

example, in the case of the very abundant *C. vicina*, the time between egg laying and the emergence of the imago determined for Warsaw (central Poland) in summer with a temperature oscillating around 21°C is 20 days. An autumn temperature of 19°C is shown to reduce the development time to 17 days (A. Draber-Mońko, unpublished data). The method used to kill insect larvae collected for forensic purposes may also be of some importance [38]. However, it is clear that most entomological evidence is strongly dependent on accurate species identification because identification allows the correct developmental data and distribution ranges to be applied in criminal and general legal investigations.

There is still a question how the proposed method may be accurate in different countries. The list of taxa occurring in Poland indicates that, in Europe, only Spain has two other species of forensic importance: *Lucilia cuprina* and *Chrysomya megacephala* (Supplementary Table 2). However, it cannot be forgotten that forensic entomology is locality-specific, and molecular studies are generally directed to the specific fauna discovered in a region. Intraspecific variation (as in the case of *L. illustris*) may influence the final result. Wells et al. [65] have already indicated a paraphyletic pattern for *L. illustris* (using specimens from Canada and Great Britain), and at least, in our opinion, it appears that this taxon represents more than just a single species.

Fortunately, even single base changes such as SNPs can be detected in the HRM analyses, and all geographic variants of the same species can be identified (it cannot be excluded, however, that some of them may represent unrecognized species). This method also needs informative nucleotide substitutions, and it cannot be used for taxa which cannot be reliably identified with the use of DNA barcoding, such as blowflies belonging to the genus *Protocalliphora* [66], known to be infected with the endosymbiotic bacteria *Wolbachia*. These bacteria can potentially influence mtDNA variation at the intra- or interspecific level and lead to the lack of within-species monophyly. In a study of forensically significant calliphorids by Harvey et al. [21], the COI sequences allowed the successful distinction of all but four of 27 studied species. Identification of phylogenetically young species will require a faster-evolving molecular marker (but see Duarte et al. [10]).

In summary, a coupling of real-time PCR and HRM analysis would seem to be suitable for successful use in identifying calliphorid species that are of forensic importance. This approach is very likely to enable the reliable identification of carrion-breeding blowflies in most (if not all) European countries. A new, fast and effective tool by which to establish times of death has thus become available. This technique is also cost-effective (e.g., when set against sequencing and Taqman SNP typing) and simple, and it may even be used by non-geneticists in any laboratory with access to an HRM-capable real-time PCR machine.

Acknowledgements We thank Dr. R. Van Den Bussche from Oklahoma State University for his comments on the previous version of the manuscript.

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