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ORIGINAL ARTICLE

Molecular identification of carrion-breeding scuttle flies (Diptera: Phoridae) using COI barcodes

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Abstract Entomological evidence is often used in forensic cases for post-mortem interval (PMI) calculation. The most dominant species present on a corpse are typically blowflies. However, several cases have been reported where access to a corpse has been restricted for blowflies (e.g., on a buried or wrapped cadavers) but species of the family Phoridae were abundant. It has also been reported that some phorid species that exploit human corpses may also feature in cases of myiasis acquired ante-mortem. In all these cases, they may provide decisive evidence. As for blowflies, the precise identification of a phorid species collected from a corpse is necessary when estimating the PMI. Since morphological determination is often hampered due to similar characteristics especially in the larval and pupal stage, we used DNA-based methods to identify six phorid species (*Megaselia scalaris*, *Megaselia giraudii*, *Megaselia abdita*, *Megaselia rufipes*, *Conicera tibialis*, and *Pulicophora borinquenensis*) on the molecular level. We focused on a 658-bp-long region of the cytochrome oxidase I gene (*COI*), the most common molecular marker in forensic entomology. The amplified fragment is also used in DNA barcode approaches and was found to be suitable for identification of a wide range of insect taxa. The present study demonstrates that this region is also sufficient to distinguish between several species of scuttle flies.

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Introduction

Medicolegal PMI estimation often involves examination of necrophagous insects developing on a corpse (reviewed in [1] and [2]). Blowflies (Diptera: Calliphoridae) are usually the first and most abundant colonizers and can therefore be used as indicators of the minimal time elapsed since death. However, several cases have been reported where small flies like the minute to medium sized scuttle flies (Phoridae) were present and even dominated the fauna of the carcass [3–10]. Although known to colonize a human corpse at a later time of decomposition, phorids may also be present at early stages of decay [7, 11, 12], especially when blowflies were unable to gain access [6, 11]. Unlike blowflies, they are able to move through the smallest openings or meters of soil, colonizing buried or concealed corpses and carcasses [3, 4, 13, 14]. Last but not least, some forensically important phorid species may also occur in cases of myiasis, the infestation of living humans or animals [6, 11]. In all these examples, they may provide decisive evidence in forensic investigations [15].

Over 3,000 of these typically humped-back species have been described so far and represent a diverse group of scavengers, herbivores, predators, and parasites. Buck [16] found over 40 necrophagous species in his study, about 50% belonging to the genus *Megaselia*. Surprisingly, only a minority has been reported to be attracted to decaying human corpses or carcasses of bigger vertebrates so far [6]; the majority infest invertebrate carrion like snails and insects [17–22]. However, as a precise identification based on morphological characteristics is hardly possible espe-

cially in the juvenile stages of these flies [15], our current knowledge about the abundance of scuttle flies on corpses may be biased [4, 6, 7, Amendt, unpublished data]. Species identification is also of major importance in forensic entomology because different species have different developmental rates at certain temperatures [7, 15, 23]. We believe that the difficult identification hampered the use of this important group in the past. Therefore, a tool which improves the identification of scuttle flies could be the starting point for research on forensically important phorid taxa, eventually leading to the routinely consideration of these flies in forensic entomology.

Within the past years, DNA-based techniques to identify forensically important fly species using either nuclear or mitochondrial DNA marker genes have been conducted [reviewed in 2]. Although there are still ambiguities about a universal marker for species identification [24], the mitochondrial COI gene is mostly used in forensic entomology and sufficient to identify many *Diptera* species [24]. Within the past years, COI became also popular for "DNA barcoding" and a 658-bp-long fragment has been suggested to use for standardized species identification [25]. Although still in their early steps, DNA barcoding approaches have been performed on a wide range of taxa so far [26–32] and it has also been applied on forensically important *Chrysomya* species (Diptera: Calliphoridae) [33].

The main purpose of this study is to characterize six forensically relevant scuttle fly taxa on the molecular level for the first time: *Megaselia scalaris*, *Megaselia giraudii*, *Megaselia abdita*, *Megaselia rufipes*, *Conicera tibialis*, and *Puliciphora borinquenensis*. The first five species have already been found on human corpses in the past [4–6, 8], only *P. borinquenensis* is still waiting to be detected in a forensic case.

We chose universal DNA barcoding primers [34] and gathered sequence data, which will be useful as reference standards for future determination of necrophagous phorid species. The need for such a reference database is subsequently described for three selected forensic cases, confirming the importance of Phoridae in forensic entomology.

Material and methods

Fly specimens

Adult flies of the necrophagous species *M. scalaris* (5), *M. giraudii* (5), *M. abdita* (8), *M. rufipes* (5), *C. tibialis* (5), and *P. borinquenensis* (6) were employed in the analysis. The majority of species originated from England. *M. abdita* and *M. scalaris* were from laboratory colonies in Cambridge and *P. borinquenensis* from a culture in Oxford. *M. giraudii* and *M. rufipes* were caught on bait in a Cambridge garden and *C. tibialis* was caught in Rüdesheim, Germany. All specimens were determined morphologically. At least five individuals of each species were used for molecular analysis. Numbers in parentheses indicate the number of individuals investigated.

DNA extraction

Genomic DNA from all specimens was extracted from the whole individual using a slightly modified phenol–chloroform extraction [35]. Subsequent ethanol precipitation a final elution in 50 µl distilled water was performed and extracts were stored at 4°C until PCR.

PCR amplification

Amplification of the COI barcoding region was performed using the primers LCO1490 (5′- GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [34]. Amplification was performed in a total reaction volume of 25 µl containing 1 unit/µl of Taq DNA polymerase, 2 mM of each dNTP, 8 mg/ml of BSA and 5 pmol of each primer. 5 µl of the DNA extracts were used as template.

All PCR amplifications were performed in a T3000 thermal cycler (Biometra). The thermal cycler program was the following: 1 min at 94°C followed by five cycles of 94°C for 1 min, 45°C for 1.5 min, and 72°C for 1.5 min followed by 35 cycles of 94°C for 1 min, 50°C for 1.5 min and 72°C for 1 min with a final extension step of 72°C for 8 min.

PCR products were detected by gel-electrophoresis in a 2.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Sequencing analysis

PCR products were directly sequenced in both directions using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The protocol included a total reaction volume of 20 µl consisting 3 µl Big Dye, 2.5 µl 5× sequencing buffer, 5 pmol primer and 1 µl PCR product. Protocol for sequencing reaction was 28 cycles of 96°C for 10 s, 50°C for 5 s, and 55°C for 4 min.

Sequencing products were purified using gel-filtrated columns (Qiagen, DyeEx 2.0 Spin Kit) and run on an ABI3130 genetic analyzer (Applied Biosystems).

Sequence data for forward and reverse DNA strands were edited and aligned manually using the software Bioedit (Version 7.0.9).

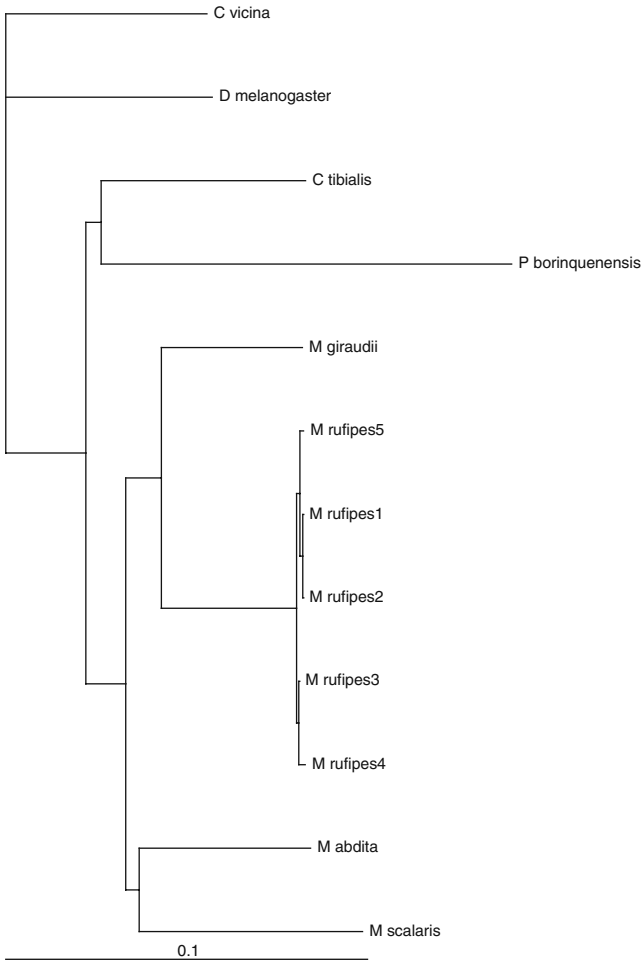

Fig. 1 Neighbor-joining tree. Relationships between the investigated scuttle fly species are presented based on the examined COI fragment. *C. vicina* and *D. melanogaster* were set as outgroups

Results and discussion

Identification

Current data present the first approach to use molecular tools to identify and distinguish between a variety of carrion-breeding scuttle flies (Phoridae). A total of 34 individuals were sequenced and aligned over 559 nucleotides of the COI barcoding fragment. A further two sequence data sets obtained from GenBank (http://www.ncbi.nlm.nih.gov/) served as outgroups for comparative purposes.

All new phorid sequences are deposited in GenBank under the following accession numbers: GU075399, GU075400, GU075401, GU075402, GU075403, GU075404, GU075405, GU075406, and GU075407.

Interspecific variation

Sequence analysis revealed a high interspecific nucleotide variation, which is sufficient for unambiguous identification and differentiation between the six species. Neighbor-joining analysis and distance matrix were performed in PAUP Version 3.1.1 [36] using default settings and are shown in Fig. 1 and Table 1, respectively.

We found 147 variable positions and levels of interspecific nucleotide divergence ranged from 7.9% (*M. giraudii* to *M. rufipes*) to 18.6% (*P. borinquenensis* to *M. rufipes*). Within the genus *Megaselia*, genetic distances are relatively small with the lowest difference between *M. giraudii* and *M. rufipes* (7.9%) and the highest genus specific sequence divergence between *M. scalaris*/*M. giraudii* and *M.*

Table 1 Pairwise sequence differences between investigated scuttle flies and two outgroup species for the analyzed COI region (upper right panel: nucleotide divergence in %, lower left: absolute nucleotide differences)

		1	2	3	4	5	6	7	8	9	10	11	12
1	*Calliphora vicina*	–	11.3	13.6	21.1	12.9	14.7	17.4	13.2	13.2	13.1	13.2	13.1
2	*Drosophila melanogaster*	63	–	14	18.4	13.8	14.1	15.4	14.3	14.3	14.1	14.3	14.1
3	*Conicera tibialis*	76	78	–	17	12.9	13.6	13.6	11.6	11.6	11.8	12	11.4
4	*Puliciphora borinquenensis*	118	103	95	–	17.5	16.3	17.5	18.6	18.6	18.4	18.4	18.4
5	*Megaselia giraudii*	72	77	72	98	–	10.2	11.4	7.9	7.9	7.7	7.9	7.7
6	*Megaselia abdita*	82	79	76	91	57	–	10.9	10	10	9.8	10	9.8
7	*Megaselia scalaris*	97	86	76	98	64	61	–	11.4	11.4	11.3	11.4	11.6
8	*Megaselia rufipes 1*	74	80	65	104	44	56	64	–	0	0.2	0.4	0.2
9	*Megaselia rufipes 2*	74	80	65	104	44	56	64	0	–	0.2	0.4	0.2
10	*Megaselia rufipes 3*	73	79	66	103	43	55	63	1	1	–	0.2	0.4
11	*Megaselia rufipes 4*	74	80	67	103	44	56	64	2	2	1	–	0.5
12	*Megaselia rufipes 5*	73	79	64	103	43	55	65	1	1	2	3	–

scalaris/*M. rufipes* (11.4%; Table 1). All these values allow a clear differentiation of the species.

Comparing scuttle fly COI sequences to those of another representative carcass associated blowfly by phylogenetic analysis using PAUP 3.1.1, *Calliphora vicina* (accession number: AJ417702) and also to the fruit fly *Drosophila melanogaster* (accession number: AJ400907), we were able to detect a clear separation (Fig. 1), confirming that the three phorid genera belong to three different tribes, with *Conicera* being in a different subfamily. *M. scalaris* has been transported around the world by man, but was almost certainly Nearctic/Neotropic in origin. *M. abdita*, *M. giraudii*, and *M. rufipes* are also tramp species. All three are showing a holarctic but predominantly palaearctic distribution, while *M. rufipes* has been the most widely transported by man.

Intraspecific variation

Almost all specimens of one species showed identical nucleotide sequence except slight differences within the sequences of *M. rufipes*. Only two individuals shared the same haplotype (*M. rufipes* 1 and 2, Fig. 1 and Table 1) while the remaining three specimens differed to each other in one to two base pairs. However, intraspecific variation did not exceed 1% which allows species association in general [37, 38]. Current analysis demonstrates that intraspecific variation may affect RFLP approaches which are commonly used as cheap and fast alternatives to sequence-based species identification; point mutations may alter restriction sites and thus lead to misidentifications if not properly interpreted [39, 40].

Case 1

On July 11th, the body of a 53-year-old man was found on a mattress in his apartment. The corpse was in an advanced stage of decay and mummification. There were no signs of a third party fault. Insect fauna was dominated just by maggots and pupae of an unknown phorid species, which was later identified as *M. abdita*. Partial mummification of the body and the absence of blowflies indicated that death occurred in the cold season, when blowflies are not active. Later investigations revealed a possible time of death in late October of the previous year.

Case 2

On October 4th, the heavily decomposed body of a newborn baby was discovered in the storage room of a charitable foundation. The cadaver was wrapped in a towel and in a jacket and placed in a plastic bag. This bag was deposited in a closed cupboard. The corpse was strongly infested by phorid flies of all life stages, which were identified via DNA analysis as *C. tibialis* and *M. scalaris*. No other insect taxa were found. While *M. scalaris* needs about 3 weeks to reach the pupal stage at 15°C (the ambient temperature in the basement) [41], there is a lack of reliable developmental data for *C. tibialis*. However, Bourel et al. [13] noted *C. tibialis* as an indicator for a minimum post-mortem interval of about 2 months. Later investigations revealed a probable time of death of approximately 4 months prior to discovery.

Case 3

On December 6th, a 65-year-old woman was found dead in her apartment. The cause of death was unknown and the partner of the woman had disappeared. As it was certain, due to witness evidence, that he was still in the apartment on November 25th, investigators wanted to know if the deceased was still alive at that time. Entomological samples showed L3 larvae of the blowfly *C. vicina* and several scuttle fly pupae. DNA analysis identified them as *M. scalaris*. While *C. vicina* needs about 5 days at the recorded temperature of 20°C to reach the measured size and stage of development, *M. scalaris* needs between 10–11 days at that temperature to reach the pupal stage [41]. This indicated a minimum post-mortem interval which reconciled with the last activity of the now disappeared partner in the apartment, who was suspected of being involved in the death of the woman.

Conclusion

Phoridae regularly infest human cadavers [5, 6, 10, 12, 15] and can serve as a very powerful evidence in the estimation of the post-mortem interval. They especially colonize buried or concealed corpses even at an early stage of decomposition and their occurrence might be helpful in death investigations when access for blowflies is restricted. Despite that advantage, their use in forensic entomology is still uncommon. One of the main reasons for this is the difficult identification of the specimens found on a corpse. Here, DNA sequence analysis can lead to unambiguous species determination. A proper species identification will also improve our knowledge of the biology and development of the scuttle flies, as one of the basic requirements in developmental studies in the laboratory is the guarantee of having an uncorrupted colony of a known species.

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References

1. Anderson GS, Cervenka VJ (2002) Insects associated with the body: their use and analysis. In: Haglund WD, Sorg MH (eds) Advances in forensic taphonomy—method, theory and archaeological perspectives. CRC, Boca Raton, pp 173–200
2. Amendt J, Krettek R, Zehner R (2004) Forensic entomology. Naturwissenschaften 91:51–65
3. Merritt RW, Snider R, de Jong JL, Benbow ME, Kimbirauskas RK, Kolar RE (2007) Collembola of the grave: a cold case history involving arthropods 28 years after death. J Forensic Sci 52:1359–1361
4. Disney RHL, Manlove JD (2005) First occurrences of the phorid, *Megaselia abdita*, in forensic cases in Britain. Med Vet Entomol 19:489–491
5. Campobasso CP, Disney RHL, Introna F (2004) A case of *Megaselia scalaris* (Loew) (Dipt., Phoridae) breeding in a human corpse. Aggrawal's Internet Journal of Forensic Medicine and Toxicology 5:3–5
6. Smith KGV (1986) A manual of forensic entomology. London and Cornell University Press, London
7. Manlove JD, Disney RHL (2008) The use of *Megaselia abdita* (Diptera: Phoridae) in forensic entomology. Forensic Sci Int 175:83–84
8. Dewaele P, Leclercq M, Disney RHL (2000) Entomologie et médecine légale: les Phorides (Diptères) sur cadavres humains. J Med Leg Droit Medl 43:569–572
9. Disney RHL, Manlove JD (2009) First report of *Triphleba nudipalpis* (Becker) (Diptera: Phoridae) in a forensic case. Forensic Sci Int 191:e1–e3
10. Thevan K, Disney RHL, Ahmad AH (2009) First records of two species of Oriental scuttle flies (Diptera: Phoridae) from forensic cases. Forensic Sci Int. doi:10.1016/j.forsciint.2009.10.020
11. Disney RHL (2008) Natural history of the scuttle fly, *Megaselia scalaris*. Annu Rev Entomol 53:39–60
12. Reibe S, Madea B (2010) Use of *Megaselia scalaris* (Diptera: Phoridae) for post-mortem interval estimation indoors. Parasitol Res. doi:10.1007/s00436-009-1713-5
13. Bourel B, Tournel G, Hédouin V, Gosset D (2004) Entomofauna of buried bodies in northern France. Int J Legal Med 118:215–220
14. Gaudry E (2010) The insects colonisation of buried remains. In: Amendt J, Campobasso CP, Goff ML, Grassberger M (eds) Current concepts in forensic entomology. Springer, Amsterdam, pp 273–311
15. Disney RHL (2005) Duration of development of two species of carrion-breeding scuttle flies and forensic implications. Med Vet Entomol 19:229–235
16. Buck M (1997) Untersuchungen zur ökologischen Einnischung saprophager Dipteren unter besonderer Berücksichtigung der Phoridae und Sphaeroceridae. Cuvillier, Göttingen
17. Lundt H (1964) Ökologische Untersuchungen über die tierische Besiedlung von Aas im Boden. Pedobiologia 4:158–180
18. Payne JA, King EW, Beinhart G (1968) Arthropod succession and decomposition of buried pigs. Nature 219:1180–1181
19. Kneidel KA (1984) Influence of carcass taxon and size on species composition of carrion-breeding Diptera. Am Midl Nat 111:57–63
20. Disney RHL (1994) Scuttle flies: the Phoridae. Chapman & Hall, London
21. Moretti TC, Thyssen PJ, Solis DR (2009) Breeding of the scuttle fly *Megaselia scalaris* (Diptera: Phoridae) in a fish carcass and implications for the use in forensic entomology. Entomol Gener 31:349–353
22. Moretti TC, Ribeiro OB, Thyssen PJ, Solis DR (2008) Insects on decomposing carcasses of small rodents in a secondary forest in Southeastern Brazil. Eur J Entomol 105:691–696
23. Greenberg B, Wells JD (1998) Forensic use of *Megaselia abdita* and *M. scalaris* (Phoridae: Diptera): Case studies, development rates, and egg structure. J Med Entomol 35:205–209
24. Wells JD, Stevens JR (2008) Application of DNA-based methods in forensic entomology. Annu Rev Entomol 53:103–120
25. Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci 270:313–322
26. Remigio E, Hebert PDN (2003) Testing the utility of partial COI sequences for phylogenetic estimates of gastropod relationships. Mol Phylogenet Evol 29:641–647
27. Hogg ID, Hebert PDN (2004) Biological identifications of springtails (Hexapoda: Collembola) from the Canadian Arctic, using mitochondrial DNA barcodes. Can J Zool 82: 749–754
28. Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc Natl Acad Sci U S A 101:14812–14817
29. Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. PLoS Comput Biol 2:1657–1663
30. Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia's fish species. Philos Trans R Soc Lond B Biol Sci 360:1847–1857
31. Ball SL, Hebert PDN, Burian SK, Webb JM (2005) Biological identification of mayflies (Ephemeroptera) using DNA barcodes. J North Am Benthol Soc 24:245–255
32. Ekrem T, Willassen E, Stur E (2007) A comprehensive DNA sequence library is essential for with DNA barcodes. Mol Phylogenet Evol 43:530–542
33. Nelson LA, Wallmann JF, Dowton M (2007) Using COI barcodes to identify forensically and medically important blowflies. Med Vet Entomol 21:44–52
34. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299
35. Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York
36. Swofford DL (1991) PAUP: Phylogenetic Analysis Using Parsimony version 3.1 Computer program. Illinois Natural History Survey, Champaign
37. Wallman JF, Donnellan SC (2001) The utility of mitochondrial DNA sequences for the identification of forensically important blowflies (Diptera: Calliphoridae) in Southeastern Australia. Forensic Sci Int 120:60–67
38. Zehner R, Amendt J, Schütt S, Sauer J, Krettek R, Povolný D (2004) Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae). Int J Legal Med 118:245–247
39. Zehner R, Zimmermann S, Mebs D (1998) RFLP and sequence analysis of the cytochrome b gene of selected animals and man: methodology and forensic application. Int J Legal Med 111:323–327
40. Wells JD, Wall R, Stevens JR (2007) Phylogenetic analysis of forensically important *Lucilia* flies based on cytochrome oxidase 1 sequence: a cautionary tale for forensic species determination. Int J Legal Med 121:229–233
41. Prawirodisastro M, Benjamin DM (1979) Laboratory study of the biology and ecology of *Megaselia scalaris* (Diptera, Phoridae). J Med Entomol 16:317–320