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SHORT COMMUNICATION

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Genetic identification of forensically important flesh flies (Diptera: Sarcophagidae)

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Abstract Unequivocal identification of fly specimens is an essential requirement in forensic entomology. However, not all species can be determined at every developmental stage, which is illustrated by the flesh flies (Diptera: Sarcophagidae), important members of the necrophagous insect fauna. Up to now no suitable key for the identification of the immature stages of this family of flies exists. DNA analysis of selected mitochondrial genes was applied to solve this problem. Sequence data of selected regions of the CO I and ND 5 genes of the most important European flesh fly taxa associated with cadavers are presented, which can act as reference standards for species determination.

Keywords Sarcophagidae · Forensic entomology · DNA analysis · Species determination

Introduction

Necrophagous insects are attracted by decaying corpses and colonize them immediately after death. The identification of these insect species and their developmental stages provides useful hints for the estimation of the postmortem interval, an important task in medico-legal investigations (Anderson and Cervenka 2002; Amendt et al. 2004). Correct species determination is crucial because the time of development of various fly species may differ, even between species of the same genus (Higley and Haskell 2001). This requires entomological expertise, especially in scenarios which deal with the immature stages of the insects. For most larval stages of flesh flies (Diptera: Sarcophagidae) identification based on morphological characteristics is still impossible (Byrd and Castner 2001). DNA analysis can be used to overcome these problems. This technique of analyzing forensically relevant insects focusses mainly on the mitochondrial encoded subunit I of the cytochrome oxidase gene (CO I, e.g. Sperling et al. 1994; Loxdale and Lushai 1998; Malgorn and Coquoz 1999; Caterino et al. 2000, Wells and Sperling 2001; Stevens and Wall 2001), a different target to the cytochrome b gene which is well established for identifying vertebrates (Kocher et al. 1989; Zehner et al. 1998; Parson et al. 2000). The present paper describes DNA sequence data of a segment of subunit I of the cytochrome oxidase (COI) gene and of a segment of subunit 5 of the NAD dehydrogenase (ND5) gene for selected necrophagous Sarcophagidae. The sequences obtained may represent reference standards for the determination of the most important European flesh flies associated with cadavers.

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Materials and methods

Specimens

Dried adult specimens of *Sarcophaga carnaria, S. subvicina, S. variegata, Parasarcophaga albiceps, Bercaea africa, Liopygia argyrostoma, L. crassipalpis, Liosarcophaga teretirostris, L. tibialis, Pandelleana protuberans, Thyrsocnema incisilobata* and *Helicophagella melanura* (all of the subfamily Sarcophaginae) were used which had been collected in the south of the Czech Republic in 2002, dried and stored at room temperature.

DNA extraction

From the adult flies parts of the thorax muscles were removed and subjected to DNA extraction using standard proteinase K digestion, phenol/chloroform extraction and ethanol precipitation (Sambrook and Russel 2001). DNA extraction from two individuals of each species was used to obtain duplicates of species-specific DNA. The DNA was dissolved in 50 µl water.

PCR

For amplification Red Taq polymerase (Sigma-Aldrich, Taufkirchen, Germany) was used according to the manufacturers recommendations in a reaction volume of 25 µl. Each primer was used at a concentration of 10 pmol per reaction and 1 µl of DNA was used per reaction.

The following primers were used:

– CO I (a): 5'-CAGCTACTTTATGATCTTTAGG-3'
– CO I (b): 5'-CATTTCAAGCTGTGTAAGCATC-3'

according to Sperling et al. (1994) and:

– ND 5 (a): 5'-CCAAAATATTCWGATCAHCCYTG-3'
– ND 5 (b): 5'-GGATTAACTGTTTGTTATWCTTTTCG-3'.

ND 5 primers were newly designed using the primer design programme Oligos v.8.31 (Kalendar 1999).

A Perkin Elmer 2400 thermocycler was used according to Sperling et al. (1994) in the case of CO I, for ND 5 the following conditions for touch-down PCR were used: 94°C for 30 s then 10 cycles of 94°C for 30 s, 58–53°C for 30 s (temperature increment reduction of 0.5°C per cycle), 72°C for 30 s, followed by 20 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 30 s with a final extension of 72°C for 15 min. The PCR products were separated on a 2.5% agarose gel and visualized after ethidium bromide staining.

Nucleotide sequencing

Taq cycle sequencing of sense as well as antisense strands was performed using the Big-Dye Terminator Cycle Sequencing Kit v 3.1 (Applied Biosystems, Foster City, CA) according to the manufacturers recommendations. The sequencing primers were the same as described for the PCR reaction. Electrophoresis and detection of the sequencing reaction products was carried out in the capillary electrophoresis system ABI Prism 3100 Genetic Analyzer using POP (Performance Optimised Polymer) 4, with a capillary length of 31 cm and diameter of 50 µm.

Data analysis

Analysis of sequence data was performed using Sequence Analysis software version 2.1.2 and Sequence Navigator software version 1.0.1 (both Applied Biosystems, Foster City, CA). For CO I the sequence of positions 2500–2795 (296 bp) and for ND 5 positions 6964–6579 (386 bp) were analyzed (nucleotide positions according to *Drosophila yakuba*, gene bank X032401, sense strand orientation). Phylogenetic analysis was also performed using PAUP 3.1.1 (Swofford 1991).

Results and discussion

For all flesh flies representative nucleotide sequences have been obtained. The estimated sequences are deposited in gene bank (http://www.ncbi.nlm.nih.gov) under the accession numbers AY315638-AY315649 (COI) and AY320054-AY320065 (ND5).

The two specimens of most species showed identical sequences. However, the following species exhibited alternative nucleotides in the two samples: *Bercaea africa* [A/G at position 2673 (COI) and T/C at position 6951 (ND5)], *Liosarcophaga tibialis* [C/(T/C, heteroplasmy) at position 2640 (COI)], *Sarcophaga carnaria* [T/C at position 2658 (COI) and A/G at position 6593 (ND5)], *Thyrsocnema incisilobata* [A/G at position 2662 (COI)]. This demonstrates that intraspecific variation occurs, but does not exceed 1%, which is in accordance with data from other necrophagous flies (Wallman and Donellan 2001; Wells and Sperling 2001). Particularly when using PCR RFLP for attempts at species determination (Sperling et al. 1994; Schröder 2003) intraspecific variation has to be taken into account. Single mutation events could alter a restriction site, thus generating the possibility of false exclusions (Zehner et al. 1998).

However, compared to the intraspecific variation, the interspecific differences are large enough to allow an unambiguous association of a maggot to a certain species by applying sequence data. Within the genus Sarcophaga the genetic distances are relatively low, i.e. 2.7–4.7% in the case of CO I and 2.3–4.1% in the case of ND5 (Table 1). The distances among the CO I and ND 5 sequences within the other Sarcophaginae investigated range from 6.1 to 10.5 and 6.5 to 13.2%, respectively. The mean distance between the Sarcophaginae, excluding the genus Sarcophaga is 8.3± 1.0% in the CO I region and 9.7±1.5% in the ND5 region. The higher divergence of the ND5 sequences illustrates the higher mutation rate of this gene and therefore the faster evolution of this gene compared to CO I (Simon et al. 1994).

Table 1 Pairwise sequence differences (%) for the analyzed regions of CO I (lower left) and ND 5 (upper right)

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Sarcophaga carnaria*	–	4.1	2.3	11.9	9.8	10.9	11.1	10.9	10.1	11.9	7.8	8.0	26.2
2	*Sarcophaga subvicina*	4.7	–	3.9	10.6	9.8	9.3	10.6	10.1	9.1	10.4	8.3	7.0	27.5
3	*Sarcophaga variegata*	2.7	4.1	–	10.9	9.3	10.4	10.6	11.1	9.3	11.1	7.5	7.0	26.9
4	*Parasarcophaga albiceps*	8.4	8.1	8.1	–	7.8	9.3	10.4	10.9	10.4	12.2	11.4	8.8	23.3
5	*Bercaea africa*	9.5	9.1	8.4	9.8	–	7.5	8.5	10.9	8.8	8.8	9.3	7.5	24.6
6	*Liopygia argyrostoma*	6.8	6.1	7.1	7.8	7.4	–	7.0	11.1	9.8	9.8	8.3	7.0	26.7
7	*Liopygia crassipalpis*	8.4	8.1	7.8	8.1	6.1	6.1	–	12.4	10.6	11.1	9.1	8.5	26.2
8	*Liosarcophaga teretirostris*	8.4	7.8	8.8	7.8	8.8	8.1	7.8	–	9.1	13.2	11.4	9.8	27.5
9	*Liosarcophaga tibialis*	9.1	8.1	8.8	6.4	9.1	7.1	8.8	7.1	–	11.7	11.1	8.0	26.9
10	*Pandelleana protuberans*	9.8	8.8	8.8	7.8	10.1	9.5	9.5	8.8	9.1	–	10.1	8.8	25.9
11	*Thyrsocnema incisilobata*	8.4	7.1	7.8	8.8	9.5	7.4	8.8	9.5	8.4	10.5	–	6.5	24.6
12	*Helicophagella melanura*	9.5	8.4	8.4	8.1	8.8	6.8	7.4	8.4	8.1	8.4	7.1	–	25.6
13	*Drosophila yakuba*	13.5	14.2	13.5	14.5	12.2	11.8	12.5	13.9	15.2	15.2	14.5	14.2	–

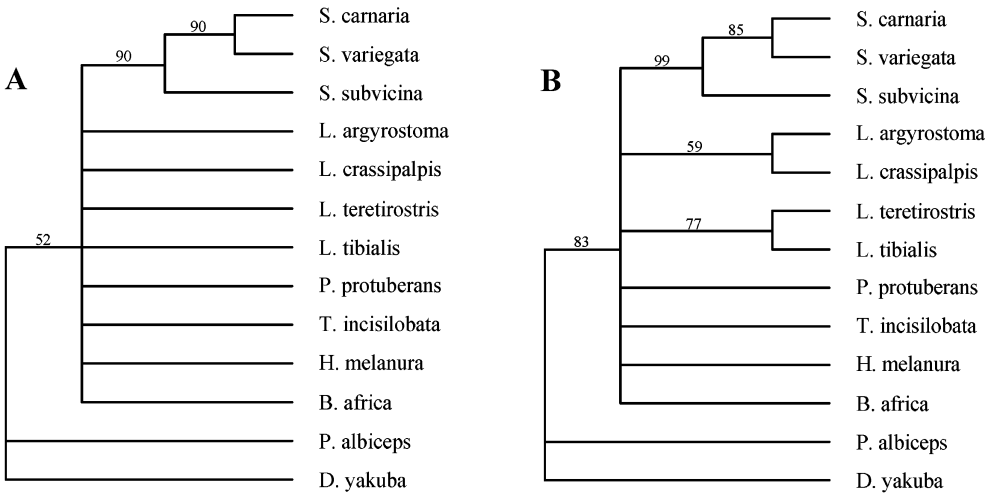

Fig. 1 Bootstrap 50% majority rule consensus tree (1,000 replicates) based on the analysed CO I **(A)** and ND 5 **(B)** regions. *D. yakuba* was defined as an outgroup

Wells et al. (2001) published sequence data of the CO I gen at nt positions 1477–2250 for the species *Bercaea africa* (geographic origin Berkeley, California), *Liopygia argyrostoma* (geographic origin Alexandria, Egypt) and *Liopygia crassipalpis* (geographic origin Berkeley, California). The sequence differences for these three flies are completely or almost identical compared to the sequence differences presented here, indicating an even distribution of nucleotide variations in this gene.

Phylogenetic analysis demonstrates the relatively close association of the genus *Sarcophaga* within the Sarcophaginae. Considering differences between the CO I and the ND5 cladogram, it is remarkable that *L. argyrostoma* and *L. crassipalpis* on the one hand and *L. teretirostris* and *L. tibialis* on the other hand are grouped together. This observation illustrates the faster emerging visible differences in the faster evolving gene ND5 (Fig. 1). The data of the phylogenetic analysis are in accordance with the traditional morphological classification, indicating its usefulness.

The study demonstrates that DNA analytical techniques are useful for identification of some important necrophagous flesh flies of Europe.

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