RESEARCH ARTICLE

Mitochondrial DNA sequence-based phylogenetic relationship among flesh flies of the genus *Sarcophaga* (Sarcophagidae: Diptera)

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Abstract

The phylogenetic relationships among flesh flies of the family Sarcophagidae has been based mainly on the morphology of male genitalia. However, the male genitalic character-based relationships are far from satisfactory. Therefore, in the present study mitochondrial DNA has been used as marker to unravel genetic relatedness and to construct phylogeny among five sympatric species of the genus *Sarcophaga*. Two mitochondrial genes viz., cytochrome oxidase subunit 1 (COI) and NAD dehydrogenase subunit 5 (ND5) were sequenced and genetic distance values were calculated on the basis of sequence differences in both the mitochondrial genes. The data revealed very few genetic difference among the five species for the COI and ND5 gene sequences.

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Introduction

Mitochondrial DNA (mtDNA) has been one of the most widely used molecular markers for phylogenetic studies in animals, because of its simple genomic structure (Avise 2004). Among insects, the maximum number of mitochondrial genomes have been characterized in the order Diptera (Cameron *et al*. 2007; Shao and Barker 2007). Though mtDNA sequence data have proved valuable in determining phylogenetic relationships, the choice of gene is also of great significance (Simon *et al*. 1994; Lunt *et al*. 1996). The size and structure of cytochrome oxidase subunit 1 (COI) gene has been well conserved in the animal groups analysed so far, a feature which makes it especially suitable for evolutionary studies (Lunt *et al*. 1996). In recent years, comparison of mitochondrial DNA sequences has been used for population genetics and phylogenetic studies in several dipterans of medical, veterinary and economic importance (Hall *et al*. 2001; Zehner *et al*. 2004; Cummings and Krafsur 2005; Segura *et al*. 2006; Angella *et al*. 2007).

The flesh flies belonging to the genus *Sarcophaga* are common filth flies with a cosmopolitan distribution. Rohdendorf (1937) established many genera in this family on the basis of external and male genitalic character. Roback (1954) for the first time emphasized the need for understanding the phylogeny for a proper classification of sarcophagids and analysed the phylogeny of Nearctic sarcophagids based on comparative morphology of the male genitalia. Sugiyama and Kano (1984) have also analysed the phylogeny of Oriental sarcophagids on the basis of male genitalic character. However, the delineation of different genera and the relationships among them are still not clear. Therefore, it is imperative that data collected from molecular markers be employed for defining the phylogenetic relationships among different taxa in the family.

In the present study, a comparison of the sequence of two mitochondrial genes—cytochrome oxidase subunit I (COI) and NAD dehydrogenase subunit 5 (ND5)—in five sympatric species of the genus *Sarcophaga* viz., *S. ruficornis, S. argyrostoma, S. dux, S. albiceps* and *S. knabi* has been carried out to unravel their phylogenetic relationships.

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Keywords. mitochondrial DNA; phylogeny; flesh flies; cytochrome oxidase subunit I (COI); NAD dehydrogenase subunit 5 (ND5); genetic distance.

Materials and methods

Genomic DNA was extracted from five species of *Sarcophaga* viz., *S. ruficornis* (Fab.), *S. argyrostoma* (R.-D.), *S. dux* (Walker), *S. albiceps* Meigen and *S. knabi* (Parker). The individuals were homogenized in 0.2 M sucrose, 0.1 M Tris, 50 mM EDTA, 0.5% SDS (pH 8.0). The extracts were digested with 2 mg/mL RNase and incubated at 37°C for 30 min. The DNA was then phenol/chloroform extracted, concentrated by ethanol precipitation and resuspended in 100 μL TE buffer (pH 8.0). Partial sequences of COI and ND5 mitochondrial genes were amplified from the genomic DNA by polymerase chain reaction (PCR).

COI gene was amplified using primers 5′ CAGCTACTTTATGATCTTTAGG 3′ and 5′ CATTTCAAGCTGTGTAAGCATC 3′ in a 25 μL reaction volume having 2.5 μL 10× buffer, 2 μL dNTP (2.5 mM each), 10 picomole of each primer, 1.5 U *Taq* polymerase, 30 ng of genomic DNA and rest milli Q water with an amplification profile of initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 1 min (denaturation), 42°C for 1 min (annealing), 72°C for 2 min (extension) and final extension of 7 min at 72°C.

The ND5 gene was amplified using primers 5′ CCAAAATATTCTGATCATCCTTG 3′ and 5′ GGATTAACTGTTTGTTATACTTTTCG 3′ in 25 μL reaction mixture, with a chemical composition which is identical to the one used for COI gene, with an amplification profile of initial denaturation at 94°C for 1 min, 30 cycles of 94°C for 30 s (denaturation), 54°C for 30 s (annealing), 72°C for 30 s (extension) and final extension of 15 min at 72°C. The amplified products were sequenced from Bangalore Genei (Bangalore, India) and submitted to GenBank (table 1). In addition to mitochondrial COI and ND5 gene sequences of five *Sarcophaga* species, COI and ND5 sequences of the same region of *Drosophila yakuba* were used in the phylogenetic analysis as an outgroup.

Table 1. List of Sarcophaga species and accession numbers of gene sequences analysed in the present study.

Species	Gene	Accession number
Sarcophaga albiceps	COI	FJ160279
Sarcophaga argyrostoma	COI	FJ160280
Sarcophaga dux	COI	FJ160281
Sarcophaga ruficornis	COI	FJ160282
Sarcophaga knabi	COI	FJ545262
Sarcophaga albiceps	ND5	FJ160283
Sarcophaga argyrostoma	ND5	FJ160284
Sarcophaga dux	ND5	FJ160285
Sarcophaga ruficornis	ND5	FJ160286
Sarcophaga knabi	ND5	FJ545263

The sequence data were aligned using Clustal X software (Thompson *et al*. 1997). Analysis of nucleotide composition, overall transition : transversion ratio (ts:tv), variable and parsimony informative positions and pairwise nucleotide distances were calculated using MEGA 4 (Tamura *et al*. 2007). Minimum-evolution (ME) analyses were performed using maximum-likelihood pairwise distance by TreePuzzle v 5.2 (Schmidt *et al*. 2002) programe implemented in Phylemon, available at http://phylemon.bioinfo.cipf.es (Tarraga *et al*. 2007). The COI and ND5 genes were analysed separately as well as in combination to assess phylogenetic relationship. Bootstrap support was calculated from 1000 replications. Modeltest programme (Posada and Crandall 1998), run under the HyPhy (Pond *et al*. 2005) environment, in Phylemon determined that the K81uf+G (Kimura 1981) provided the best fit to the data according to Akaike information criterion (AIC).

Results and discussion

Cytochrome oxidase I (COI) and NAD dehydrogenase 5 (ND5) genes, amplified in the present study, were 296-bp and 386-bp long, respectively, in all the five species. The COI and ND5 genes of sarcophagids analysed in the present study correspond to the positions 2500–2795 for COI and 6579–6964 for ND5 genes, respectively, of *D. yakuba* (Clary *et al*. 1982). The COI gene revealed 71 variable sites in 296-bp long sequence and only 26 sites were found to be parsimony informative. The average nucleotide composition across all the species was T = 40, A = 31, C = 15, G = 14. The ts:tv ratio was 0.63. The ND5 amplicon which is 386-bp long shows 55 variables and 26 parsimony–informative sites. The amplicon is characterized with an average nucleotide composition of T = 47, A = 31, G = 14, C = 8 and ts:tv ratio was 1.44 across the five species. The phylogenetic relationships inferred for both the genes separately and together using *D. yakuba* as an outgroup are represented in figures 1–3. Bootstrap values higher than 50% are indicated in figures. Phylogenetic trees based on COI and ND5 gene sequences separately reveal different picture with some of the nodes showing weak bootstrap support. However, combination of COI and ND5 gene sequences provides an improved resolution of relationship among the five species supported by bootstrap values greater than 70%.

The five species of *Sarcophaga* analysed in the present study show A–T bias in both the genes. Thus, the ratio was 40:31:15:14 for COI gene and 47:31:8:14 for ND5 gene, respectively. These findings are in conformity with the findings in other dipterans (Wolstenholme and Clary 1985; Segura *et al*. 2006). It seems that there might be some kind of selection favouring A+T nucleotides. All the sarcophagids are interestingly endowed with regions of A+T rich DNA as evidenced by bright fluorescence with AT base specific fluorochromes (Samols and Swift 1979; Kaul *et al*. 1989; Parise-Maltempi and Avancini 2000).

A smaller ts:tv ratio has been ascribed to the increase of distances among dipterans (Segura *et al*. 2006). Thus the av-

erage ts:tv ratio of 0.63 for COI and 1.44 for ND5 in *Sarcophaga* species indicates that ND5 gene sequence shows larger distance as compared to the COI gene sequence. The pairwise genetic distance among the five species of *Sarcophaga* ranges from 0.037–0.106 and 0.049–0.207 for COI and ND5 genes, respectively (tables 2 and 3). Analysis of genetic distance on the basis of sequence difference for both the mitochondrial genes shows very little genetic difference. The discrepancy in the phylogenetic trees based on individual genes may be due to the fact that these genes are evolving at different rates. The combinations of such genes are supposed to react positively and maximize the informative and explanatory power of sequences to resolve phylogenetic relationships (Mousson *et al*. 2005). The present findings reaffirm a very close genetic similarity among sarcophagids as evidenced by earlier studies at chromosomal and molecular level (Kaul *et al*. 1981; Thakur 1992; Zehner *et al*. 2004). Thus, our results show that analysis based on mitochondrial genes can be useful for unravelling phylogenetic relationships in the family Sarcophagidae.

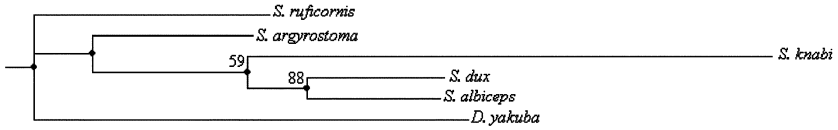

Figure 1. Minimum-evolution (ME) tree for COI gene sequence based on maximum-likelihood pairwise distance. Numbers indicate bootstrap values for nodes retained by more than 50% of bootstrap replicates (1000 replications).

Figure 2. Minimum-evolution (ME) tree for ND5 gene sequence based on maximum-likelihood pairwise distance. Numbers indicate bootstrap values for nodes retained by more than 50% of bootstrap replicates (1000 replications).

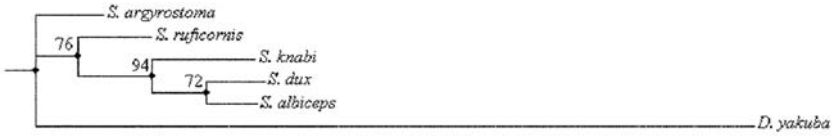

Figure 3. Minimum-evolution (ME) tree for the combined sequences of COI and ND5 genes, using K81uf+G evolutionary model. Numbers indicate bootstrap values for nodes retained by more than 50% of bootstrap replicates (1000 replications).

Table 2. Pairwise genetic distance for COI gene sequence.

	S. knabi	*S. albiceps*	*S. dux*	*S. argyrostoma*	*S. ruficornis*
S. knabi	–				
S. albiceps	0.037	–			
S. dux	0.060	0.040	–		
S. argyrostoma	0.090	0.100	0.106	–	
S. ruficornis	0.096	0.098	0.094	0.064	–

Table 3. Pairwise genetic distance for ND5 gene sequence.

	S. knabi	*S. albiceps*	*S. dux*	*S. argyrostoma*	*S. ruficornis*
S. knabi	–				
S. albiceps	0.170	–			
S. dux	0.162	0.049	–		
S. argyrostoma	0.207	0.103	0.102	–	
S. ruficornis	0.172	0.115	0.123	0.101	–

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