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DNA-based identification of forensically important species of Sarcophagidae (Insecta: Diptera) from Rio de Janeiro, Brazil

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ABSTRACT. Sarcophagidae, or flesh flies, are of great importance in forensic entomology, but their effective application requires precise taxonomic identification, which relies almost exclusively on characteristics of the male genitalia. Given that female flies and larvae are most abundant in animal carcasses or on corpses, precise morphological identification can be difficult; therefore, DNA sequencing can be an additional tool for use in taxonomic identification. This paper analyzes part of the mitochondrial cytochrome c oxidase subunit I (COI) gene

from three Sarcophagidae species of forensic importance in the City of Rio de Janeiro: *Oxysarcodexia fluminensis*, *Peckia chrysostoma*, and *Peckia intermutans*. COI fragments of 400 bp from 36 specimens of these three species were sequenced. No intraspecific differences were found among specimens of *O. fluminensis*, but *P. chrysostoma* and *P. intermutans* each had two haplotypes, ranging from 0 to 0.7%. The interspecific divergence was 8.5-11.6%, corroborating previously reported findings.

Key words: COI gene; Forensic entomology; Sarcophagidae

INTRODUCTION

Flies represent the most important insect order used for forensic purposes, especially because members of the Calliphoridae, Muscidae, and Sarcophagidae families are the first and most frequent colonizers of corpses (Smith, 1986). Morphological examination is used to identify characteristics of the male genitalia, and there are few taxonomic features that can be easily used by non-specialists for species identification (Carvalho and Mello-Patiu, 2008; Giroux et al., 2010). Furthermore, few tools are available for the identification of forensically important fly species (Giroux et al., 2010) and these are constantly outdated in regions where the diverse insect fauna is understudied. These difficulties limit the use of entomological data by the forensic investigator.

Sarcophagid flies (flesh flies) are potentially useful dipterans for forensic purposes because most of these species have necrophagous larvae. However, their performance has been underestimated because most individuals that are attracted to carcasses are females, which use them to lay larvae (Byrd and Castner, 2011). The identification of females and immature flies is difficult because of their conservative morphology and there are very few or no morphological differences between species.

As compared with Calliphoridae, only a few studies on community succession on carcasses in Brazil have included data on Sarcophagidae (Moura et al., 1997; Carvalho et al., 2000; Carvalho et al., 2004; Barbosa et al., 2009) in Rio de Janeiro (Barbosa et al., 2009). Some species have been suggested as possible indicators for forensic purposes, including: *Oxysarcodexia fluminensis* (Lopes, 1946), *Peckia* (*Pattonella*) *intermutans* (Walker, 1861), and *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830).

DNA-based techniques have been used as successful alternatives to morphological examination for taxonomic identification of species of forensic interest (Wells et al., 2001; Schroeder et al., 2003; Wells and Stevens, 2008; Saigusa et al., 2009; Meiklejohn et al., 2011; Tan et al., 2010; Guo et al., 2012). Molecular identification is advantageous because it can be applied to any stage of development and to adult flies of any gender. The large number of publicly available sequences of the mitochondrial gene cytochrome c oxidase subunit I (COI) gene has made this marker the most widely used in forensic studies.

Nevertheless, DNA-based identification relies on an extensive database of sequences derived from correctly identified individuals. Therefore, because no molecular data are available on Sarcophagidae from Rio de Janeiro, our goal was to analyze part of the mitochondrial COI

gene from three Sarcophagidae species of forensic importance in the Rio de Janeiro County in order to initiate the formation of a molecular database for these species.

MATERIAL AND METHODS

We selected a total of 36 specimens comprising three species that were of potential forensic importance (Carvalho et al., 2000; Barbosa et al., 2009; Barbosa et al., 2010; Oliveira and Vasconcelos, 2010), namely: *O. fluminensis*, *P. intermutans,* and *P. chrysostoma*. To determine possible haplotype variations of the population, these species were sampled at three sites in the City of Rio de Janeiro. Collected material was preserved in 95% ethanol and maintained at 4°C until analysis. DNA was extracted from male specimens to assure the correct identification at the species level. Terminalia of these males were preserved in 70% ethanol and stored as vouchers at the Laboratory of Diptera at Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro.

Samples were transferred to a microtube and dried on a heating block at 65°C for ~30 min. DNA was extracted using 200 µL DNAzol® (Invitrogen), following the manufacturer instructions, and purified with Microcon® (Millipore). DNA was amplified using primers and conditions described previously by Amorim et al. (2014).

Amplicons were purified using AxyPrep™ PCR Cleanup Kit (Axygen Biosciences®), following the manufacturer protocol. The sequencing reaction was performed with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®) and analyzed using a Genetic Analyzer AB3130 (Applied Biosystems®), along with the program Sequencing Analysis Software v1.0. Sequence chromatograms were edited using Geneious® v4.7.5 (www.geneious.com) and deposited in GenBank. Several COI sequences from Sarcophaginae and Paramacronychiinae flesh flies and other fly families were obtained from GenBank to analyze the genetic distance. Consensus sequences were analyzed using the program MEGA 5 (Tamura et al., 2011) to obtain the multiple alignment, pairwise uncorrected distances (**S1 Table**), and the neighbor joining (NJ) tree, supported by 1000 bootstrap replicates.

RESULTS AND DISCUSSION

This study constitutes the first attempt to initiate a molecular reference database for Sarcophagidae species from the Rio de Janeiro area that are commonly found at crime scenes. This was especially the case for *O. fluminensis*, which is endemic to Brazil (Pape, 1996) and this study presents the first reported COI sequence for this species. Previous studies (Pape, 1996; Amorim et al., 2014) in São Paulo and the Bahia States have presented sequence data of the COI gene of *P. intermutans* only.

From 36 individuals, we obtained five different sequences comprising a single haplotype for *O. fluminensis* (JQ009192), two haplotypes for *P. chrysostoma* (JQ009193, JQ009194), and two haplotypes from *P. intermutans* (JQ009195, JQ009196). Interestingly, one of the *P. intermutans* haplotypes (JQ009195) is identical to one specimen (GQ409345) identified by Kutty et al. (2010), but not to one from São Paulo, Brazil (HM069340). The second *P. intermutans* haplotype (JQ009196) was identical to that from São Paulo, Brazil (HM069341). These findings confirm the reliability and demonstrate confidence in our

obtained sequences.

Concerning intrageneric divergences (**S1 Table** and Table 1), the five *Oxysarcodexia* species analyzed have an average pairwise divergence of 0.081, while the three *Peckia* species analyzed have an average divergence of 0.049. Divergences between genera of Sarcophagidae show an average pairwise divergence of 0.103. Divergence values among *Peckia* and *Oxysarcodexia* were 0.120 and within *P. chrysostoma* this value was 0.003 and for *P. intermutans* was 0.007; these values correspond to those found for other forensically important fly species (Wallman and Donellan, 2001; Wells and Sperling, 2001; Zehner et al., 2004; Meiklejohn et al., 2011).

Table 1. Intra- and interspecific mean genetic divergences (uncorrected p-distances) of three species analyzed for the mitochondrial cytochrome c oxidase subunit I (COI), with the correspondent standard error of mean (means ± SEM). The minimum and maximum values are shown in parentheses.

	Calliphoridae	Sarcophagidae	Oxysarcodexia	*O. fluminensis*	Peckia	*P. chrysostoma*	*P. intermutans*
Calliphoridae	**	**0.123 ± 0.0141**	**0.134 ± 0.013**	**0.128 ± 0.025**	**0.113 ± 0.008**	**0.104 ± 0.006**	**0.118 ± 0.003**
		(0.088 - 0.165)	(0.110 - 0.153)	(0.110 - 0.145)	(0.098 - 0.123)	(0.098 - 0.110)	(0.113 - 0.123)
Sarcophagidae		**0.103 ± 0.0241**	**0.123 ± 0.013**	**0.118 ± 0.014**	**0.104 ± 0.015**	**0.104 ± 0.016**	**0.098 ± 0.022**
		(0.000 - 0.180)	(0.088 - 0.158)	(0.075 - 0.155)	(0.065 - 0.153)	(0.030 - 0.140)	(0.000 - 0.153)
Oxysarcodexia			**0.081 ± 0.044**	**0.094 ± 0.013**	**0.120 ± 0.011**	**0.117 ± 0.006**	**0.121 ± 0.013**
			(0.000 - 0.130)	(0.075 - 0.105)	(0.098 - 0.153)	(0.105 - 0.128)	(0.108 - 0.153)
O. fluminensis				**	**0.113 ± 0.006**	**0.116 ± 0.002**	**0.113 ± 0.004**
					(0.098 - 0.118)	(0.113 - 0.118)	(0.108 - 0.118)
Peckia					**0.049 ± 0.04**	**0.079 ± 0.017**	**0.048 ± 0.040**
					(0.000 - 0.093)	(0.030 - 0.090)	(0.000 - 0.093)
P. chrysostoma						**0.003 ± 0.001**	**0.085 ± 0.003**
						(0.003 - 0.005)	(0.080 - 0.090)
P. intermutans							**0.007 ± 0.005**
							(0.000 - 0.015)

**Data not available.

In order to implement a molecular comparison, NJ analysis was performed with our sequences and those from members of Sarcophagidae and outgroups (Calliphoridae and Muscidae). As shown in Figure 1, specimens of *P. intermutans* formed a monophyletic group, separated from *P. chrysostoma*, although both species are closely related, and *Oxysarcodexia* was separate and distinct. *Peckia*, *Oxysarcodexia*, *Boettcheria*, and *Ravinia* formed a group separate from *Sarcophaga* and the outgroup, including *Oxysarcodexia* as a sister group of *Ravinia*, as found by Giroux et al. (2010), based on larval biology and adult morphology. The monophyletic separation of *P. chrysostoma* and *P. intermutans* in the phylogenetic tree, which is supported by strong bootstrapping values, confirms the sufficient resolution of the 400-bp COI fragment. This is the first report for *O. fluminensis*, which we could only compare among the genera.

In summary, sequences obtained from the mitochondrial COI gene fragment of *O. fluminensis*, *P. chrysostoma*, and *P. intermutans* are now available and can be used as an additional tool for the identification of populations from the City of Rio de Janeiro, especially in forensic applications, as previously described for West European *Sarcophaga* species (Jordaens et al., 2013).

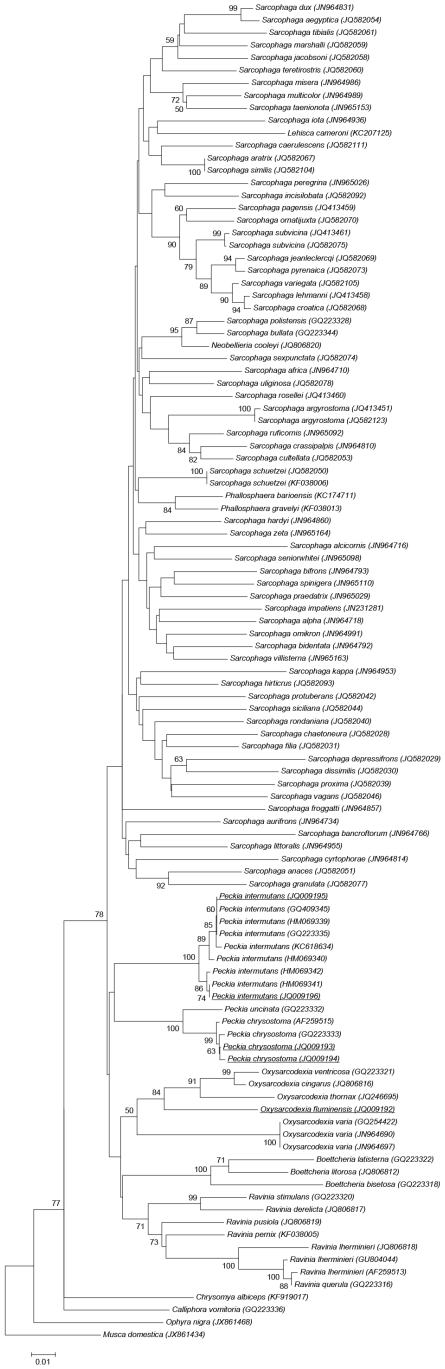

Figure 1. Neighbor joining (NJ) phylogram for 107 COI sequences of flesh flies and outgroup families. Values below branches correspond to NJ bootstrap and each sample with respective accession number in GenBank. The five sequences from Rio de Janeiro are underlined.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

S1 Table. Pairwise uncorrected p-distance matrix of COI sequences from flesh flies and outgroup families (GenBank Accession Number).