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Molecular Characterization of *Peckia* (*Pattonella*) *intermutans* (Walker, 1861) (Diptera: Sarcophagidae) based on the Partial Sequences of the Mitochondrial Cytochrome Oxidase I Gene

Jandui A Amorim[1], Carina M Souza[2] and Patricia J Thyssen[3]*

[1]Federal Institute of Education, Science and Technology of São Paulo, State of São Paulo, Brazil
[2]State University of Campinas, UNICAMP, Campinas, State of São Paulo, Brazil 13083-970
[3]Federal University of Pelotas, UFPel, Pelotas, State of Rio Grande do Sul, Brazil 96010-900

Abstract

We analyzed nucleotide sequences that constitute a part of the mitochondrial cytochrome oxidase subunit I (COI) gene from individuals of *Peckia* (*Pattonella*) *intermutans* (Walker, 1861) (Diptera: Sarcophagidae) of different populations from the States of São Paulo and Bahia, Brazil. Indices of genetic variability were calculated and phylogenetic tests (Maximum Parsimony) were applied. The presence of at least five haplotypes among sampled individuals was observed. Pairwise sequence differences within *P.* (*P.*) *intermutans* haplotypes were lower than the pairwise sequence differences between the haplotypes and outgroup species. This finding, in addition to the phylogenetic analyzis performed in this study, validates the use of molecular tools for distinguishing species of *P.* (*P.*) *intermutans* from other sarcophagids already molecularly characterized in Brazil so far. The greatest number of haplotypes (n = 3), genetic diversity (π = 0.01), and nucleotidic differences (k = 2.38) were found from specimens in Ubatuba, reflecting the low rate of genetic flow in this population compared to those from other locations; this was probably influenced by the local ecotope, i.e., the tropical rainforest called "Mata Atlântica". At collection areas within the rainforest, the vegetal cover is well preserved and this could significantly influence evolutive factors such as population size and selection towards genetic drift, supporting variability maintenance. Further investigations should be conducted for a better understanding of this finding and for expanding knowledge about the close relationships among *P.* (*P.*) *intermutans* and other sarcophagids of forensic importance.

Keywords: Flesh flies; mtDNA; Identification; Forensic entomology; Postmortem interval

Introduction

Species identification is the primary step in forensic entomology for obtaining information that is valuable for the development or conclusion of an investigative proceeding [1,2]. Since insects exhibit distinct growth rates and biological characteristics, a clear differentiation is essential. However, there are some obstacles for clear differentiation between insects, such as availability of only a scant number of specialists and few taxonomic keys that include a limited number of species, particularly Neotropical insects.

The Sarcophagidae family has a worldwide distribution, with a higher number of species in warm continental regions. These dipterans are included in 108 valid genera, with approximately 2,510 cataloged species, over 750 of which can be found in the Neotropical region [3]. Their life cycles are highly related to feeding resources, comprising decaying animal organic matter such as carrions or animal feces. Therefore, many of them are either important from a forensic standpoint, for example, in estimation of the postmortem interval (PMI) [4] or are relevant to public health because they carry human-disease-causing pathogens [5].

These species exhibit external morphologic characters, which are highly variable or very homogeneous, thus restricting species identification, almost exclusively, to detailed analyses of the male genitalia [6,7]. Consequently, identification of closely related species and/or females, found frequently associated with corpses looking for breeding sites, has become limited [1]. Similar issues occur with their immature stages, which are not identified routinely because of minuscule morphological differences among the species, low number of descriptions, and absence of keys. Thus, molecular approaches that enable species-specific identification are gaining importance as useful methodologies for this purpose [8-14].

Mitochondrial DNA (mtDNA) has been used as a suitable molecular marker because of the simple and uniform organization of the genome, the lack of recombination, and the high rate of nucleotide substitutions. In addition, the ability to retrieve genetic information efficiently from damaged or poorly preserved samples also facilitates the use of mtDNA markers in forensic investigations [15], particularly the cytochrome oxidase subunit I (COI) [eg., see 8–14]. Indeed, analysis of COI has provided diagnostic markers for the identification of species in many different groups [16].

Peckia (*Pattonella*) *intermutans* (Walker, 1861) (Diptera: Sarcophagidae) is strictly Neotropical. The occurrence of this species has been recorded in the Federal District and in the Brazilian States of Amazonas, Bahia, Ceará, Goiás, Mato Grosso, Minas Gerais, Pará, Paraná, Pernambuco, Rio de Janeiro, Rondônia, Roraima, Santa Catarina, and São Paulo [3,7,17–27]. This fly is commonly collected from cadavers and carrions due to its necrophagous behavior [25,26,28–32]. Despite this, the insect is rarely used to estimate the PMI because the larvae of this insect have to be reared until they reach adult stage to confirm their identity, and this is not a practical option.

In this study, we analyzed partial sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene of *P.* (*P.*) *intermutans* species to start a database that can be useful to facilitate identification of this and other flesh fly species of forensic importance in Brazil.

***Corresponding author:** Patricia J Thyssen, Federal University of Pelotas, UFPel, Pelotas, State of Rio Grande do Sul, Brazil 96010-900, Tel: 55 19 99707564; E-mail: thyssenpj@yahoo.com.br

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Material and Methods

Samples and areas of collection

Adult specimens were collected from natural environments by using an entomological net and by using rat carrion, chicken entrails, raw fish, and human feces as bait. Specimens were collected from the Brazilian municipalities of Campinas (22°54′21″S: 47°03′39″W), Jundiaí (23°11′09″S: 46°53′02″W), Mogi Guaçu (22°22′19″S: 46°56′31″W), Ubatuba (23°26′02″S: 45°04′15″W), belonging to São Paulo state, and Salvador (12°58′15″S: 38°30′39″W), belonging to the Bahia state (Figure 1). Collected specimens were dead by low temperature (-20°C for 1 h) and identified using traditional taxonomic keys [6]. Species of interest were individually stored in eppendorf tubes with 99.3% alcohol, coded using the locality of collection (Table 1), and kept at -20°C until further use for molecular analysis.

DNA extraction

DNA extraction was carried out by using a Qiagen DNeasy blood and tissue Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's protocol, but with an extra addition of 20 µL of proteinase K. DNA was extracted only from thoracic tissues. Other body parts were stored in eppendorf tubes with 99.3% alcohol as voucher.

DNA amplification

A partial COI sequence of *P.* (*P.*) *intermutans* was amplified using a primer set that was designed and synthesized based on the published sequence of *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) (GenBank accession nº AF259515), with Gene Runner 3.05 (Hastings Software Inc. 1994) software. The forward primer (Peckia For-1) sequence was 5′-CGAGCHGAATTAGGWCAYCC-3′ and the reverse primer was (Peckia Rev-1) 5′-GGGTGTCCGAAAAATCAGAA-3′. The above methodology was used because for this taxa, it was not possible to amplify the COI gene by using previously published primers.

For *Oxysarcodexia thornax* (Walker, 1849) and *Sarcodexia lambens* (Wiedmann, 1830), the COI fragment was amplified using the universal primers LCO1490 and HC02198 [33].

PCR was performed using the protocol mentioned by Thyssen et al. [34], and it consisted of a total reaction volume of 25 µL containing 12.5 µL of GoTaq™ Colorless Master Mix (Promega, Madison, WI, USA), 1.0 µL (10 pmol) of each primer, 1–4 µL (10–30 ng) of template DNA, and the remaining was double-distilled water. All DNA amplifications were carried out in a T-Gradient Thermoblock (Biometra, Goettingen, Germany). The reaction cycle consisted of an initial denaturing step of 3 min at 94°C followed by 34 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. The last cycle included an extended elongation step of 5 min at 72°C.

The PCR products were separated by electrophoresis on 1% agarose gels (Sigma-Aldrich, St. Louis, MO, USA) in 1×TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) at 80 V, stained with GelRed™ (Biotium Inc., Hayward, CA, USA) and observed under ultraviolet light using a transilluminator UVB (Ultra-Lum, Claremont, CA, USA). The sizes of the amplified fragments were estimated by comparison with Low DNA Mass™ Ladder (Invitrogen, Carlsbad, CA, USA) used as the molecular weight standard.

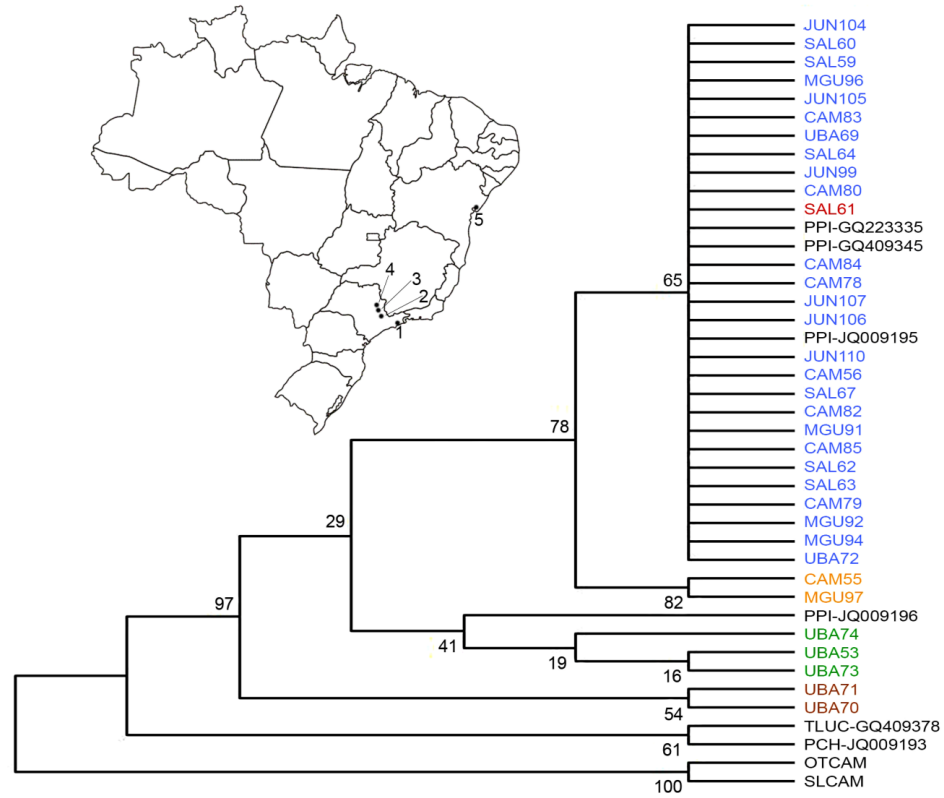

Figure 1: Collection sites in Brazil (where 1 = Ubatuba, 2 = Jundiaí, 3 = Campinas, 4 = Mogi Guaçu, and 5 = Salvador) and the most parsimonious tree illustrating the relationship among haplotypes of *Peckia* (*Pattonella*) *intermutans* and other species of Sarcophagidae (cut-off value for consensus = 10%). Blue haplotype, 1; Orange haplotype, 2; Green haplotype, 3; Brown haplotype, 4; and Red haplotype, 5. Black: represents other species of flesh flies non-*Peckia* (*Pattonella*) *intermutans*.

Species name	Locality	Voucher code	GenBank accession number
Peckia (*Pattonella*) *intermutans*	Ubatuba	UBA69, UBA72	HM069339 (haplotype 1)
		UBA53, UBA73, UBA74	HM069341 (haplotype 3)
		UBA70, UBA71	HM069342 (haplotype 4)
	Campinas	CAM56, CAM78, CAM79, CAM80, CAM82, CAM83, CAM84, CAM85	HM069339 (haplotype 1)
		CAM55	HM069340 (haplotype 2)
	Mogi Guaçu	MGU91, MGU92, MGU94, MGU96	HM069339 (haplotype 1)
		MGU97	HM069340 (haplotype 2)
	Jundiaí	JUN99, JUN104, JUN105, JUN106, JUN107, JUN110	HM069339 (haplotype 1)
	Salvador	SAL59, SAL60, SAL62, SAL63, SAL64, SAL67	HM069339 (haplotype 1)
		SAL61	KC618634 (haplotype 5)
Oxysarcodexia thornax	Campinas	OTCAM	KC618632
Sarcodexia lambens	Campinas	SLCAM	KC618633

Table 1: List of taxa, specimen collection locations, voucher codes, and GenBank accession nº of the *Peckia* (*Pattonella*) *intermutans* haplotypes and *Oxysarcodexia thornax* and *Sarcodexia lambens*.

Sequencing and sequence alignments

The amplified products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions. Direct sequencing was performed on the purified product with the same primers used for PCR and a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The obtained sequences were aligned with Clustal× 2.0.12 [35] and edited in BioEdit version 7.1.3.0 [36]. Other sequences of *P.* (*P.*) *intermutans* (= PPI) (GenBank accession nº GQ223335, JQ009195, JQ009196, and GQ409345) were included at the alignment for further analysis. *Peckia* (*P.*) *chrysostoma* (= PCH) (GenBank accession n° JQ009193) and *Titanogrypa* (*Cuculomyia*) *luculenta* (Lopes, 1935) (GenBank accession nº GQ409378), were chosen and included in the phylogenetic analysis, considering a previous phylogenetic approach for flesh flies [37]. Sequences of *O. thornax* and *S. lambens* obtained in this study were designated as outgroups.

Data analyses

The resulting alignment was analyzed using MEGA 5.1 [38]. It was built using a maximum parsimony (MP) tree, and the reliability of the tree topology was assessed through bootstrapping with 1000 replicates. Genetic variability, including number of polymorphic sites, haplotype number, diversity of nucleotides, mean difference of nucleotides, and diversity of haplotypes among *P.* (*P.*) *intermutans* specimens from different populations were estimated using MEGA 5.1. An analysis of a pairwise sequence differences was calculated for all haplotypes of *P.* (*P.*) *intermutans* and for *O. thornax*, *S. lambens*, *T.* (*C.*) *luculenta,* and *P.* (*P.*) *chrysostoma* species by p-Distance method using MEGA 5.1.

Results

For *P.* (*P.*) *intermutans* specimens, there was a 419-bp sequence of the partial COI gene amplified and a 426-bp sequence amplified for *O. thornax* and *S. lambens*.

The most parsimonious tree obtained represents the relationship among different populations of *P.* (*P.*) *intermutans* and other species, namely, *O. thornax*, *S. lambens*, *T.* (*C.*) *luculenta*, and *P.* (*P.*) *chrysostoma*, where the low divergence among *P.* (*P.*) *intermutans* sequences can be clearly observed by topology-shaped rake (Figure 1). Outgroups were clustered together with a supporting bootstrap value of 100% for the clades, including *O. thornax* and *S. lambens*, and 61% for the others, including *T.* (*C.*) *luculenta* and *P.* (*P.*) *chrysostoma*.

Five different haplotypes within the different populations of *P.* (*P.*) *intermutans* emerged from the analyses, and the mean difference of nucleotides among the haplotypes was 1.399 (Table 2). The analyses of the sequences retrieved from GenBank indicated that *P.* (*P.*) *intermutans* with accession number JQ009196 was the same as haplotype 3, while *P.* (*P.*) *intermutans* accession numbers JQ009195, GQ223335, and GQ409345 were similar to haplotype 1 (Figure 1).

A higher number of polymorphic sites were recognized in sequences from the specimens recovered from Ubatuba compared to the sequences of specimens recovered from Campinas and Mogi-Guaçu. In addition, the nucleotide diversity and the mean difference of nucleotides were also significantly higher than that in other analyzed populations, distinguishing Ubatuba from the others (Table 2).

Pairwise sequence differences within *P.* (*P.*) *intermutans* haplotypes were lower than the pairwise sequence differences between these haplotypes and the outgroup species (Table 3).

Discussion

Although molecular analyses of mitochondrial DNA can be more robust with longer base-pair fragments (>1 kb), which allows easier identification of patterns of nucleotide divergence and solve greater ranges of divergence [14,39], short sequences have also been reported to be useful in identifying fly species of forensic importance [11,14], especially considering time, ease, and economy [14].

Intraspecific variation found in *P.* (*P.*) *intermutans* haplotypes (0.30%–1.50%) was slightly higher than the values reported in literature for other flesh fly species, (<1.00%–1.35%). However, the minimum interspecific variation among *P.* (*P.*) *intermutans* haplotypes and *O. thornax*, *S. lambens*, *T.* (*C.*) *luculenta*, and *P.* (*P.*) *chrysostoma* corresponds to that observed in-between different species in other studies [9,10,13,14]. Furthermore, no overlap between minimum interspecific variation and maximum intraspecific variation allows easy distinction between species [13].

Less divergence of the COI sequence within compared species has already been recognized as a possible way for recognizing an unknown species, considering the percentage similarity with standards, when previous sequences and comparisons with large numbers of species are available [9]. The amino acid variation of analysed sequences was restricted to a unique base for individuals of different haplotypes, e.g. haplotypes 1 and 5 (which appear together in the phylogenetic tree due to the cutoff value of 10% chosen for performing of consensus in order to improve graphic exposition of our results), or was completely equal in individuals of the same haplotype. This can be explained by the fact the COI gene is considered conserved in terms of amino acid

Locality	N	S	H	π	k	H_d	D
Campinas	9	2	2	0.001	0.444	0.222	-1.362*
Mogi-Guaçu	5	2	2	0.002	0.800	0.400	-0.973*
Jundiaí	6	0	1	0	0	0	0*
Ubatuba	7	5	3	0.006	2.381	0.762	0.826*
Salvador	7	1	2	0.001	0.286	0.286	-1.006*
Total	34	7	5	0.003	1.3993	0.412	-1.162*

Table 2: Genetic variability indexes within different populations of *Peckia (Pattonella) intermutans.* Where: N = number of specimens; S = number of polymorphic sites; h = haplotype number; π = diversity of nucleotides; k = mean difference of nucleotides; H_d = diversity of haplotypes. * Statistical differences were considered non-significant at p > 0.10.

	#1	#2	#3	#4	#5	#6	#7	#8	#9
1. HAP 1 - *P.* (*P.*) *intermutans*	...								
2. HAP 2 - *P.* (*P.*) *intermutans*	0.5	...							
3. HAP 3 - *P.* (*P.*) *intermutans*	1.0	1.0	...						
4. HAP 4 - *P.* (*P.*) *intermutans*	1.3	1.3	0.3	...					
5. HAP 5 - *P.* (*P.*) *intermutans*	0.3	0.8	1.3	1.5	...				
6. *Oxysarcodexia thornax*	46.1	45.9	45.6	45.4	46.1	...			
7. *Sarcodexia lambens*	46.6	46.4	46.1	45.9	46.6	10.3	...		
8. *Titanogrypa* (*C.*) *luculenta*	11.5	11.3	11.5	11.3	11.8	49.9	51.9	...	
9. *Peckia* (*P.*) *chrysostoma*	8.5	8.5	8.5	8.3	8.8	46.1	47.4	11.5	...

Table 3: Pairwise sequence differences (%) for a 400-bp region of the COI gene of the 5 haplotypes (HAP) of *Peckia* (*Pattonella*) *intermutans* from different locations in Brazil and other species of Sarcophagidae.

evolution [40] or because the length of the analyzed fragment was not long enough to cover all the substitutions present in the COI gene [11].

In any case, the analyzed sequences were long enough to illustrate genetic variation within the Ubatuba population, as evidenced by the presence of three haplotypes and conspecific divergence, inferred from the formation of two non-shared clades, tough the low bootstrapping values. The occurrence of different polymorphisms within the Ubatuba population can be correlated to the local ecotope, i.e., a tropical rainforest called "Mata Atlântica," which is considered a hotspot of biodiversity due to high biodiversity index [41]. At the locations of specimen collection, this vegetal cover is quite preserved, and thus it could significantly influence evolutive factors, such as population size and selection towards genetic drifts, supporting variability maintenance [42]. A broader study using distinct molecular markers for a better comprehension of the genetic structure and flow may lead to a more consistent phylogenetic hypothesis for answering questions more appropriately in the light of systematics.

The recognition of haplotype variation within *P.* (*P.*) *intermutans* from different geographical Brazilian locations can be useful for the identification of species of forensic interest, since a wide knowledge of intraspecific haplotype diversity within the carrion-fly species has been recommended in order to avoid misidentification and validate molecular identification of species [9,43]. Furthermore, phylogenetic approaches based on gene sequences should be commonly used to achieve identification of unknown species or of haplotypes that are not yet available in a reference database [43].

The results of this work confirm the usefulness of molecular markers in differentiating and identifying flies of forensic importance and represent one more step toward a more detailed investigation of the molecular identification of flies. Such approaches could prove useful in the routine analysis of endemic Diptera species of forensic importance in Brazil. However, for specimens from Central America or other regions of South America, further studies are needed.

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