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Phenotypic polymorphism of *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) may lead to species misidentification

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ABSTRACT

Species identification is an essential step in the progress and completion of work in several areas of biological knowledge, but it is not a simple process. Due to the close phylogenetic relationship of certain species, morphological characters are not always sufficiently distinguishable. As a result, it is necessary to combine several methods of analysis that contribute to a distinct categorization of *taxa*. This study aimed to raise diagnostic characters, both morphological and molecular, for the correct identification of species of the genus *Chrysomya* (Diptera: Calliphoridae) recorded in the New World, which has continuously generated discussion about its taxonomic position over the last century. A clear example of this situation was the first record of *Chrysomya rufifacies* in Brazilian territory in 2012. However, the morphological polymorphism and genetic variability of *Chrysomya albiceps* studied here show that both species (*C. rufifacies* and *C. albiceps*) share very similar character states, leading to misidentification and subsequent registration error of species present in our territory. This conclusion is demonstrated by the authors, based on a review of the material deposited in major scientific collections in Brazil and subsequent molecular and phylogenetic analysis of these samples. Additionally, we have proposed a new taxonomic key to separate the species of *Chrysomya* found on the American continent, taking into account a larger number of characters beyond those available in current literature.

1. Introduction

Calliphoridae (Diptera: Muscomorpha) species are distributed worldwide, from the Northern limits of land to New Zealand and the sub-Antarctic islands (Shewell, 1987). In this taxon, there are approximately 1500 registered species in 150 genera; at least 80% of which are restricted to the Old World (Thompson, 2013). James (1970) has listed about 100 species in the Neotropical region; however, the actual number of species may exceed 130, due to predictions of new records from the Andes (Carvalho and Mello-Patiu, 2008).

Adult specimens are commonly found feeding and breeding on meat, fish, dairy products, animal carcasses, garbage, and excrement (Linhares, 1981; Guimarães and Papavero, 1999; Vianna et al., 2004), and because of this behavior they are vectors of numerous pathogens for humans and domestic animals (Greenberg, 1973; Thyssen et al., 2004). Some species also cause injuries to the skin of vertebrates, larval infestations known as myiasis (Zumpt, 1965; Guimarães and Papavero, 1999). In addition to their medical and veterinary importance, they have an economic impact due to the expensive measures that are usually taken for their control (Linhares and Thyssen, 2007).

Eggs, larvae, pupae or adult insects collected from carcasses have been used as evidence in forensic investigations; not only for estimating the postmortem interval (PMI) (Erzinçlioglu, 1983; Marchenko, 2001), but also to determine the causes and circumstances of deaths (Smith, 1986; Catts and Goff, 1992; Byrd

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and Castner, 2010). Particularly, carrion-breeding blowflies of the genus *Chrysomya* (Diptera: Calliphoridae) (Robineau-Desvoidy, 1830) have an important role in forensic entomology because they are usually the first to colonize and the most abundant flies found feeding on carrion where they occur (Tomberlin et al., 2012; Moretti and Godoy, 2013).

Approximately 35 years ago, three species of Old World *Chrysomya*, *C. albiceps* (Wiedemann, 1819), *Chrysomya putoria* (Wiedemann, 1818) and *Chrysomya megacephala* (Fabricius, 1794), were introduced and became established in the Neotropical region (Guimarães et al., 1978; Guimarães et al., 1979; Guimarães and Papavero, 1999). Presently, they are widely distributed in South America and they have dispersed rapidly to the rest of the American continent (Richard and Ahrens, 1983; Baumgartner and Greenberg, 1984; Greenberg, 1988; Wells, 1991; Shahid et al., 2000; Tomberlin et al., 2001). Moreover, the establishment of these species in the New World has affected the native fauna, by displacing several native species (Wells and Greenberg, 1992; Faria et al., 1999).

Recently, the occurrence of *Chrysomya rufifacies* (Macquart, 1843) was reported in the State of Maranhão, Northern Brazil (Silva et al., 2012). This species is native to the Australasian region and it was first recorded in Central America in 1978 (Jirón, 1979), and a few years later in North America (Baumgartner, 1993; Rosati and VanLaerhoven, 2007). The first South American record of *C. rufifacies* was in Argentina (Mariluis and Schnack, 1989), subsequently in Colombia (Barreto et al., 2002; Pape et al., 2004) and recently in mainland Ecuador (Tantawi and Sinclair, 2013). In Argentina and Ecuador, *C. rufifacies* now overlaps ranges with *C. albiceps* (Tantawi and Greenberg, 1993; Tantawi and Sinclair, 2013).

The close morphological similarity between *C. albiceps* and *C. rufifacies* (Wells and Sperling, 1999) may lead to an inaccurate identification of these two species in areas where they overlap, and an unambiguous diagnostic character for these species is of particular importance for forensic entomologists and ecological studies.

Adults of *C. albiceps* and *C. rufifacies* are usually separated by a few diagnostic characters (Holdaway, 1933; Guimarães and Papavero, 1999; Whitworth, 2010). The presence of the proepimeral seta in *C. rufifacies*, and the shape of male terminalia are the most commonly used structures (Bezzi, 1927; Holdaway, 1933; Guimarães et al., 1978; Carvalho and de Ribeiro, 2000; Mello, 2003). Nevertheless, proepimeral seta may be present in a small percentage of *C. albiceps* individuals, making this character doubtful and questionable (Zumpt, 1965; Tantawi and Greenberg, 1993). In this case, molecular analysis can be an efficient complementary taxonomic tool (Vincent et al., 2000; Wallman and Donnellan, 2001; Hajibabaei et al., 2007; Nelson et al., 2007; Nelson et al., 2008; Wells and Stevens, 2008; Chen et al., 2011; Nelson et al., 2012) and it has been used to aid in the identification of forensically important blowflies when the use of morphological characters is not reliable (Wells and Sperling, 1999; Harvey et al., 2003a; Marinho et al., 2011).

The present study used a combined morphological and multigene molecular analysis to investigate when the morphological polymorphism and genetic variability observed in *C. albiceps* may cause misidentification of *C. albiceps* and *C. rufifacies*. With this purpose, a new taxonomic key for the *Chrysomya* species occurring in the American continent is proposed.

2. Material and methods

2.1. Obtaining samples for analysis

Accessed specimens for morphological and molecular analysis are deposited in the following institutions from Brazil:

CEIOC—Entomological Collection, Oswaldo Cruz Institute (Rio de Janeiro State).

MPEG—Entomological Collection, Museum Paraense Emílio Goeldi (Pará State).

L2B-DBA—scientific collection of the Laboratory of Entomology (L2B-DBA), Department of Animal Biology, Campinas State University, UNICAMP (São Paulo State).

Field collection was also performed in different localities of Brazil (Campinas, São Paulo State; Manaus, Amazonas State; Terenos, Mato Grosso do Sul State) and Indonesia (Tanggamus Camping, South West Sumatra and East Sumatra) between 2012 and 2013 in natural environments using, in some cases, appropriate traps (Moretti et al., 2009) or entomological sweep-nets. All collected flies were taken to the lab for identification by taxonomic keys (Holdaway, 1933; Guimarães et al., 1978; Dear, 1985; Guimarães and Papavero, 1999) and comparison with identified vouchers.

In addition, specimens of *C. albiceps*, *C. megacephala* and *C. putoria* were borrowed from several entomologists and samples of *C. rufifacies* were obtained from a laboratory lineage from Homestead, Florida, USA.

For detailed information on the species, institutions/localities of collection, label data from examined materials, and GenBank sequence accession ID, see Table A.1.

2.2. Preparation of material, photographic records, and terminology

Some of the adult specimens were pinned, and others were dissected for externalization of the male genitalia and separation of other body parts. Structures with taxonomic significance, such as sternites, were cleared in 10% KOH for 24 h, washed in distilled water, and fixed in 70% ethanol to allow better visualization.

All photographic records were made using a stereomicroscope Zeiss™ Discovery V.12 with image capture system AxioCam 5.0™ and software ZEN™ version 2.0. Scale bars (in mm) were inserted with the support of ZEN™ software. Arrows were added to some photographs to indicate relevant details.

Terminology for the external characters follows McAlpine (1981) and Merz and Haenni (2000).

2.3. PCR amplification and sequencing

Genomic DNA extractions were carried out with an Invisorb® Spin Tissue Mini Kit (Stratec Molecular) using up to three legs from each specimen analyzed, or the abdomen when no legs were available. Extractions of head or thorax tissues were avoided in order to preserve the morphological characters of the collected specimens.

Four molecular markers were amplified by PCR and subsequently sequenced, comprising the mitochondrial (1) 5′ region of the cytochrome *c* oxidase subunit I (COI), and (2) the whole gene of the cytochrome *c* oxidase subunit II (COII); the complete nuclear region (3) of the internal transcribed spacer 2 (ITS2), and (4) the region containing the short intron 2 and the homeobox coding region in exon 3 of the developmental gene *bicoid* (*bcd*).

The PCR for the COI and COII regions were done in 25-μL reaction volumes containing 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1 mM $MgCl_2$, 80 μM dNTPs, 0.2 μM of each primer, 1.5 U *Taq* DNA polymerase (Thermo Scientific), and 1–2 μg of extracted DNA. The following universal primers (Simon et al., 1994) were used to amplify each region: C1-N-2329 and TY-J-1460 for COI, TL2-J-3034, and TK-N-3785 for COII. PCR conditions were an initial denaturation step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 1 min, and 72 °C for 2 min, and a final elongation step of 7 min at 72 °C.