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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.

The ITS2 amplifications were done in 25-μL reaction volumes containing 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 2 mM $MgCl_2$, 80 μM dNTPs, 0.4 μM of each primer, 2.5 U *Taq* DNA polymerase (Thermo Scientific), and 1.5–2.0 μg of extracted DNA. The following primers were used: 5.8S (5′-ATCACTCGGCTCGTGGGATTCGAT-3′) and 28S (5′-GTTAGTTTCTTTTCCTCCCCT-3′). PCR reactions were performed with an initial denaturation step of 95 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 45 sec, and 72 °C for 2 min, and a final elongation step of 3 min at 72 °C.

The PCR reactions for the *bcd* partial region were done in 25-μL reaction volumes containing 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1 mM $MgCl_2$, 40 μM dNTPs, 0.25 μM of each primer, 2.5 U *Taq* DNA polymerase (Thermo Scientific), and 1.0–2.5 μg of extracted DNA. The following primers were used: Chry-bcd-Intr2 (5′-CCAAATCAATTACCAAAGCCAGGTG-3′) and Chry-bcd-Exo3 (5′-CATTTGGTGTTGATCCACCACTGCC-3′), adapted from primers described for *Chrysomya* species analyzed by Park et al. (2013) and designed in this study. PCR conditions were the same as those described for the COI and COII regions.

All PCR amplicons were visualized by electrophoresis in 1× TAE (40 mM Tris-acetate, 1 mM EDTA) 1% agarose gels stained with GelRed™ dye, and purified using an Illustra GFX PCR DNA and a Gel Band Purification kit (GE Healthcare, UK).

In order to evaluate the intragenomic variability in ITS2 and *bcd* sequences, a cloning step was conducted before sequencing for each specimen of *C. albiceps*, *C. rufifacies*, and polymorphic *C. albiceps*. This step was carried out using purified PCR products cloned into a pGEM-T Easy Vector (Promega), as described by Marinho et al. (2012).

The automatic bidirectional sequencing was conducted in an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) with the respective primers used in PCR's reactions, and 40–60 ng of DNA. The sequencing of the clones was performed with universal primers M13-Forward and M13-Reverse. At least three clones of each species were submitted to sequencing.

2.4. DNA analysis

After sequencing, the reads were confirmed by a homology search in GenBank using the Blastn tool (Altschul et al., 1990), and the bidirectional reads assembled with CAP3 online software (Huang and Madan, 1999). The base quality of each read was accessed with the Phred tool available at the MetAmiga database (Feijão et al., 2006).

Multiple sequence alignments were performed using Clustal Ω (Sievers et al., 2011) and, with the exception of COI and COII, some manual adjustments were made on the final alignments. The predicted secondary-structures of ITS2 described by Marinho et al. (2011, 2012) were used in order to improve the final alignment of this marker.

Uncorrected sequence divergences (*p*-distances) were calculated in MEGA 5.1 software (Tamura et al., 2011), and employed for clustering analyses using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987), performed for either the four molecular markers separately and concatenated. Node supports were measured by 5000 replicates of bootstrap.

To evaluate whether or not the NJ results were robust to changes in the analytical method, a partitioned Bayesian Inference (BI) was also performed. The best-fitted substitution model selection for each dataset was carried out using MrAIC 1.4.4 (Nylander, 2004) software. The favored model for COI and COII was the GTR + I (General Time Reversible; I= invariable sites). HKY + G (Hasegawa, Kishino, and Yano, G = gamma distribution rate) was the favored model for ITS2 and the GTR for *bcd* region.

Partitioned BI analysis was conducted using mrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Each of the four genes was treated as a different partition, evolving under the best model selected previously. Two independent analyses were run for 10×10^6 generations (sample frequency = 1000), with 25% burning after checking for convergence. Node supports were analyzed by their posterior probabilities in the 50% majority rule consensus tree.

To ensure more consistency in both analyses (NJ and BI), sequences of *Chrysomya megacephala*, *Calliphora vomitoria* L., *Lucilia sericata* (Meigen), and *Musca domestica* L., available in GenBank, were included (accession numbers in Table A.1). Only *C. vomitoria*, *L. sericata*, and *M. domestica* were set as outgroup.

After the distance and phylogenetic analyses, the resultant phylogenetic hypothesis was evaluated using the likelihood-map (Strimmer and Von Haeseler, 1997) implemented in Tree-Puzzle 5.2 software (Schmidt et al., 2002). Concatenate sequences were grouped into four taxa: *C. albiceps*, *C. putoria*, *C. rufifacies*, and the polymorphic *C. albiceps*. The percentage of quartets favoring each one of the three possible tree topologies was conducted by evaluating all possible quartets of the dataset with accurate parameter estimation, Quartet sampling + NJ tree option, and GTR as general substitution model.

3. Results

3.1. Diagnosis and distribution of Chrysomya species introduced in the Americas

Chrysomya albiceps (**Wiedemann**)

Head. Inner face of the third antennal segment dark brown; palpus yellowish with dark distal region; gena with lower half blackish and upper half pale brown. Male: outer vertical setae present; parafrontalia with pale, short, and sparse setulae, shorter in length than the frontal setae. Female: proclinate orbital setae absent. **Thorax**. From 5 to 7 proepisternal (propleural) setae; proepimeral (prostigmatic) seta usually absent. The proepimeral seta is present in the specimens termed by us as polymorphic, and is the only state of character that differ polymorphic specimens of non-polymorphic. Anterior spiracle whitish; lower calypter whitish with darkish setulae; the branch of vein M1 + 2 forming acute angle as it approaches the wing edge. **Abdomen**. Male: sternite IV with similar width and height; sternite V "V-shaped"; cercus and surstylus with almost the same length of the aedeagus. Female: tergite V with dorsal cleft.

New World distribution. Argentina, Bolivia, Brazil, Colombia, Dominica, Ecuador, Guatemala, Nicaragua, Paraguay, Peru, Puerto Rico, Uruguay, Venezuela.

Chrysomya putoria (**Wiedemann**)

Head. Inner face of the third antennal segment dark brown; palpus yellowish; gena totally blackish. Male: outer vertical setae absent; parafrontalia with pale, short and sparse setulae, with length smaller than the frontal setae. Female: two pairs of proclinate orbital setae. **Thorax**. From 1 to 2 proepisternal (propleural) setae; one proepimeral (prostigmatic) seta present; anterior spiracle whitish; lower calypter and setulae whitish. Wing with the branch of vein M1 + 2 forming smooth angle as it approaches the wing edge. **Abdomen**. Male: sternite IV with similar width and height, and sternite V "V-shaped"; cercus and surstylus smaller than the length of the aedeagus. Female: tergite V without a dorsal cleft.

New World distribution. Argentina, Bolivia, Brazil, Colombia, Panama, Paraguay, Peru.

Chrysomya rufifacies (**Macquart**)

Head. Inner face of the third antennal segment pale brown; palpus yellowish; gena totally blackish. Male: outer vertical setae present; parafrontalia with pale, long and broadly distributed setulae, similar in length to the frontal setae. Female: proclinate orbital setae absent. **Thorax**. From 2 to 4 proepisternal (propleural) setae; one proepimeral (prostigmatic) seta present; anterior spiracle whitish; lower calypter whitish with darkish setulae; the branch of vein M1 + 2 forming smooth angle as it approaches the wing edge. **Abdomen**. Male: sternite IV with width broader than the height, and sternite V "V-shaped"; cercus and surstylus with almost the same length as the aedeagus. Female: tergite V with a dorsal cleft.

New World distribution. Argentina, Canada, Colombia, Cuba, Dominica, Ecuador, Guatemala, Jamaica, Mexico, Panama, Puerto Rico, United States of America.

Chrysomya megacephala (**Fabricius**)

Head. Third antennal segment, palpus, and gena, yellowish to orange. Male: ommatidia in the compound eyes enlarged on the upper half; outer vertical setae absent. Female: 2 pairs of orbital setae. **Thorax**. One proepisternal (propleural) seta well developed; 1 proepimeral (prostigmatic) present; anterior spiracle blackish; lower calypter with setulae, darkish; the branch of vein M1 + 2 forming acute angle as it approaches the wing edge. **Abdomen**. Male: cercus and surstylus almost the same length as the aedeagus. Female: tergite V without a dorsal cleft.

New World distribution. Argentina, Brazil, Colombia, Dominica, Dominican Republic, Ecuador, Greater Antilles, Jamaica, Nicaragua, Peru, Puerto Rico, United States.

3.2. Morphological analysis

3.2.1. Analysis of the characters described in identification keys

3.2.1.1. Head

Ommatidia of males: (0) enlarged *versus* (1) not enlarged.

Remarks. The most widespread characteristic used in the dichotomous keys to distinguish males of *C. megacephala* from the others species of *Chrysomya* introduced in the New World.

Outer vertical seta of males: (0) absent *versus* (1) present.

Remarks. Zumpt (1956) proposed the use of the outer vertical seta on the male head to distinguish *C. albiceps* from *C. putoria* and *C. chloropyga* originated from the Ethiopic region. Later, he admitted the existence of *C. rufifacies* as a valid species, thus using this characteristic to differentiate *C. rufifacies* from *C. putoria* (Zumpt, 1965). Guimarães and Papavero (1999) and Kosmann et al. (2013) also proposed the use of this characteristic on their identification keys. Silva et al. (2012) pointed out that for *C. putoria* the seta is usually absent, but considering the specimens that we observed, together with the descriptions that have been made by other authors, we can assert that the seta is always absent in *C. putoria and C. megacephala*, which makes this character useful in distinguishing these two species from *C. albiceps* and *C. rufifacies*.

Relation between frons and anterior ocellus of males: (0) frons width twice the width of anterior ocellus *versus* (1) frons width less than the width of anterior ocellus.

Remarks. The proposition appears for the first time in Zumpt (1956), as the authors made a detailed characterization of *C. albiceps* and *C. putoria*. In this case, the analysis was based on morphometric characteristics, where the measure was related to the total head width with that of the frons. Dear (1985) took a new approach to this character, with the objective to make the interpretation more practical and began to qualitatively use the relation between the frons width and the anterior ocellus diameter. The use of this new information became more widely reported among authors (Dear, 1986; Amat, 2009; Whitworth, 2010; Silva et al., 2012). We also found out that the compound eyes are closer in *C. putoria*, consequently the frons width is smaller. In *C. albiceps* and *C. rufifacies* the compound eyes are further apart from each other, and because of this, the frons width can be twice as much as the anterior ocellus width.

Orbital seta of female: (0) absent *versus* (1) present, being small and proclinate, or outer lateral.

Remarks. The setae were firstly studied by Guimarães et al. (1978), who registered the introduction of *Chrysomya* into the American continent, and later described again by Dear (1985), Amat et al. (2008) and Amat (2009) in their respective studies.

Although it can be easily seen, we propose here the use of this characteristic associated with at least another one into the same key branch, since the character is found only in females. None of the previous works presented the combination of the orbital setae with the outer vertical in the same branch, which in our judgment may be the ideal way to distinguish the species in question, *C. rufifacies* from the other three *Chrysomya* species.

Third antennal segment: (0) dark brown *versus* (1) inner face, pale brown *versus* (2) yellowish.

Remarks. This was one of the first characteristics proposed to identify *C. rufifacies*, when it was accepted as a valid species. Holdaway (1933) suggested that the colour of the antenna and the difference of the wing venation M1 + 2 are two characteristics that may be used in the original description to distinguish *C. albiceps* from *C. rufifacies*. Although the author observed different colour patterns regarding the antenna, he did not include the results of his analysis in the identification key drawn in his work.

Since the first proposition, only Guimarães et al. (1978) and Guimarães and Papavero (1999) referred back to this characteristic, however they used it to indicate a possible separation between *C. megacephala* and other species, such as *C. albiceps* and *C. putoria*, pointing that this antennal segment is reddish brown in the first species, and dark brown to blackish in the others.

After examination of our specimens, we corroborate the original descriptions of Holdaway (1933), wherein *C. albiceps* and *C. putoria* present a dark brown colour on flagellomere I, and *C. rufifacies* presents a pale brown colour on the inner side of the dorsal part of the antenna. Although the colours of the anatomical structures of insects may present a high level of variation depending on their preservation conditions, we are confident in highlighting this character to aid in the diagnosis of *C. rufifacies* due to the varying conditions of preservation exhibited by the specimens we examined.

Setulae and parafrontalia setae of males: (0) setulae whitish, short and sparse, smaller than the frontal setae *versus* (1) setulae whitish, long, widely spread and with a similar size to the frontal setae *versus* (2) setulae darkish, long, widely spread and smaller than the frontal setae.

Remarks. This character, proposed only in males, was used only once in an identification key (James, 1948). However, in our study, we noticed that it may be applied also to females, and we consider that this is a valid characteristic for the diagnosis of *Chrysomya* species, since the setulae length did not present much intraspecific variation among the examined specimens.

Gena: (0) pale brown, darker in the inferior half *versus* (1) blackish *versus* (2) yellowish/orange.

Remarks. Historically, the facial, parafacial, and gena regions may be interpreted in different ways concerning the specific delimitation of each anatomical structure and the colour pattern. The synonymization of some terms has been proposed recently by Merz and Haenni (2000), referring to "bucca," "cheek," and "genal

dilation" merely as "gena." In the present study, we adopted the same terminology.

James (1948) added the analysis of the parafacial and facial colour to distinguish *C. putoria* from *C. rufifacies* on the terminal node, generalizing the facial and parafacial colour of *C. rufifacies* as extensively reddish, whereas for *C. putoria* he refers to it as blackish in the major part. Based on the denominations of this character proposed by James (1948) for *C. putoria*, Zumpt (1956) did not accept *C. rufifacies* as a valid species and, for this reason, did not describe the characteristics inherent to this character. Only later did Zumpt (1965) mention that the anterior part of the gena of *C. rufifacies* varies between yellow and orange colour.

For *C. albiceps*, the gena is described both as yellow/orange, though it may be partially blackish sometimes (Zumpt, 1956), and as yellow/orange or more darkish or less darkish (Zumpt, 1965). There is a lot of inconsistency on these two works, especially when compared with James (1948), Guimarães et al. (1978), Guimarães and Papavero (1999) and Tantawi and Sinclair (2013).

Guimarães and Papavero (1999), for example, manage to maintain coherence in the description of the colour of the gena for all *Chrysomya* species of the New World, except *C. megacephala*, but for *C. rufifacies*, they keep the analysis only for the anterior region, without concluding if the colour of gena is similar between *C. rufifacies* and *C. putoria*. After examining our samples, we observed that the background colour of the inferior half of the gena in *C. albiceps* is partially blackish and partially pale brown next to the oral cavity, whereas in *C. rufifacies* and *C. putoria* the gena is darkish in almost its totality.

For *C. rufifacies* and *C. putoria*, the separation is more consistently done through the analysis of the face region that is closer to the oral cavity, as has been pointed out by other authors. Therefore, while this region is slightly reddish in *C. rufifacies*, in *C. putoria* it has a darker brown colour. Since it is a very narrow band of colouring, which extends from the parafacialia to the gena, this is a characteristic that requires some care to be properly observed, although it is a useful character to separate these two species.

3.2.1.2. Thorax

Proepisternal (propleural) setae: (0) from 1 to 2 *versus* (1) from 2 to 4 *versus* (2) from 5 to 7.

Remarks. This was one of the first characters used to differentiate the *Chrysomya* species analyzed on this study (Holdaway, 1933) due to the consistent numbers of setae within the same species (1–2 for *C. putoria;* 2–4 for *C. rufifacies* and 5–7 for *C. albiceps*). Therefore, this character continues to be used in identification keys, whether or not they include *C. rufifacies* (Zumpt, 1965; Guimarães et al., 1978; Mariluis, 1981; Carvalho and de Ribeiro, 2000; Greenberg and Kunich, 2002; Amat et al., 2008; Amat, 2009; Carvalho and Mello-Patiu, 2008).

Among the three possible groups of variation, it was observed that the numerical abundance of proepisternal setae presented a greater variation for *C. albiceps* than for other species, from 5 to 7 setae, usually 6. The literature shows variation from 4 to 6 (Holdaway, 1933; Carvalho and de Ribeiro, 2000; Greenberg and Kunich, 2002; Amat et al., 2008; Amat, 2009). For *C. putoria*, the number of setae may vary from 1 to 2, frequently with a strong seta accompanied with a small seta. In *C. rufifacies*, we observed that the number of setae may range from 2 to 4, usually 3, in accordance with Holdaway (1933).

Proepimeral (prostigmatic) seta: (0) absent *versus* (1) present.

Remarks. Probably because it is easily visualized, this has been the most widespread characteristic used on identification keys of the New World's *Chrysomya* species, especially to distinguish *C. albiceps* from *C. putoria*, since it is always observed on the latter; it occurs on *C. rufifacies* as well. However, as our study of polymorphism related to *C. albiceps* revealed the existence of individuals in some populations that may present the seta unilaterally or bilaterally, we suggest that this character should not be used as a determinant to settle the identification of the species, as it has been used previously (Carvalho and de Ribeiro, 2000).

Since the proposition of this character, a few authors have noted the possibility of the presence of the seta in *C. albiceps* (Holdaway, 1933; Guimarães et al., 1978; Guimarães and Papavero, 1999). Patton (1925) discussed the historical and geographical aspects that surround the separation and acceptance of *C. albiceps* and *C. rufifacies* in different continental regions, without presenting enough taxonomic support to safely differentiate these *Chrysomya* species. When making reference to the type-material of *C. albiceps* analyzed in Patton (1925), the author himself wonders about the rigor of the use of the seta as a diagnostic character, when the studied specimen presented seta on only one side of its body. Contesting what was presented by Patton, Holdaway (1933) registered that the type-material of *C. albiceps* had a miniscule seta at the subspiracular region, but its size never approaches that of the stigmatal seta of *C. rufifacies*. Although Holdaway (1933) has added the presence of the setae in the identification key he prepared, he left note that it may be absent is some specimens.

Bezzi (1927), the first author to propose the acceptance of *C. albiceps* and *C. rufifacies* as valid species, also pioneered the use of the presence/absence of the proepimeral seta to differentiate these species. Zumpt (1956) agreed that the stigmatic seta could be the main character in distinguishing *C. albiceps* from *C. rufifacies*, but based on the taxonomic surveys of Holdaway (1933), he speculated about the validity of *C. rufifacies* being a separate species and proposed that if there were lineages of *C. albiceps* with symmetrically developed setae, as in *C. rufifacies*, they should be considered merely as a subspecies of *C. albiceps*, due to the close resemblance of the genitalia (cercus, and surstylus) of both species.

By reviewing Holdaway's (1933) propositions and observing our samples, we assert that these characters are not as variable as stated by Zumpt (1956) and, although the use of the proepimeral seta to differentiate *Chrysomya albiceps* from *C. putoria* is valid, it should not be used to differentiate *C. albiceps* from *C. rufifacies*.

Anterior spiracle: (0) white *versus* (1) blackish.

Remarks. A character used in every identification key for the New World species that intends to separate *C. albiceps, C. putoria*, and *C. rufifacies* from *C. megacephala*, since the latter is the only one with a darkish spiracle, though may have other interpretations concerning the coloration as brown, dark brown, or grayish (Guimarães et al., 1978; Guimarães and Papavero, 1999; Carvalho and de Ribeiro, 2000; Mello, 2003; Amat, 2009; Silva et al., 2012; Kosmann et al., 2013). The range of colours is probably related either to the preservation conditions of specimens or to natural population variations, although this does not lead to problems of misidentification.

Lower calypter: (0) white *versus* (1) blackish.

Remarks. Proposed only by Guimarães et al. (1978) this character clearly separates *C. albiceps*, *C. rufifacies*, and *C. putoria* from *C. megacephala*, since the latter is the only one that presents a blackish lower calypter; on the other species this structure is white.

Lower calypter setae: (0) whitish *versus* (1) blackish.

Remarks. This is the first proposition of how to separate the *Chrysomya* species of the New World. In *C. albiceps* and *C. rufifacies*, the setae of the dorsal surface of the lower calypter are blackish, while in *C. putoria* these setae are whitish. Since all of our *C. albiceps* specimens have blackish lower calypter setae, this is an additional character that can be used to separate *C. albiceps* and *C. rufifacies* from *C. putoria*.

Vein M1 + 2: (0) more acute angle *versus* (1) smoother angle.
Remarks. In his attempt to clarify the taxonomical status of *C. albiceps* and *C. rufifacies*, Holdaway (1933) analyzed the original descriptions from Wiedemann (1819) and Macquart (1843), respectively, acknowledging that the difference in the angle formed by the vein M1 + 2 is one of the few characters that are useful to distinguish these two species.

By examining wings from more than one hundred specimens, Holdaway (1933) concluded that the curvature in the M1 vein, from its junction with M1 + 2 to the border of the wing, forms either an acute angle (in *C. albiceps*) or a more rounded and smooth one (in *C. rufifacies*). This characteristic, the venation, although discussed in the original description, was not considered by the author as non-diagnostic for both species, and was not included in his identification key.

A brief comment on the variability of the characters used by Holdaway (1933) is found in James' (1948) work, however, without mentioning which characteristics were variable. Our analyses were similar to those of Holdaway (1933) and, although they are not especially intuitive, the differences in the curvature of the veins at the wings edge are visible. For a better understanding of what we propose, we suggest the observation of the characteristic from a parallel line drawn between the point of the wingspan of the M1 vein when leaving the M1 + 2 until it reaches the border of the wing.

3.2.1.3. Abdomen

Sternite V of males: (0) "C" shape *versus* (1) "V" shape.
Remarks. Studies involving the final portion of the abdomen, including sternites and genitalia, are common, especially for the diagnoses of closely related species. Rognes and Paterson (2005), for example, in a detailed study to confirm that *C. putoria* and *C. chloropyga* are different species used the shape of sternites IV and V.

Sternite V is a more reliable character when we compare it with other sternites, because it is related to the perfect fit with the others structures from male terminalia. The shape may vary between *C. putoria* and *C. chloropyga*, especially regarding the width and length.

Our findings for *C. putoria* corroborate of Rognes and Paterson (2005), in which the opening from the sternite is grooved and deep, with a "V" shape. For *C. albiceps*, as for *C. rufifacies*, the opening from the sternite is wide and shallow, both presenting a "C" shape, however the sternite amplitude of *C. rufifacies* is bigger.

Despite requiring a small degree of preparation and dissection, we have added this character to the identification key because it contributes to a good diagnosis. The shapes of the sternites are described here for the first time for *C. albiceps* and *C. rufifacies*, and we emphasize that this character can only be used for males.

Cercus and surstylus: (0) well developed *versus* (1) weakly developed.
Remarks. Examining our specimens, we realized that these characteristics did not differ and were consistent with those described by Holdaway (1933) and Rognes and Paterson (2005), that produced detailed studies of male genital segments for *C. albiceps, C. putoria*, and *C. rufifacies*, among other *Chrysomya*.

In *C. rufifacies*, the attachment of the penis occurs by a continuity with the base of apodeme, more dilated in its insertion, while in *C. albiceps* there is a theca articulating the penis to this structure, which is far less robust. Furthermore, the cercus (that can be seen dorsally) are wider in *C. rufifacies* than in *C. albiceps*. In the lateral view, the cercus and surstylus of *C. albiceps* and *C. rufifacies* are long and well developed, whereas in *C. putoria*, they are short and weakly developed.

Even though the characteristics of the genitalia may not be easy to view due to the inconvenience of handling and preparation of the material, we emphasize that this is one of the characteristics which best allows unambiguous species identification.

Dorsal surface of tergite V: (0) with cleft *versus* (1) without cleft.
Remarks. Character proposed and included in identification key for the first time by Dear (1985) for *Chrysomya* species from the New World, afterwards widespread in other papers (Amat et al., 2008; Amat, 2009; Whitworth, 2010; Silva et al., 2012; Kosmann et al., 2013; Tantawi and Sinclair, 2013).

The surface of tergite V is dorsally fissured and is a very easy characteristic to see in *C. albiceps* and *C. rufifacies; C. putoria* does not have this cleft, so the tergite is almost continuous in its extension. Although it is not possible to use this characteristic to distinguish *C. albiceps* and *C. rufifacies*, we indicate its use to separate both these species from *C. putoria*.

3.2.2. Additional characters observed that did not reliably separate the species or were not included in the keys

3.2.2.1. Head

Palpus: (0) yellowish *versus* (1) yellowish, with dark distal region.
Remarks. So far, there are no reports on the use of this character as a diagnostic source. In our samples, we observed that *C. albiceps*, *C. putoria*, and *C. rufifacies* present a yellowish palpus, but only *C. albiceps* have a dark distal region. Although this character is useful for separating *C. albiceps* and *C. rufifacies*, we do not recommend its use due to the possibility of colour change over time in many preserved specimens.

3.2.2.2. Thorax

Shiny and microtrichose (pollinosity) from mesonotum when observed from a dorsal angle: (0) mostly shining, with little pollinosity *versus* (1) more whitish, with more pollinosity on the borders
Remarks. Whitworth (2010) and Tantawi and Sinclair (2013) proposed this character; however, the interpretation may vary due to the difficulty of visualization. Some factors can contribute to the unreliability of this character, including bad preservation of the insect, dirt deposited on the surface of the integument, or variation of the stereomicroscope light (some equipment may be equipped with a yellow or white light).

In analyzing our samples, we observed that *C. putoria* has little shine to its mesonotum and a discrete microtrichose (pollinosity) on the mesonotum borders, whereas *C. albiceps* and *C. rufifacies* are more shiny with less microtrichose.

3.2.2.3. Abdomen

Sternite IV of males: (0) as wide as high *versus* (1) more wide than high.
Remarks. We observed in our samples that, generally, sternite IV from *C. putoria* has the same width and height, with rounded corners. The sternites from *C. albiceps* are very similar to those of *C. putoria*. In *C. rufifacies*, sternite IV is wider than its high and the corners are not so round, nearly forming a right angle. This is the first time that this character has been observed for *C. albiceps* and *C. rufifacies*, but we did not use this character in the identification key because it may have greater variation than we observed.

Transverse bands of the abdomen: (0) abdominal bands narrow *versus* (1) abdominal bands approximately equal to one-third of the segment *versus* (2) abdominal bands considerably less than one-third of the segment, almost linear.
Remarks. Highly variable and therefore unreliable. *Chrysomya putoria* presents more blackish bands and more highlighted when

compared to *C. albiceps* and *C. rufifacies*, but these differences are impossible to measure in the way proposed by Holdaway (1933); since this character is also hard to distinguish.

3.3. Molecular analysis

A total of 74 *Chrysomya* specimens were analyzed in this study at the molecular level (Table A.1). Taxon sampling included *Chrysomya* individuals from Brazil presenting complete or partial proepimeral seta, which were previously identified as *C. rufifacies*. From their characteristics, we designated them as polymorphic *Chrysomya albiceps*. Other samples included *C. rufifacies* collected in different localities throughout Indonesia and from a laboratory lineage from the USA, and more individuals of *Chrysomya* species collected from the Midwest, Southeast, and North of Brazil.

3.3.1. Amplification and sequencing of molecular markers and genetic distances

A total of 560-bp and 667-bp were sequenced for COI and COII, respectively, for the majority of blowflies sampled. The absence of stop codons in open read frames and high Phred quality scores of the chromatogram peaks indicated that no contamination with nuclear pseudogenes had occurred (Zhang and Hewitt, 1996). For ITS2 and bicoid, a total of 305-bp and 482-bp, respectively, were recovered. The intragenomic variations for ITS2 and *bcd* cloned amplicons were very low, and, thus, it is unlikely that they have disturbed the subsequent analyses. The final concatenated alignment dataset of all genetic regions used was 2,156 characters length, ranging of 441 (ITS2) up to 667 (COII) aligned characters.

The interspecific sequence divergence was calculated between the *Chrysomya* species investigated and 31 specimens of polymorphic *C. albiceps*. The mtDNA markers showed no variation between "standard" *C. albiceps* and the polymorphic *C. albiceps*, while a significant average sequence divergence was observed between *C. rufifacies* and the polymorphic *C. albiceps*, with 2.4% and 3.1% for COI and COII, respectively (Table A.2). The highest average variation was observed when these species were compared to *C. putoria* (4.1–6.5%). According to Hebert et al. (2003), among species of the same taxonomic group an interspecific sequence divergence greater than 2% for COI marker is expected, as well as an intraspecific sequence divergence that does not exceed this percentage, generally less than 1% (Avise, 2000). Although this seems not to be a general rule for some dipteran groups (Meier et al., 2006; Whitworth et al., 2007; Meier and Zhang, 2009), the genetic variation observed between *C. albiceps* and the polymorphic *C. albiceps* is within an acceptable range with regard a intraspecific variation. The high sequence divergence found in COII, when compared with COI, may explain the preferential usage of this marker as a complement to COI analysis (Wells and Sperling, 2001; Harvey et al., 2003b; Chen et al., 2004), indeed the highest genetic variations, including the nuclear markers, was observed for COII.

ITS2, which has been evaluated for species identification (Nelson et al., 2007; Song et al., 2008a), phylogenetic inferences (Song et al., 2008b; Marinho et al., 2011, 2012), and *bcd*, which was recently evaluated as a molecular identification marker (Park et al., 2013), showed a low sequence divergence when compared with mtDNA regions (Table A.2). These may be a consequence of the higher mutation rate of mtDNA than nuclear DNA (Harvey et al., 2003b). Nevertheless, no overlap was observed in the range of variations between *C. rufifacies* and *C. albiceps* against the polymorphic *C. albiceps* specimens (Table A.2). Indeed, these results suggest a conspecificity of polymorphic *C. albiceps* species and *C. albiceps*, and agree with the observations that extremely low sequence divergences between species may indicate a recent common origin (Funk and Omland, 2003; Tautz et al., 2003), as expected for *C. albiceps* and *C. rufifacies* (Wells and Sperling, 1999; Singh et al., 2011).

3.3.2. Clustering method, phylogenetic inference, and likelihood-map analysis

Five different clustering analyses were conducted using NJ distance and a phylogenetic inference (BI), both with single and concatenated genetic regions. In all inference methods, *Chrysomya* species were recovered as monophyletic groups (Fig. 5), with *C. albiceps* and *C. rufifacies* forming a well-supported monophyletic clade with a common original lineage, as expected (Harvey et al., 2008; Singh et al., 2011; Marinho et al., 2012). *C. albiceps* plus polymorphic *C. albiceps* were recovered in all phylogenetic reconstructions with high values of posterior probabilities (PP = 1.0) and bootstrap (BS > 90%). One of the polymorphic *Chrysomya* specimens, previously identified as *C. rufifacies*, was reclassified as belonging to the *C. putoria* species supported by both molecular and morphological analyses.

Besides the low genetic variation among nuclear markers compared with mtDNA ones, the ITS2 and *bcd* provide sufficient phylogenetic support for differentiating all *Chrysomya* species. Indeed, this result corroborates with those of Park et al. (2013) that this region of the developmental *bcd* may be a suitable molecular marker for forensic science and entomology. In addition, the multigene phylogenetic analyses were able to differentiate the *C. putoria* individuals collected in Manaus, Amazonas, from those collected in Campinas, SP, Brazil. The same result was observed for *C. rufifacies* individuals from Homestead, Florida and Indonesia (Fig. 5).

Once no differences in topologies were observed between NJ and BI, another analysis was included based on Maximum-Likelihood (ML) method. The Likelihood-map results (Fig. A.1) were congruent with NJ and BI results, indicating that *C. albiceps* and "polymorphic" *C. albiceps* species form a monophyletic clade.

3.4. Key to species of Chrysomya of the New World

1. Anterior spiracle (Fig. 2H) and lower calypter blackish (Fig. 4D); male, ommatidia enlarged on the upper half (Fig. 1H)
– ***C. megacephala*** (Fabricius, 1794)

1′. Anterior spiracle (Fig. 2A) and lower calypter whitish (Fig. 4A); male, ommatidia not enlarged (Fig. 1F)
– **2**

2. Lower half of the gena blackish and upper half pale brown (Fig. 1A); 5 to 7 proepisternal setae (Fig. 2C); proepimeral seta usually absent (Fig. 2B), but may be present in polymorphic specimens (Fig. 2A); more acute angle formed by the branch of vein M1 + 2 approaching the edge of wing (Fig. 4E)
– ***C. albiceps*** (Wiedemann, 1819)

2′. Gena totally blackish (Fig. 1B and E); 1 to 4 proepisternal setae (Fig. 2E and G); proepimeral seta always present (Fig. 2D and F); smoother angle formed by the branch of vein M1 + 2 approaching the edge of wing (Fig. 4F and G)
– **3**

3. Lower calypter with darkish setulae (Fig. 4B). Two to four proepisternal setae (Fig. 2E). Female: tergite V with a dorsal cleft (Fig. 3B); proclinate orbital setae absent. Male: outer vertical setae present (Fig. 1D); parafrontalia with long and broadly distributed setulae, similar in length to the frontal setae (Fig. 1C); cercus and surstylus with almost the same length of aedeagus (Fig. 3E); sternite V "C-shaped" (Fig. 3E). Inner face of the third antennal segment pale brown (Fig. 4I)
– ***C. rufifacies*** (Macquart, 1843)

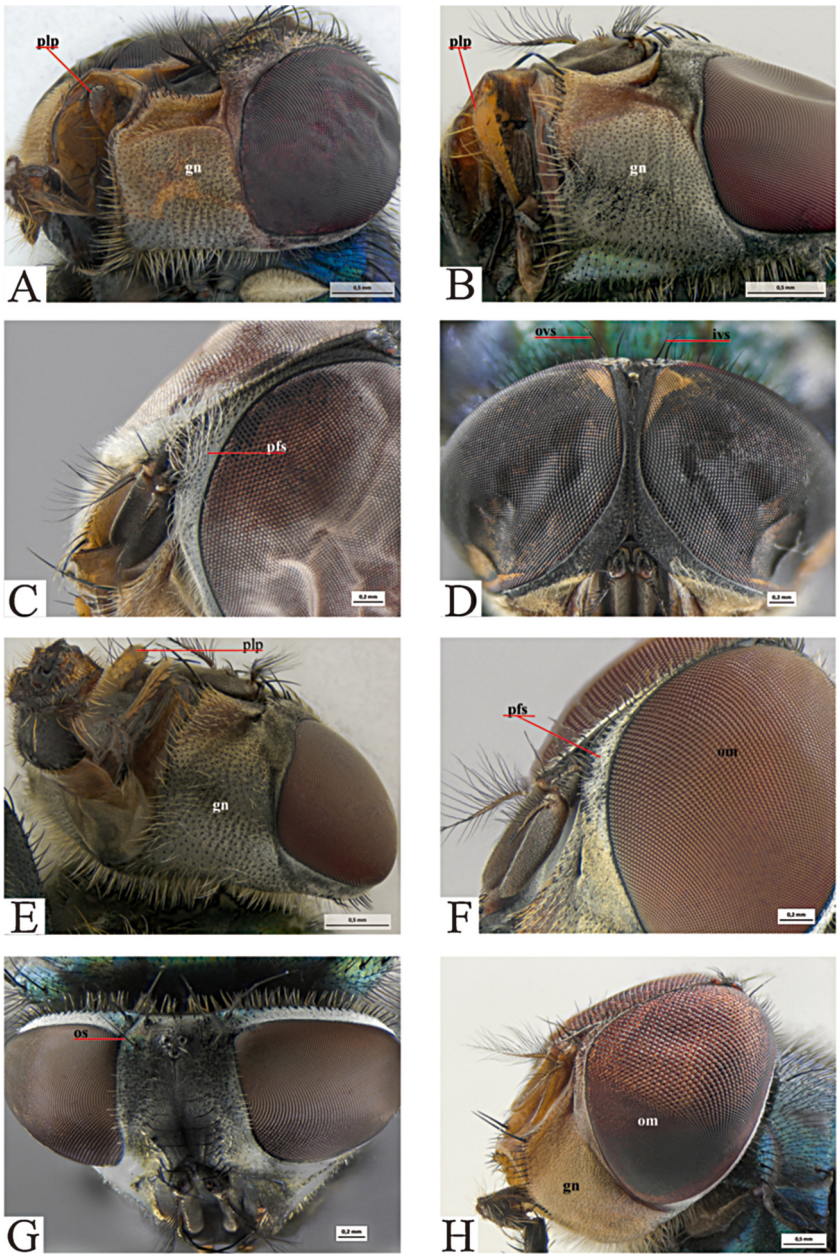

Fig. 1. Head. In detail, gena (gn), palpus (plp), parafrontal setula (pfs), inner (ivs) and outer (ovs) vertical seta, orbital seta (os) and ommatidia (om). In: (A) *C. albiceps*; (B)–(D) *C. rufifacies*, with long and broadly distributed parafrontal setulae; (E)–(G) *C. putoria*, with short and sparse parafrontal setulae and ommatidia not enlarged; (H) *C. megacephala*, with ommatidia enlarged.

3′. Lower calypter with whitish setulae (Fig. 4C). From 1 to 2 proepisternal setae (Fig. 2G). Female: tergite V without a dorsal cleft (Fig. 3C); one pair of proclinate orbital setae (Fig. 1G); Male: outer vertical setae absent; parafrontalia with short and sparse setulae, smaller in length than the frontal setae (Fig. 1F); cercus and surstylus smaller than the length of the aedeagus (Fig. 3F); sternite V "V-shaped" (Fig. 3I); Inner face of the third antennal segment dark brown (Fig. 4J)
– ***C. putoria*** (Wiedemann, 1830)

4. Discussion

4.1. Problems with identification and reports in the literature

We present a detailed study for the four species of *Chrysomya* introduced into the Americas aimed at refocusing the correct limits of geographic distribution of these representatives of the family Calliphoridae. With respect to *C. albiceps*, particularly those individuals characterized as polymorphic, after all morphological and molecular analyses carried out, the presence of the proepimeral seta was the most significant intraspecific variation detected, but not the only one. Previously, it had been believed and widely reported in the literature that the proepimeral seta was nonexistent in *C. albiceps* (Dear, 1985; Carvalho and de Ribeiro, 2000; Mello, 2003; Carvalho and Mello-Patiu, 2008; Silva et al., 2012; Kosmann et al., 2013). This has led to reports of the introduction of *C. rufifacies* into Brazil (Silva et al., 2012; Kosmann et al., 2013; Ribeiro et al., 2013). In part, this was due to the lack of a more thorough review of the literature, for previous records had alerted to the existence of phenotypic polymorphism involving *C. albiceps* (Holdaway, 1933; Guimarães et al., 1978; Guimarães and Papavero, 1999). Furthermore, there is a lack of consistent investigation about other characters that could ensure the correct identification of the species.

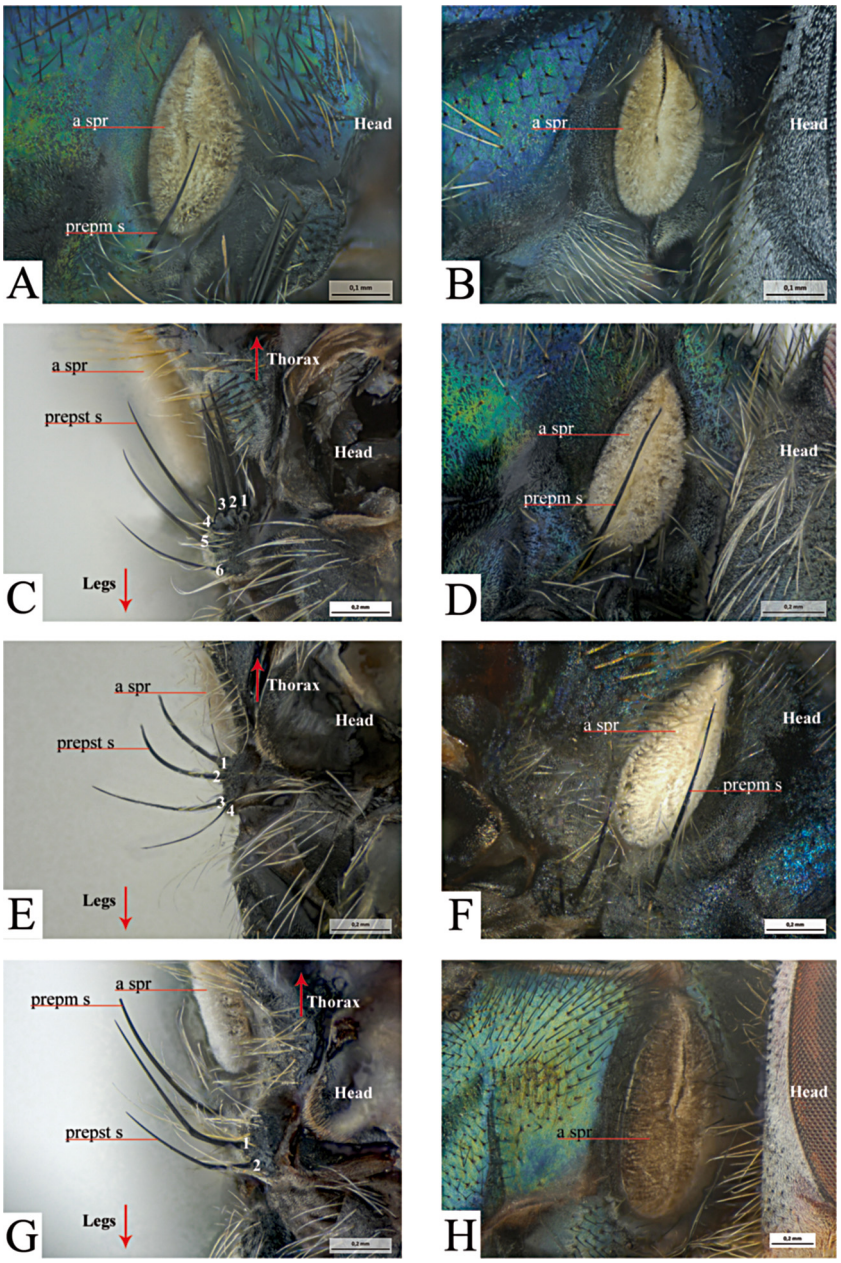

Fig. 2. Thorax. In detail, proepimeral seta (prepm s), anterior spiracle (a spr) and proepisternal seta (prepst s). In: (A) Polymorphic *C. albiceps*; (B) and (C) *C. albiceps*, without proepimeral seta and with 6 proepisternal setae, respectively; (D) and (E) *C. rufifacies*, with proepimeral seta and 4 proepisternal setae, respectively; (F) and (G) *C. putoria*, with proepimeral seta and 2 proepisternal setae; (H) *C. megacephala*, with a blackish spiracle.

Additionally, it is also important to note that the number of recovered and gathered polymorphic individuals examined in this study is low ($N = 34$), considering the sampling universe of blowflies that have been recently collected from carcasses in Brazil (on the average 20,000 specimens—see Souza and Linhares, 1997; Rosa et al., 2009; Biavati et al., 2010; Ururahy-Rodrigues et al., 2013). And, at least, part of them could have been identified as *C. putoria*, due to the available identification keys for Brazilian species of *Chrysomya*, based on the presence/absence of proepimeral seta.

More attention should have been given to the survey of a larger number of diagnostic characters to separate and correctly identify *Chrysomya* species that are present in the Americas, once the entrance of *C. rufifacies* was reported in South America, in Argentina by Mariluis and Schnack (1989), in Colombia by Barreto et al. (2002) and in Ecuador by Tantawi and Sinclair (2013). This fact highlights the negligence and lack of incentive for studies involving the field of taxonomy.

4.2. Proposals to help identify the species at issue

In the almost one hundred years in which taxonomists have discussed the validity of Calliphoridae species, whether they are closely related or not, and the elaboration of identification keys that could contribute to minimizing the taxonomic impediment, seven authors (Bezzi, 1927; Holdaway, 1933; James, 1948; Zumpt, 1956; Guimarães et al., 1978; Dear, 1985; Whitworth, 2010) have contributed to the presentation and proposition of new characters that could help in the process of identification of those species. At present, we have discussed, character by character, the proposals of these authors, before arranging them to present our proposal for

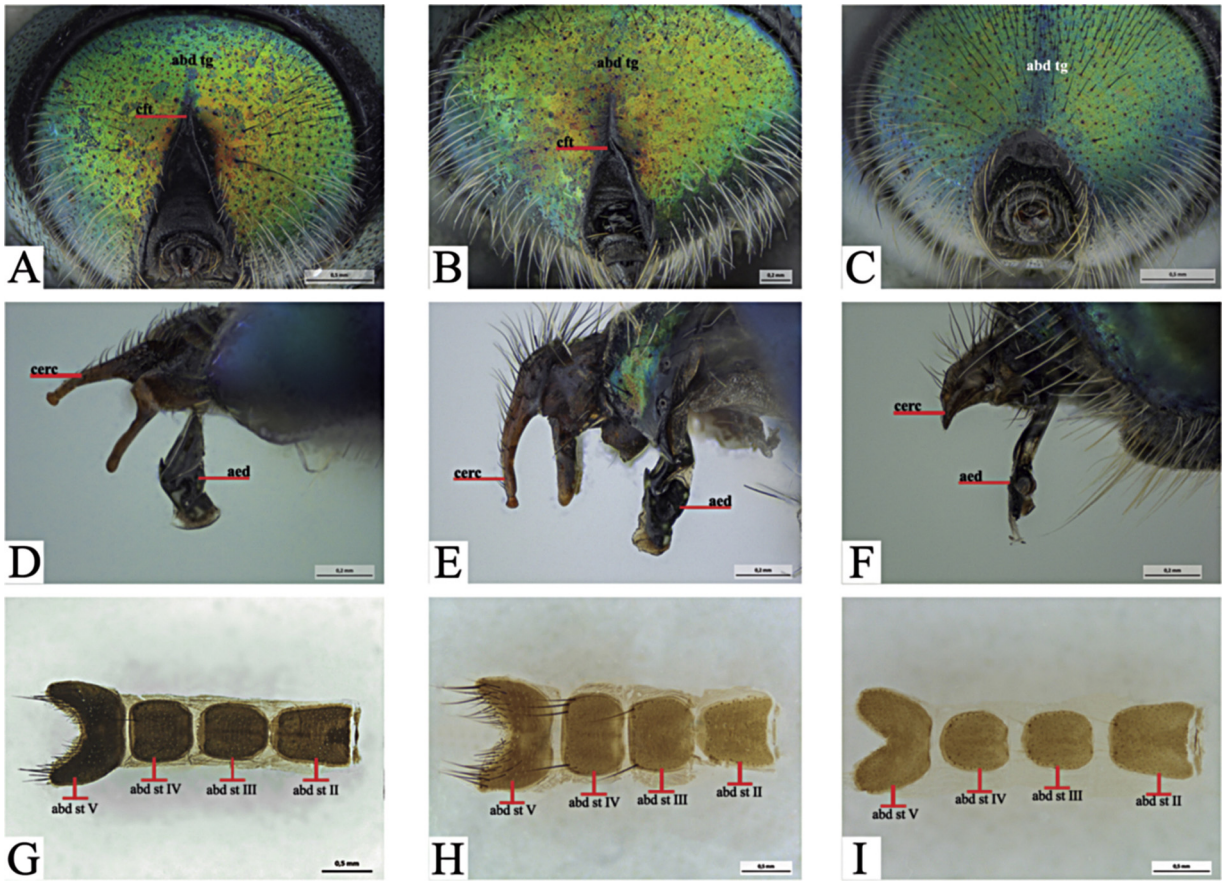

Fig. 3. Posterior region of the abdomen. In detail, the female abdominal tergite V (abd tg), cercus (cerc), aedeagus (aed) and male abdominal sternite (abd st). In: (A) and (B) tergite V for female with a dorsal cleft (cft) in *C. albiceps* and *C. rufifacies*, respectively; (C) tergite V for female without a dorsal cleft in *C putoria*. (D)–(F) male terminalia of *C. albiceps, C. rufifacies* and *C. putoria*, respectively. (G) and (H) *C. albiceps* and *C. rufifacies*, with a C-shaped sternite V, respectively; (I) *C. putoria*, with a V-shaped sternite V.

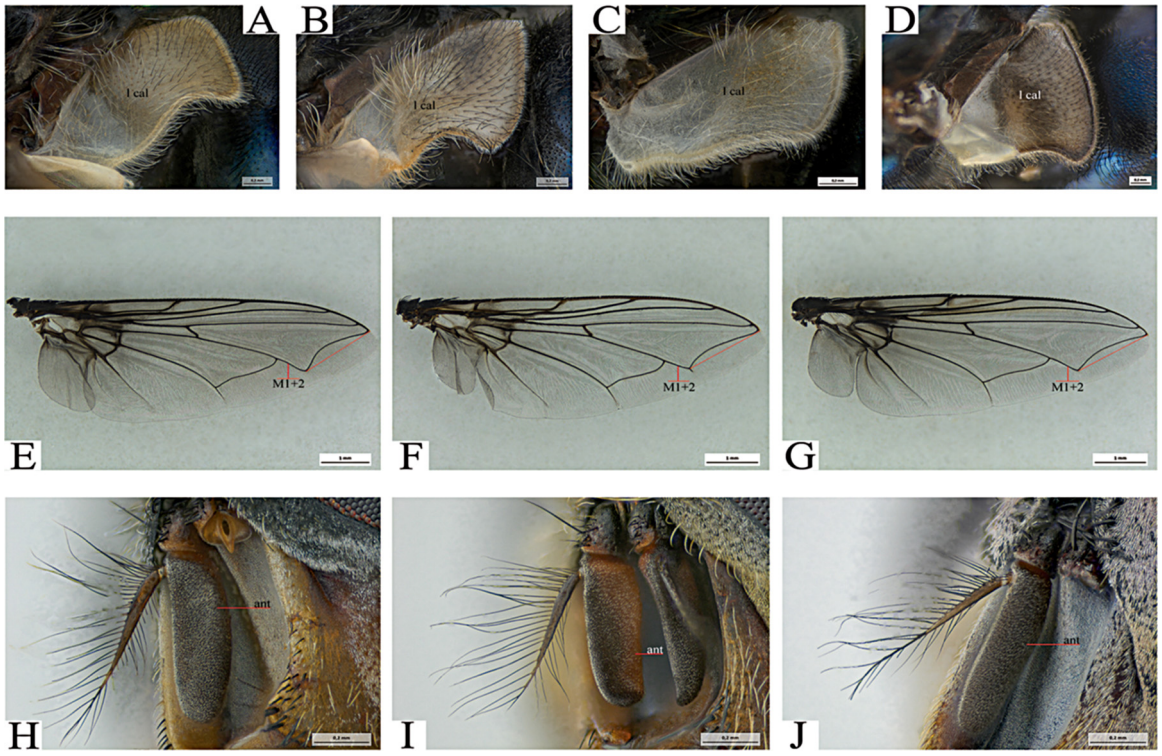

Fig. 4. Thorax and head. In detail, lower calypter with colour of setulae (l cal s); wing, with curvature of vein M1 + 2, and colour of antenna (ant). In: (A), (E) and (H) *C. albiceps*, respectively; (B), (F) and (I) *C. rufifacies*, respectively; (C), (G) and (J) *C. putoria*, respectively; For *C. megacephala*, detail only for lower calypter (D). For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of the morphological characteristics to separate the species of *Chrysomya* of the New World. Identical symbols (* or ■) in the same row indicate identical states of characters.

	Characters	Species			
		C. albiceps	*C. rufifacies*	*C. putoria*	*C. megacephala*
1.	Size of cercus and surstylus	Long and well developed *	Long and well developed *	Short and weakly developed ■	Short and weakly developed ■
2.	Shape of tergite V, for females	With a dorsal cleft *	With a dorsal cleft *	Without a dorsal cleft ■	Without a dorsal cleft ■
3.	Third antennal segment	Dark brown *	Inner face pale brown	Dark brown *	Yellowish/orange
4.	Number of propleural setae	5 to 7 setae	2 to 4 setae	1 to 2 setae	Only 1 seta
5.	Colour of the gena	Lower half of the gena blackish, and upper half pale	Totally blackish *	Totally blackish *	Yellowish / orange
6.	Curvature of the wing vein M1 + 2	Forming an acute angle *	Forming a smoother angle ■	Forming a smoother angle ■	Forming an acute angle *
7.	Shape of sternite V, for males	"C-shaped" *	"C-shaped" *	"V-shaped" ■	"V-shaped" ■

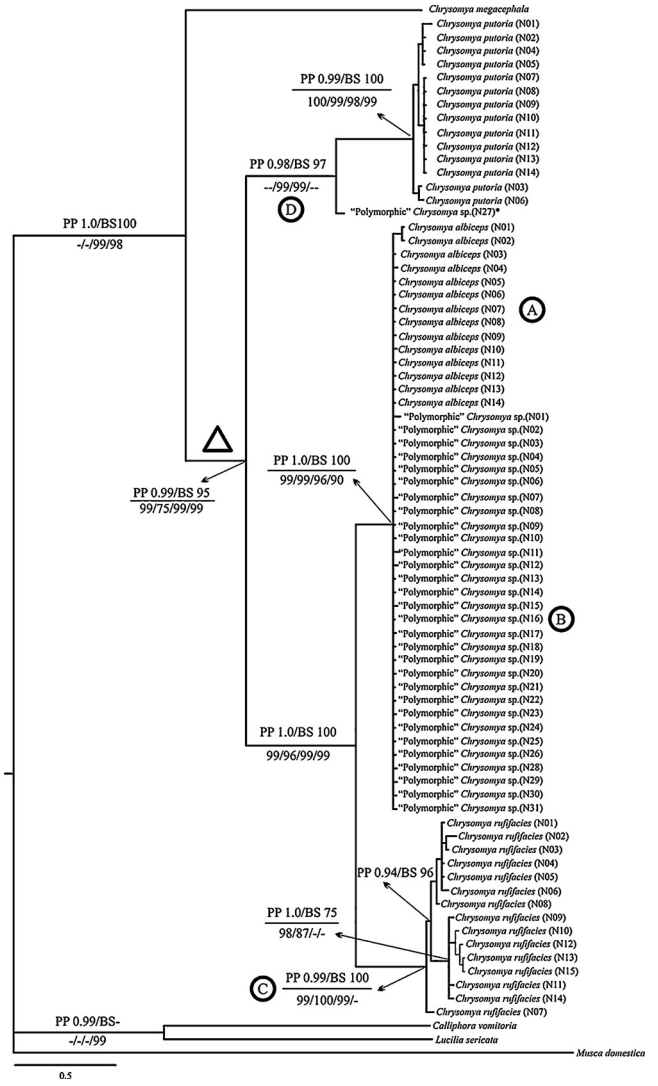

Fig. 5. Bayesian tree inferred using concatenated dataset of mitochondrial and autosomal molecular markers from *Chrysomya* blowflies species. *C. vomitoria*, *L. sericata*, and *M. domestica* were set as outgroup. Number above branches refers to node supports of concatenated analyses: (PP) Bayesian inference posterior probability, where each one of the genetic regions used was treated as different partitions (see Section 2 for more details), and (BS) Neighbor-Joining bootstrap proportions among 5000 replicates. Numbers below branches refers to bootstrap values among 5000 replicates for COI, COII, ITS2 and *bcd* datasets correspondently. Values below 0.7 PP and/or 70% BS support are not recorded on the tree. (*) indicates the unique polymorphic *Chrysomya* sp. specimen previously identified as *C. rufifacies* that belong to *C. putoria* species. Δ indicates the subtree and A–D the monophyletic defined groups used for likelihood-map analysis.

a new identification key that includes new characters and others which are reinterpreted.

In summary, we pointed out the morphological characteristics to reliably separate the species of *Chrysomya* of the New World in the Table 1. To avoid misidentification, we recommend that the characters are always observed in a combined manner, not in isolation.

4.3. Molecular aspects

Comparing all genetic regions and methods utilized, the results presented agree with the assumption that a multigene approach is stronger than single gene analyses (Stevens et al., 2002; Wallman et al., 2005; McDonagh and Stevens, 2011; Zaidi et al., 2011; Nelson et al., 2012) leading to more robust and unambiguous conclusions. The distance method, the phylogenetic topologies, and the likelihood-map results are in agreement with previous observations (Zumpt, 1965; Tantawi and Greenberg, 1993) that the isolated proepimeral seta, in fact, is a weak morphological diagnostic character to differentiate *C. albiceps* from *C. rufifacies*, since a few *C. albiceps* individuals may present it in a polymorphic status.

5. Conclusions

Based on the findings and clarification of morphological variations of *C. albiceps* and reviews presented, in conjunction with the morphological characteristics of the molecular markers used, following the examination of all specimens we collected and also those deposited in accredited scientific collections, it is possible to confirm that *C. rufifacies* is not in the Brazilian territory until the present time.

In this study, we provide a key for identification of New World species of *Chrysomya*. We also point out that the proepimeral seta is not over emphasized as the main characteristic of the dichotomous key to separate *C. albiceps* and *C. rufifacies* or *C. putoria*. Other characters have been proposed to support accurate diagnosis and correct identification of species.

We expect that this work can help minimizing the risk of misidentification of *Chrysomya* species, as well as stress the need of a more careful use of identification characters, particularly in those cases involving new records of species for a given location.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2014.09.011.

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