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Crane flies (Diptera, Tipuloidea) from southern Neotropical salt marshes: survey with DNA barcoding

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ABSTRACT. Crane flies are the most diverse group within Diptera, but they are rarely studied in coastal ecosystems. Considering the scarcity of information on the biology and ecology of this group in the Neotropics, and the sparse literature available for taxonomic identification, we developed a descriptive checklist that incorporates morphology and DNA barcoding. We also created a generic identification key for crane flies of southern Brazilian salt marshes. We sampled crane flies continuously at three areas along the Patos Lagoon salt marshes over one year. A total of 14 genera/subgenera, 6 species, and 12 morphotypes belonging to Limoniidae and Tipulidae were identified. Distribution ranges of *Symplecta cana* (Walker, 1848) and two *Ormosia* Rondani, 1856 species were expanded. mtDNA COI sequences were compared to the BOLD and NCBI databases, but were matched only at the family level. Therefore, we provided sequences to both platforms, updated to the genus level. We found low (0.00-0.03) intraspecific and high (0.11-0.25) interspecific molecular differences indicating that the mtDNA COI region is adequate for distinguishing species within the Tipuloidea. The *Dicranomyia* Stephens, 1829 species complex showed low genetic difference, indicating that they could be one species with high morphological plasticity. This study will serve as a basis for future research on insects of Neotropical salt marshes.

KEYWORDS. Barcoding gap, coastal insects, Cytochrome Oxidase I, distribution range.

The superfamily Tipuloidea is the richest within the order Diptera. It is subdivided into Cylindrotomidae, Limoniidae, Pediciidae, and Tipulidae *sensu stricto*. Approximately 3,500 crane fly species are recognized throughout the Neotropical region, of which around 99.6% are in the families Limoniidae and Tipulidae (De Jong *et al.*, 2008; Oosterbroek, 2018). Crane flies are non-hematophagous flies that are extremely important in the trophic webs they participate in, generally exhibiting a detritivorous diet during their larval phase. Adults generally do not feed, but some will take nectar, and perhaps pollen, and water. The large corporal mass of crane flies, along with their occupation of transitional ecosystems (*e.g.*, salt marshes), make these insects a potential and important trophic link between aquatic and terrestrial environments (*e.g.*, Baxter *et al.*, 2005). This group is widely distributed over almost all environments, and includes species that occupy wetlands such as salt marshes (Rogers, 1932; Autio *et al.*, 2013).

Salt marshes are typical coastal habitats of mid- and high-latitude areas (Stevens *et al.*, 2006), including the southern Neotropical region. They are characterized as transition areas between estuaries and land, display few but numerous plant species, and are dominated by a fauna and flora that tolerate rapid variations in salinity (Costa & Marangoni, 2010). At Patos Lagoon Estuary, southern Brazil, estuarine flow and precipitation levels tend to increase salinities at these salt marshes during the summer, and decrease them in the winter (D'Incao *et al.*, 1992). The margins of this estuarine environment are dominated by halophyte plant species such as *Spartina alterniflora* and *S. densiflora* (Poaceae), as well as *Myrsine parvifolia* (Primulaceae) shrubs (Costa *et al.*, 1997).

Other than their original descriptions, few studies have reported and updated the occurrence of crane fly species across the Neotropical region (see Ribeiro *et al.*, 2007; Ribeiro & Santos, 2016). Additionally, research at salt marshes is commonly focused on species such as fish (*e.g.* Contente *et al.*, 2010; Campos *et al.*, 2015), polychaetes, crustaceans (*e.g.* Mackenzie *et al.*, 2015) and birds (*e.g.* Britto & Bugoni, 2015), but there are rare studies on insect groups conducted at these environments (see Giberson *et al.*, 2001; Mackenzie, 2005; Dummel *et al.*, 2011; Bolico *et al.*, 2012; Gantes *et al.*, 2013; Rodrigues *et al.*, 2017). In order to support studies on the classification, biogeography, and ecology of these groups, it is necessary to identify the insect species that inhabit these environments, preferably with high taxonomic resolution, as suggested by Lenat & Resh (2001). Morphological and DNA barcoding methods are frequently combined for taxonomic determination, the latter based mainly on mitochondrial DNA Cytochrome Oxidase Subunit I (COI) sequences for specific

identification of animals (Hebert *et al.*, 2004). Therefore, we conducted morphological identification, provided species-specific COI barcode sequences, and developed an identification key for the Tipuloidea genera that occur at salt marshes of the southern Neotropical Region.

MATERIAL AND METHODS

Study area and sampling. The salt marshes of the Patos Lagoon Estuary are one of the largest in Brazil, covering over 70 km² (Costa *et al.*, 1997). We sampled three salt marsh areas: upper estuary (31°53'33"S; 52°14'33"W), middle estuary (32°02'01"S; 52°10'45"W), and lower estuary (32°10'65"S; 52°08'52"W) (Figure 1). Two Malaise traps with ethanol-filled collection cups were set up at each sampling area, close to the dominant vegetation, for a total of six traps. Malaise traps are flight intercept traps known for efficacy in capturing winged insects, including crane flies. Sampling was conducted continuously over twelve months, from August 2015 to July 2016. Collection cups were changed every 15 days, and collected insects were removed for identification and preservation in 70% ethanol. Specimens were deposited at the *Fundação Zoobotânica do Rio Grande do Sul* - FZB/RS. Sampling was conducted under the Brazilian system for biological sampling (SISBIO) license number 50253-1.

Morphological identification. Genus identification was done using Alexander & Byers (1981) and Gelhaus (2009) based on wing venation and body morphology. When possible, species identification was done using Alexander (1912, 1913, 1962), Andrew (2000), and Starý & Brodo (2009) based on male genitalia. The morphological terminology adopted here follows McAlpine (1981).

Species distribution. Large scale species distributions are from the *Catalogue of the craneflies of the World* (Oosterbroek, 2018). However, this catalogue does not differentiate between different regions within Brazil, which is important for such a large country with pronounced regional variations.

DNA barcoding. Genomic DNA of approximately three specimens of each morphologically distinct species was extracted through phenol:chlorophorm or salt extraction protocols adapted from Sambrook *et al.* (1989) and Aljanabi & Martinez (1997), after initial cryogenic grinding with liquid nitrogen. Polymerase Chain Reactions (PCR) were conducted in a Veriti thermocycler to amplify approximately 690 bp of the mitochondrial DNA Cytochrome Oxidase I (COI) gene, using universal primers LCO1490 (5'-GGGTCAACAAAT-CATAAAGATATTGG-3') and HCO2198 (5'-TAAACTT-CAGGGTGACCAAAAAATCA-3') [Folmer *et al.* (1994)]. The reaction conditions were adapted from Pilipenko *et al.* (2012): 4.0 ng DNA; 2.5 U Taq DNA polymerase; 0.4 Mm dNTP mix; 3 Mm MgCl2; and 10% buffer, in a final volume of 25 µl. Cycling conditions were 1 min at 94 ºC; 35 cycles of 1 min at 94 °C, 1 min at 51 °C, and 2 min at 72 °C; followed by a final extension of 5 min at 72 °C. Amplified products were purified with Polyethylene Glycol (PEG) 8000 15% (Hartley & Bowen, 1996) and sequenced in both directions at Macrogen (http://dna.macrogen.com/eng/). Sequences were visually checked for errors, edited and aligned using BioEdit 7.2.5 (Hall, 1999), and deposited in two online public databases (The Barcode of Life Data System – BOLD – http://www.barcodinglife.org/ and National Center for Biotechnology Information – NCBI – http://www.ncbi.nlm.nih.gov/Genbank/) with corresponding genus and/or species names. Intra and inter-specific distances were calculated with pairwise genetic distance K2P in MEGA 7, and a histogram created using *BarcodingR* package available inhttps://github.com/zhangab2008/BarcodingR.

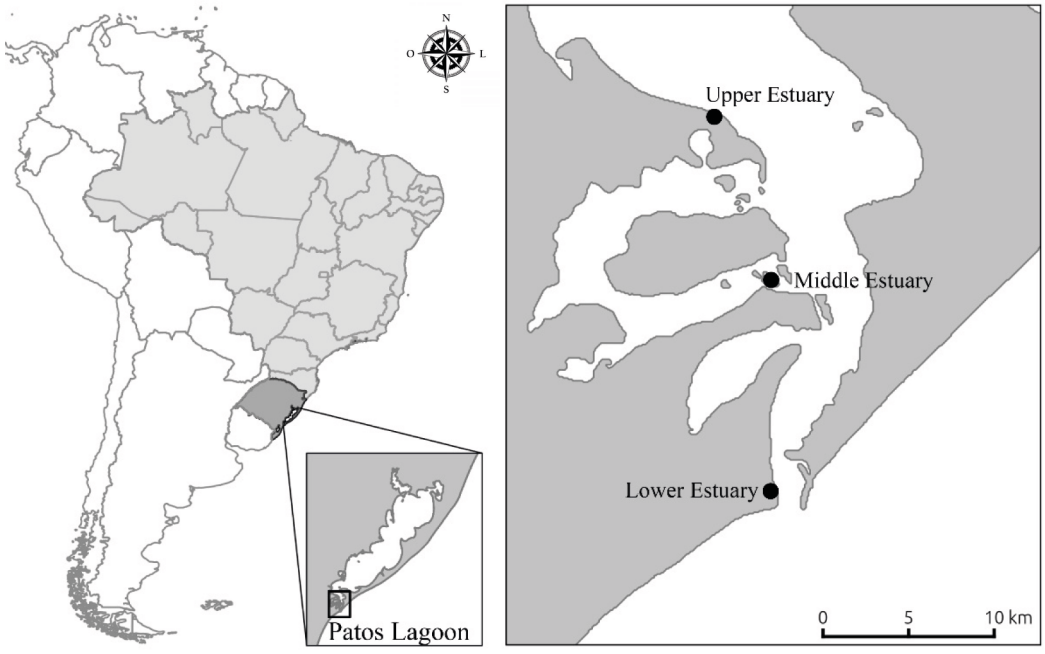


Fig. 1. Sampling areas at salt marshes of the Patos Lagoon Estuary, Rio Grande do Sul, southern Brazil.

RESULTS

We collected a total of 5,248 specimens distributed over 18 crane fly species/morphospecies at the salt marshes of Patos Lagoon Estuary. Three species belonged to the family Tipulidae and the other 15 to Limoniidae. This included members of a *Dicranomyia* species complex. We also found one taxon that we could not identify to genus. *Symplecta pilipes* (Fabricius, 1787) and *Rhipidia domestica amazonensis* Osten Sacken, 1860 represented 68% of all crane flies collected. A total of 43 sequences, corresponding to the 18 species, were compared with those in BOLD. Thirty of these presented ≥ 97% similarity compared to the existent sequences in the database, but none had specific identification. The majority of these matched sequences are from Argentina, but matches included sequences of *Nephrotoma* sp. from Ecuador and *Geranomyia* from the U.S.A., Dominican Republic, and Haiti. All haplotypes of the *Dicranomyia* complex (~91.43%), *Gonomyia* (*Neolipophleps*) sp. (~91.37%), *Polymera inornata* Alexander, 1913 (~88.53%), *P. obscura* Macquart, 1838 (88.11%), and *Symplecta cana* (95.05%) showed the lowest similarities with BOLD sequences, and were therefore considered new. We observed overall high interspecific COI differences among Tipuloidea species (between 0.11 and 0.25) and low intraspecific differences (between 0.00 and 0.03) (Figure 2). *Gonomyia* (*Neolipophleps*) sp. was the exception, with intraspecific variation ranging from 0.03 to 0.12. A checklist of taxa listing their authors, references, and FZB/NCBI deposit numbers is shown in Table 1. It was hard, and in some cases impossible, to identify at species-level because C. P. Alexander, whom mostly described them, used mainly coloration or other non-usual taxonomic characters. *Geranomyia*, *Ormosia* and the unidentified genus were not included. Three listed species – *Ozodicera* sp., and the two morphotypes of *Toxorhina* – did not have enough specimens for molecular analysis. An identification key (modified after Gelhaus, 2009) and list of genera/species description is presented below.

Key to Tipuloidea of Patos Lagoon Estuary, Rio Grande do Sul, Brazil

1. Well-developed rostrum, longer than the remainder of the head, usually with anteriorly projecting and sharply pointed nasus (Fig. 3)…….....[Tipulidae] 2
1’. Nasus absent. Rostrum short (Fig. 4), but occasionally lengthened as in *Geranomyia* and *Toxorhina* ……..……..Limoniidae] 4
2. Flagellomeres 5-10 each with branches (Fig. 5) ……
…….....*Ozodicera*
2’. Antennae without branched flagellomeres ……..3
3. Vein Sc ending nearly opposite of origin of Rs; Rs short, oblique; cell dm four-sided with rectangular aspect; bm-cu present (Fig. 6) …….....*Nephrotoma*
3’. Vein Sc longer, ending after the origin of Rs; cell dm five-sided, all sides almost equilaterals; bm-cu absent (Fig. 7) ……
…….....……*Zelandotipula*
4. Head with long and slender rostrum and mouthparts, greatly exceeding length of head, usually half of body length or more (Figs 8, 9) …….....……...5
4’. Head with non-elongated rostrum and mouthparts; maximum length little more than length of head (Fig. 10) …….....……..6
5. Mouthparts fused (Fig. 8).....……*Toxorhina*
5’. Mouthparts not fused (Fig. 9) ……....……..*Geranomyia*
6. Rostrum as long as or longer than remainder of head (Fig. 10) ….....…*Teucholabis*
6’. Rostrum inconspicuous or absent …….....……..7
7. Antennae of males as long as or larger than the entire body …….....….*Polymera*
7’. Antennae of males shorter than the entire body .....……8
8. Rs absent (Figs 11, 12) …….....…….……9
8’. Rs present (Fig. 13) …….....……..10
9. Cell dm absent (Fig. 11) ……...…*Gonomyia* (*Neolipophleps*)
9’. Cell dm present; apex of R_{4+5} very close to M_{1+2} (Fig. 12) …….....……*Gonomyia* (*Paralipophleps*)
10. Cell dm absent (Fig. 11) …….....……..11
10’. Cell dm present (Fig. 12)……..……...12
11. R_4 and R_5 beginning after of bt CuA_1 (Fig. 13) ……
….....……*Molophilus*
11’. R_4 and R_5 beginning nearly opposite origin of bt CuA_1 (Fig. 14) ….....….*Ormosia*
12. Antennae with 12 flagellomeres …….....……..13
12’. Antennae with 13-14 flagellomeres …..……...14
13. Flagellomeres unipectinate or subpectinate (Fig. 15) ……
…….....*Rhipidia*
13’. Flagellomeres of males and females oval to elongated shape (Fig. 16) ....…….*Dicranomyia*
14. A_2 “S” shaped distally; cell r_3 with crossvein (Fig. 17) ……
…….....……..*Symplecta* (*Symplecta*)
14’. A_2 straight (Fig. 18) .....*Symplecta* (*Trimicra*)

LIMONIIDAE Speiser, 1909

Dicranomyia Stephens, 1829

Diagnosis. Morphology of antennae usually simple, antennae with elongated flagellomeres (Fig. 16). Wings, if well developed, with relatively short Sc vein, usually ending approximate to the base of Rs or before mid-length of Rs, never reaching the fork of Rs.

Geranomyia Haliday, 1833

Diagnosis. Its members are characterized by both having a supernumerary crossvein at about midlength of wing cell Sc and the elongate mouthparts of the adult fly (Fig. 9).

Gonomyia (*Neolipophleps*) Alexander, 1947

Diagnosis. Two branches of Rs strongly divergent, at least at wing margin. Cell dm absent. Wing hyaline.

Gonomyia (*Paralipophleps*) Alexander, 1947

Diagnosis. Two branches of Rs strongly divergent, at least at wing margin. Cell dm present. Wing with conspicuous dark-brown pterostigmal spot.

Tab. I. List of genera/species, deposit numbers, sequence nomenclature, and references of crane flies found in salt marshes of the Patos Lagoon Estuary, Rio Grande do Sul, Brazil. *, According to Chakrabarty *et al.* (2013); **, First record in Brazil.

TAXA	FZB deposit number	NCBI deposit number	GenSeq nomenclature*	References
LIMONIIDAE				
Dicranomyia sp. 1 "species complex"	69853	MF176169; MF176170; MF176171	genseq-4	-
Dicranomyia sp. 2	69856	MF176172; MF176173; MF176174	genseq-4	-
Geranomyia sp. 1	69842	MF176175; MF176176	genseq-4	-
Geranomyia sp. 2	-	MF176177; MF176178; MF176179	genseq-4	-
Gonomyia (*Neolipophleps*) sp.	69854	MF176183; MF176184; MF176185	genseq-4	-
Gonomyia (*Paralipophleps*) sp.	69852	MF176180; MF176181; MF176182	genseq-4	-
Molophilus sp.	-	-	no classification	-
Polymera (*Polymera*) *obscura* Macquart, 1838	69844	MF176197	genseq-4	Alexander,1913
Polymera (*Polymera*) *inornata* Alexander, 1913**	69845	MF176194; MF176195; MF176196	genseq-4	Alexander, 1913
Rhipidia domestica amazonensis Osten Sacken, 1860	69843	MF176198; MF176199; MF176200	genseq-4	Alexander, 1912
Symplecta (*Symplecta*) *cana* (Walker, 1848)**	69857	MF176201	genseq-4	Stáry & Brodo, 2009
Symplecta (*Trimicra*) *pilipes pilipes* (Fabricius, 1787)	69849	MF176202; MF176203; MF176204	genseq-4	Andrew, 2000
Teucholabis (*Teucholabis*) sp.	69848	MF176205; MF176206; MF176207	genseq-4	-
Toxorhina (*Toxorhina*) sp. 1	69855	-	no classification	-
Toxorhina (*Toxorhina*) sp. 2	69846	-	no classification	-
TIPULIDAE				
Nephrotoma sp.	69847	MF176186; MF176187; MF176188	genseq-4	-
Ozodicera (*Ozodicera*) sp.	69850	-	no classification	-
Zelandotipula neurotrichia (Alexander, 1962)**	69851	MF176210; MF176211	genseq-4	Alexander, 1962

Molophilus Curtis, 1833

Diagnosis. Rs forking into R_{2+3} and R_{4+5} while in the other Limoniidae species Rs forks into R_{2+3+4} and R_5.

Polymera Wiedemann, 1821

Diagnosis. Males with extremely elongated antennae, at least as long as body, and usually clothed with long, delicate, outstretched hairs. Antennal segments elongate-cylindrical or bi-nodose.

Polymera obscura Macquart, 1838

Diagnosis. Antennal segments bi-nodose, darker on the nodes, lighter at the constriction. Wings gray with indistinct rounded clouds at the origin of Rs.

Previous geographical distribution: Argentina, Bolivia, Brazil, Guyana, Mexico, Panama, Peru.

Polymera inornata Alexander, 1913

Diagnosis. Antennal segments bi-nodose, lighter at the base and apex of each segment, but not producing an annulated effect as in *P. obscura*. Differs from other species in the extreme recession of the cross-vein *r* (*i.e.*, *r* equidistant between tip of Sc2 and tip of R_1).

Previous geographical distribution: Guyana.

Rhipidia Meigen, 1818

Diagnosis. The most important and conspicuous diagnostic feature of the genus is that the male flagellomeres

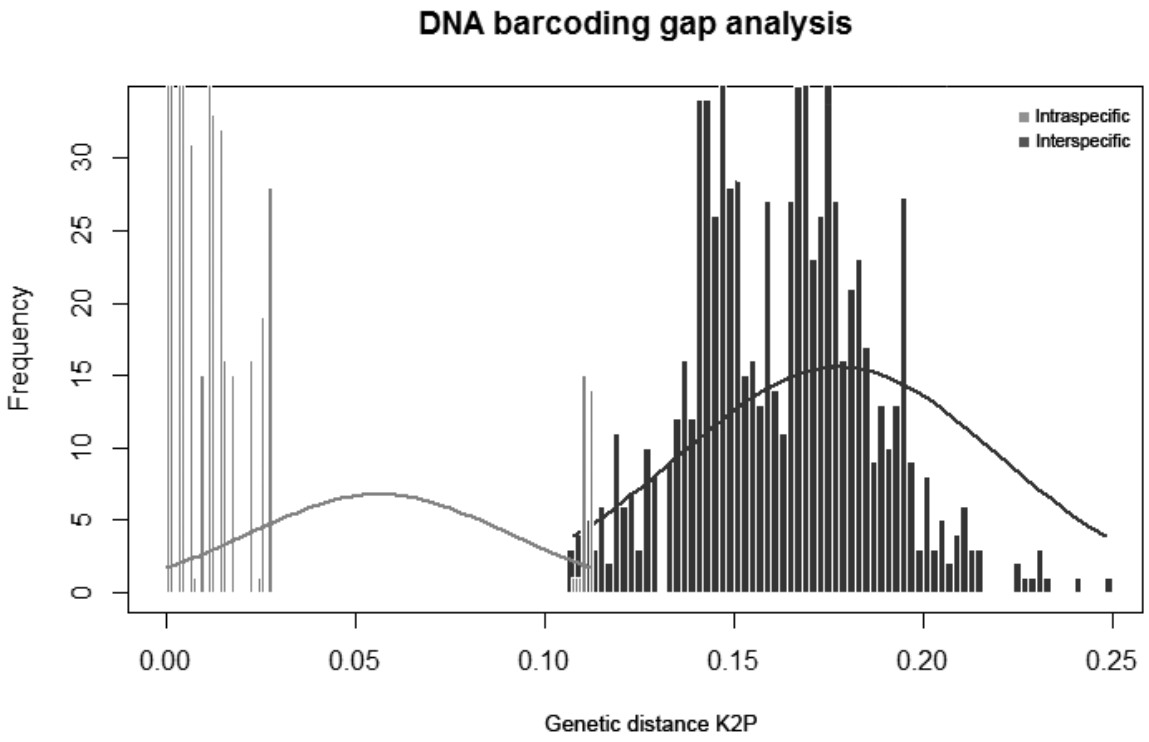


Fig. 2. DNA barcoding gap analysis, with frequency of intra and interspecific distances in COI sequences among Tipuloidea species.

are bipectinate, unipectinate, or subpectinate (Fig. 15).

***Rhipidia domestica amazonensis* Osten Sacken, 1860**

Diagnosis. Mesotonal praescutum with dark longitudinal lines with wing markings large, scanty, confined to the neighborhood of veins. Wings tinged with brown in *R. domestica amazonensis* compared to the hyaline wings of *R. domestica domestica*.

Previous geographical distribution: East Brazil

***Symplecta* (*Symplecta*) Meigen, 1830**

Diagnosis. Wings usually with spots and seams on cross-veins, base of Rs, tips of Sc and R_1, and middle and tip pf A_2. Supernumerary cross-vein in cell R_3. A_2 more or less sinuous (Fig. 17).

***Symplecta* (*Symplecta*) *cana* (Walker, 1848)**

Diagnosis. A_2 "S" shaped distally. The ventral tip of the gonocoxite extended, placing the gonostyli in a subapical position.

Previous geographical distribution. Canada, U.S.A., Guatemala, Mexico.

***Symplecta* (*Trimicra*)**

Diagnosis. Subgenera *Trimicra* is distinguished by the antennae, in which the apical three segments are abruptly slenderer than the preceding ones.

***Symplecta* (*Trimicra*) *pilipes* (Fabricius, 1787)**

Diagnosis. On male, vein Rs with dense row of long hairs. Mainly larger size, unpatterned wings and hairy legs distinguish this species from others in *Trimicra*.

Previous geographical distribution. The only cosmopolitan species crane fly (Andrew, 2000).

TIPULIDAE Latreille, 1802

***Zelandotipula* Alexander, 1922**

Diagnosis. Wing with cell r_3 constricted at midlength. Wing often with spot or cloud over origin of Rs, and at base of middle of cell bm.

***Zelandotipula neurotrichia* (Alexander, 1962)**

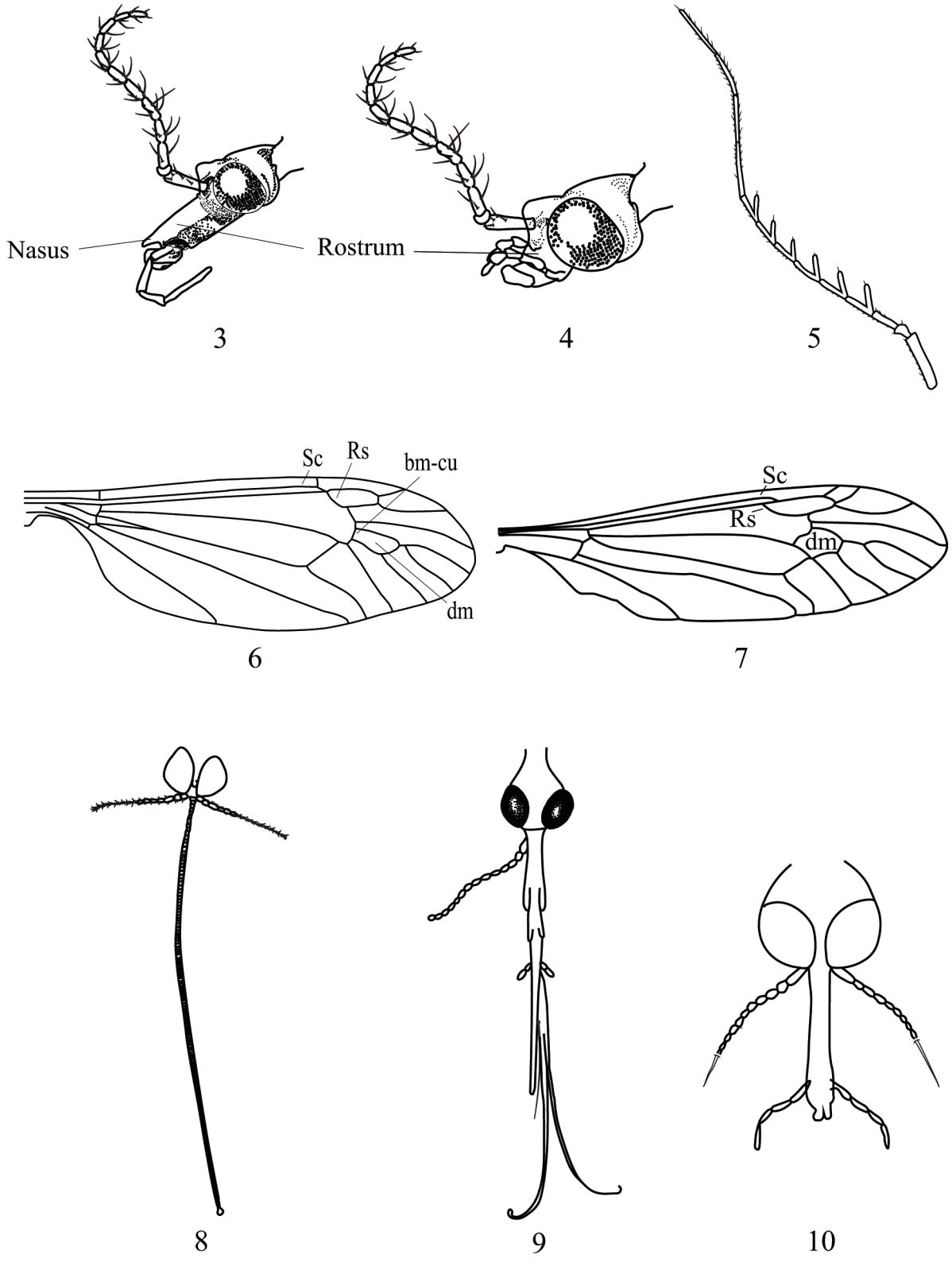
Diagnosis. Wings gray, conspicuously patterned with brown, including cord and adjacent veins. Veins beyond cord with conspicuous macrotrichia, including all veins from R_{2+3} to distal section of vein Cu_1. Abdomen with segments conspicuously bicolored, disk yellow and with brown margins.

Previous geographical distribution: Bolivia.

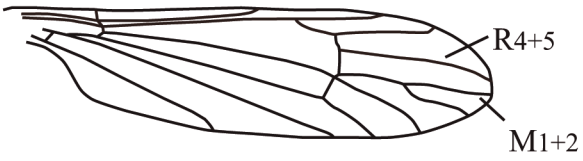
DISCUSSION

The richness of insects in salt marsh environments is commonly underestimated because of the general assumption that this environment is hostile for insects with aquatic/semi-aquatic larval phases. However, previous studies have shown that relatively high salinity does not hinder the presence of Diptera species in these habitats (Williams & Williams, 1998; Giberson *et al.*, 2001; Mackenzie, 2005; Silberbush *et al.*, 2005; Boix *et al.*, 2008). This is supported by our results, particularly for the species richness in Limoniidae.

Some of the taxa we identified are noteworthy: *Dicranomyia* is the largest genus in the Limoniidae,



Figs 3-10. Morphological characteres for Tipuloidea genera identification: (3) Tipulidae*, (4) Limoniidae, head; (5) *Ozodicera**, antennae. (6) *Nephrotoma** and (7) *Zelandotipula*, wings; (8) *Toxorhina**, (9) *Geranomyia** and (10) *Teucholabis**, head (Sc, Subcostal vein; Rs, Radial sector vein; bm-cu, Basal-medial cubital vein; dm, Discal-medial cell). *Modified from Gelhaus (2009).



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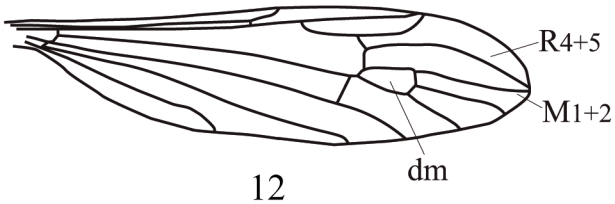

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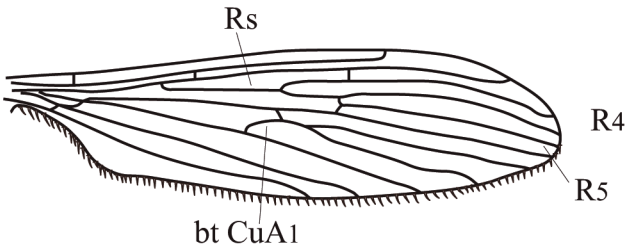

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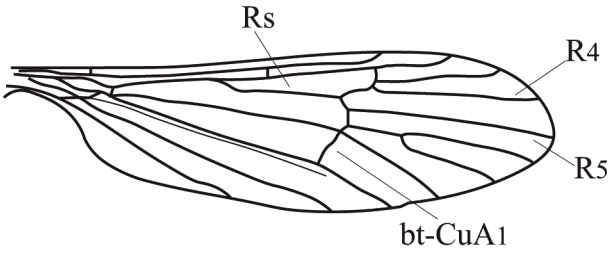

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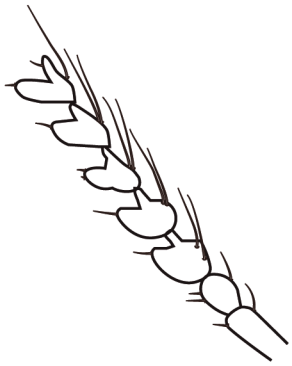

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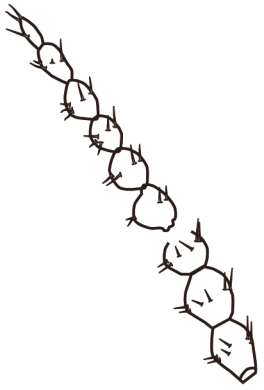

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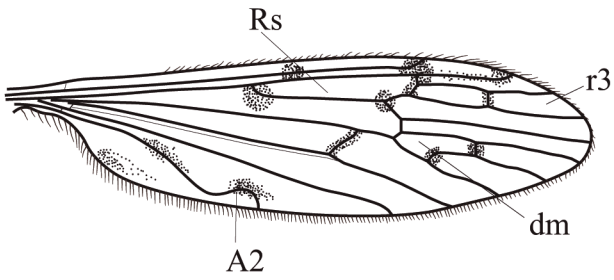

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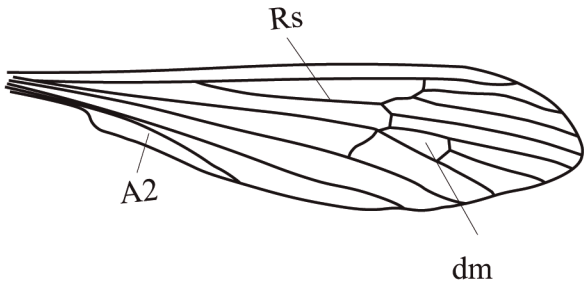

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Figs 11- 18. Morphological characteres for Tipuloidea genera identification. (11) *Gonomyia* (*Neolipophleps*), (12) *Gonomyia* (*Paralipophleps*), (13) *Molophilus* and (14) *Ormosia*, wings; (15) *Rhipidia* and (16) *Dicranomyia*, antennae; (17) *Symplecta* (*Symplecta*) and (18) *Symplecta* (*Trimicra*), wings. Modified from Gelhaus (2009).

representing around 10% of Neotropical species (Oosterbroek, 2018). Representatives often display significant morphological variability, confusing species distinctions, so it is common for these to be treated as species complexes (Nitta & O'Grady, 2008; Goodman & O'Grady, 2013; Salmela *et al.*, 2014; Starý & Stubbs, 2015). Species within our *Dicranomyia* complex were all genetically similar, which suggests that their different morphologies, especially in terms of wing venation, are a morphological plasticity of the group. This is the first report of the genus *Ormosia* for the Neotropical region, and consequently for Brazil. We expanded the ranges for *Polymera inornata* and *Symplecta cana*, with the latter now for both continents of the New World (previously only reported in North America). *Symplecta pilipes* is a cosmopolitan species, and combined with the American *Rhipidia domestica amazonensis*, these two species exhibit high densities in Neotropical salt marshes (see also Rodrigues *et al.*, 2017).

De Jong *et al.* (2008) argue that all Tipuloidea species/subspecies are restricted to determined biogeographic regions, with only a few sharing neighboring regions. Most mtDNA COI sequences found in this work were highly similar to those from Argentina, Costa Rica, Dominican Republic, Ecuador, and Nicaragua (Neotropical region), and different from those from regions such as Canada and U.S.A. (Nearctic region), which corroborates a relatively limited range distribution of these crane fly species, and reinforces the endemic characteristic of this group in the Neotropics. Floyd *et al.* (2009) alert that using COI sequences for identification of insect species can lead to overestimation of richness. However, we found low intraspecific (< 0.03) and high interspecific molecular differences (0.11-0.25) among the sampled insects, indicating that the mtDNA COI region is a useful tool for species distinction in the Tipuloidea. This has also been observed by Hebert *et al.* (2004). This molecular marker has been shown to be useful for the identification of other Diptera species with non-conspicuous morphological diagnostic characteristics, such as blackflies (Diptera: Simuliidae; Pramual *et al.*, 2016), mosquitoes (Diptera: Culicidae; Kumar *et al.*, 2007), and sandflies (Diptera: Psychodidae; Nzelu *et al.*, 2015). *Gonomyia* (*Neolipophleps*) sp. was the only species with high intraspecific distance (0.03-0.12), because of one or more divergent specimens. This differentiation between specimens was not detected morphologically by wing venation and male genitalia, but could indicate the existence of another *Gonomyia* (*Neolipophleps*) species.

The current literature on this superfamily at the Neotropical region is sparse and fragmented, consisting primarily of the original descriptions of C. P. Alexander. In this manner, it becomes necessary a complete review for regional Tipuloidea species. The insertion of new and generically-identified sequences in NCBI is an important step that will provide support for researches using molecular tools for identification and/or phylogenetic studies of Tipuloidea. Additionally, mtDNA COI barcoding is a useful tool for Tipuloidea species identification. This is one of the first works to list and barcode Tipuloidea species from Neotropical salt marshes, decreasing the large gap in information on crane flies at the region, and aiding in future studies aimed towards exploring the life cycles and/or ecology of these insects.

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