



Systematics, Morphology and Biogeography

## Can sibling species of the *Drosophila willistoni* subgroup be recognized through combined microscopy techniques?



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### ABSTRACT

In several arthropod groups, male genitalia is the most important feature for species identification, especially in cryptic species. Cryptic species are very common in the *Drosophila* genus, and the Neotropical *Drosophila willistoni* species group is a good example. This group currently includes 24 species divided into three subgroups: *alagitans*, *bocainensis* and *willistoni*. There are six sibling species in the *willistoni* subgroup – *D. willistoni*, *D. insularis*, *D. tropicalis*, *D. equinoxialis*, *D. pavlovskiana* and *D. paulistorum*, which is a species complex composed of six semispecies – Amazonian, Andean-Brazilian, Centroamerican, Interior, Orinocan and Transitional. The objective of this study was to characterize male genitalia of the *willistoni* subgroup, including the *D. paulistorum* species complex, using scanning electron microscopy and light microscopy. We also tried to contribute to the identification of these cryptic species and to add some comments about evolutionary history, based on male genitalia characters. Despite being cryptic species, some differences were found among the siblings, including the *Drosophila paulistorum* semispecies.

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### Introduction

Male terminalia is one of the most important traits used to identify cryptic species, which are very common in the *Drosophila* Fallén genus. The Neotropical *Drosophila willistoni* species group is a good example of cryptic speciation. This group includes 24 species, which are divided into three subgroups: *alagitans*, *bocainensis* and *willistoni* (Bachli, 2015); the last of them showing various taxonomic levels with successive degrees of reproductive isolation (Robe et al., 2010). The *willistoni* subgroup includes six sibling species: *D. willistoni* Sturtevant, 1916, *D. equinoxialis* Dobzhansky, 1946, *D. insularis* Dobzhansky, 1957, *D. tropicalis* Burla and Da Cunha, 1949, *D. pavlovskiana* Katritsis and Dobzhansky, 1967 and *D. paulistorum* Dobzhansky and Pavan, 1949. These species are almost morphologically indistinguishable based on external morphology, exhibit varying degrees of premating isolation and usually do not cross-hybridize (Ehrmann and Powell, 1982). Within the subgroup, *Drosophila paulistorum* is a species complex, or also referred as a superspecies, composed of six semispecies (Dobzhansky and Spassky, 1959; Perez-Salas et al., 1970). The *willistoni* subgroup also shows taxonomic differentiation at the subspecies level:

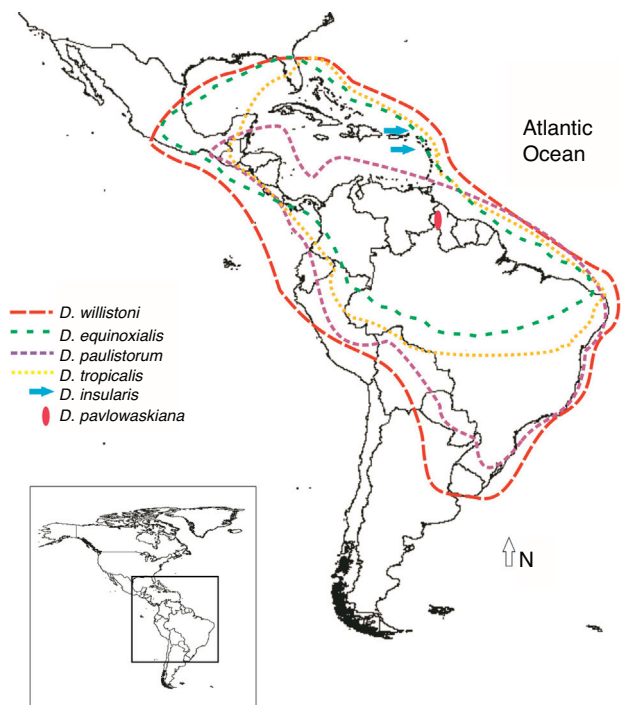
*D. willistoni* differentiates into the *willistoni* and *quechua* subspecies (Ayala and Tracey, 1973); *D. tropicalis* contains the *tropicalis* and *cubana* subspecies (Townsend, 1954); and *D. equinoxialis* is divided into the *equinoxialis* and *caribbensis* subspecies (Ayala et al., 1974).

According to the review of Cordeiro and Winge (1995), the sibling group is still in an active process of speciation and all levels of this process can be observed. The authors suggest two steps of speciation: incipient isolation, represented by the subspecies. The second step of speciation is exemplified by the semispecies, which show several degrees of reproductive isolation ranging from complete isolation to the presence of fertile offspring (Cordeiro and Winge, 1995) which was observed in the crossings of the Transitional semispecies with the Andean-Brazilian and the Centroamerican semispecies (Ehrman, 1961, 1965).

*D. willistoni* has the broader distribution of the group (Fig. 1), spanning from Central Mexico and Florida to Southern Brazil and Northern Argentina, and from the Atlantic to the Pacific Ocean (Dobzhansky and Powell, 1975; Ehrmann and Powell, 1982), even in areas of human disturbance (Valiati and Valente, 1996). *D. willistoni* is uninterruptedly distributed over this area, except in deserts and high altitudes (Ehrmann and Powell, 1982). Other sibling species have narrower distributions within the distribution of *D. willistoni*, except *D. insularis*, which is endemic to Saint Kitts and Saint Lucia of the Antilles Islands (Dobzhansky et al., 1957), and *D. pavlovskiana*, which has been found only once in

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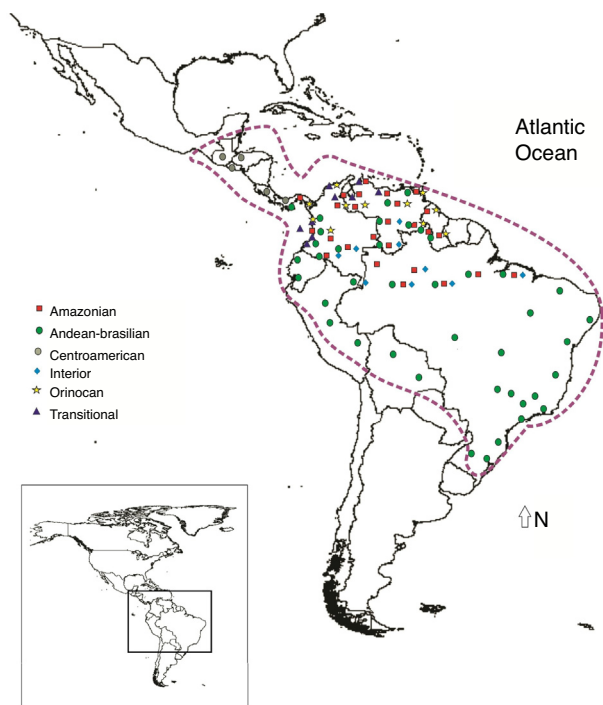
E-mail: [vera.valente@pq.cnpq.br](mailto:vera.valente@pq.cnpq.br) (V.L. da Silva Valente).



**Fig. 1.** Geographic distribution of the *D. willistoni* species subgroup, according to Spassky et al. (1971), Winge (1971), Dobzhansky and Powell (1975), Ehrmann and Powell (1982), Santos and Valente (1990).

Guyana (Spassky et al., 1971) and has not been collected since then (Fig. 1).

The *D. paulistorum* semispecies occur from Southern Brazil to Central America (Guatemala) and Trinidad (Dobzhansky and Spassky, 1959) (Fig. 2). According to Dobzhansky et al. (1964),



**Fig. 2.** Geographic distribution of the *D. paulistorum* superespecies, according to Spassky et al. (1971), Winge (1971), Dobzhansky and Powell (1975), Ehrmann and Powell (1982), Santos and Valente (1990).

when the semispecies' territories overlap, they apparently do not interbreed. Previous studies suggested that the differences among morphological, physiological and ecological traits within the semispecies are too small to distinguish each semispecies (Pasteur, 1970; Perez-Salas and Ehrmann, 1971) and could only be recognized by examination of the gene arrangements on their chromosomes (Kastritsis, 1967; Rohde et al., 2006) and by crossing tests (Perez-Salas and Ehrmann, 1971).

Despite being a traditionally studied group with an exciting evolutionary history, there is a lack of studies concerning the morphology in the early stages of development and also in adults. Some morphological studies of adults were made after the species were described. Burla et al. (1949) presented illustrations of maxillar palpi, vaginal plates, spermatechae and hypandria of *D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. paulistorum*. Hsu (1949) briefly described *D. willistoni* and *D. equinoxialis* male genitalia, in addition of some species of the *alagitans* and *bocainensis* subgroups. Malogolowkin (1952) provided a very detailed description of the male and female genitalia of *D. willistoni*, *D. equinoxialis*, *D. tropicalis*, *D. paulistorum* and some species of the *bocainensis* subgroup. Spassky (1957) shown illustrations of hypandria, aedeagi, surstyly and preniseta of five sibling species – *D. willistoni*, *D. tropicalis*, *D. equinoxialis*, *D. paulistorum* and *D. insularis*. Pasteur (1970) made a biometrical comparison of four semispecies of *D. paulistorum* regarding wing and tibia lengths, wing-to-tibia ratios, wing size and number of preniseta on surstyly. Vilela and Bächli (1990) redescribed *D. willistoni* and provided several illustrations of the male terminalia of this species. Eberhard and Ramirez (2004) presented several Scanning Electron Microscopy (SEM) images of male and female terminalia of *D. willistoni*. Rohde et al. (2010) provided photos of the hypandria of *D. willistoni*, *D. equinoxialis*, *D. tropicalis* and *D. paulistorum* without indicating the semispecies while suggesting the importance of this structure for their identification. Recently, Souza et al. (2014) provided SEM images of *D. willistoni*, which was used as outgroup of the phylogenetic reconstruction of the *D. saltans* group. Civetta and Gaudreau (2015) shown photos of external male genitalia and aedeagus of *D. willistoni* and the subspecies *D. willistoni quechua*. Also, there are SEM images of male terminalia of four sibling species (*D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. paulistorum*) available in Emilio Goeldi Museum database (mar.te.museu-goeldi.br).

Burla et al. (1949) only found slight morphological differences that were insufficient for the identification of single individuals, while Spassky (1957) noted differences in the male genitalia that did permit such identification. In other way, Malogolowkin (1952) described the general morphology of the male genitalia of four sibling species (*D. willistoni*, *D. equinoxialis*, *D. tropicalis* and *D. paulistorum*) as very similar but completely different from non-sibling species. In addition, this author found no differences in the penises of these four sibling species. Spassky (1957), however, observed that the shapes of the penises and their gonapophyses differed in the sibling species.

With respect to *D. insularis*, only a few illustrations were presented by Spassky (1957). The previous descriptions and illustrations of *D. paulistorum* were based on Andean-Brazilian specimens once the specimens were collected in areas where only this semispecies is found (Mogi das Cruzes, São Paulo, Brazil in Burla et al. (1949) and Malogolowkin (1952); Tamandaré, Pernambuco, Brazil in Rohde et al. (2010)). The remaining species, *D. pavlovskiana*, has not been collected and is no longer available in Stock Centers. There is no description in the literature of the male genitalia of this species.

In this scenario, our objective was to characterize and compare the male terminalia of the species of the *D. willistoni* subgroup, including the *D. paulistorum* semispecies complex, using

**Table 1**  
Species and strains used in this study. The numbered strains are represented in the figures.

Species	Semispecies	Population sample	Localities	Source
<i>D. equinoxialis</i> [1]		Mexico	Apazapán/Mexico	Lee Ehrman and Yong Kyu Kim
<i>D. equinoxialis</i>		FNT12	Belterra/Brazil	Mário Josias Müller
<i>D. equinoxialis</i>		FNT19	Belterra/Brazil	Mário Josias Müller
<i>D. equinoxialis</i>		FNT32	Belterra/Brazil	Mário Josias Müller
<i>D. equinoxialis</i>		Tape03	Santarém/Brazil	Mário Josias Müller
<i>D. equinoxialis</i>		Tape31	Santarém/Brazil	Mário Josias Müller
<i>D. equinoxialis</i>		Tape32	Santarém/Brazil	Mário Josias Müller
<i>D. equinoxialis</i>		Tape63	Santarém/Brazil	Mário Josias Müller
<i>D. equinoxialis</i>		Tape89	Santarém/Brazil	Mário Josias Müller
<i>D. equinoxialis</i>		207018	Belém do Pará/Brazil	Marlucia Martins
<i>D. insularis</i> [2]		SL03	Saint Lucia/Lesser Antilles	Jeffrey R. Powell
<i>D. insularis</i>		SL15	Saint Lucia/Lesser Antilles	Jeffrey R. Powell
<i>D. tropicalis</i> [3]		0801	San Salvador/El Salvador	Tucson Stock Center
<i>D. willistoni</i> [4]		WIP4	Salvador/Brazil	Antônio R. Cordeiro and Helga Winge
<i>D. willistoni</i>		Gdh4-1	Guadalupe/Caribe	Tucson Stock Center
<i>D. willistoni</i>		Ribeirão Preto	Ribeirão Preto/Brazil	Cláudia Rohde
<i>D. willistoni</i>		Mexico	Apazapán/Mexico	Margaret Kidwell
<i>D. willistoni</i>		DW-Pool Ita	Itapuã/Brazil	André Schnorr
<i>D. willistoni</i>		DW-Pool Faz	Serra do Cipó/Brazil	Carlos Vilela
<i>D. willistoni</i>		CIP	Serra do Cipó/Brasil	Carlos Vilela
<i>D. willistoni</i>		COR	Coronilla/Uruguay	Beatriz Goñi
<i>D. willistoni</i>		ISC	Santa Catarina/Brazil	Daniela de Toni
<i>D. willistoni</i>		DLA	Dois Lajeados/Brazil	Cláudia Rohde
<i>D. willistoni</i>		MAS	Morror Santana/Brazil	Ana Lauer Garcia
<i>D. willistoni</i>		Joinville	Joinville/Brazil	Jonas Döge
<i>D. willistoni</i>		17A2	Eldorado do Sul/Brazil	Vera L.S. Valente
<i>D. paulistorum</i> [5]	Amazonian	Pará q3	Belém do Pará/Brazil	Lee Ehrmann and Yong Kiu Kim
<i>D. paulistorum</i>		Tape 60	Santarém/Brazil	Mário Josias Müller
<i>D. paulistorum</i> [6]	Andean-Brazilian	MLC	Santa Catarina/Brazil	Marco Gottschalk
<i>D. paulistorum</i>		RIB	Ribeirão Preto/Brazil	Vera L.S. Valente
<i>D. paulistorum</i>		RMT	Porto Alegre/Brazil	Ana Lauer Garcia
<i>D. paulistorum</i>		Pool-Bur	Brasília/Brazil	Rosana Tidon
<i>D. paulistorum</i>		Játon-Sacha	Jaton Sacha/Equador	Margaret Kidwell
<i>D. paulistorum</i> [7]	Centroamerican	Lancetilla	Lancetilla/Honduras	Lee Ehrman and Yong Kyu Kim
<i>D. paulistorum</i> [8]	Interior	INT	Llanos/Colombia	Lee Ehrman and Yong Kyu Kim
<i>D. paulistorum</i> [9]	Orinocan	ORI	Georgetown/Guyana	Lee Ehrman and Yong Kyu Kim
<i>D. paulistorum</i> [10]	Transitional	Santa Marta	Santa Marta/Colombia	Lee Ehrman and Yong Kyu Kim

Scanning Electron Microscopy and Light Microscopy. We attempt to describe this morphological trait within species and find features that differentiate the sibling species and semispecies of the *willistoni* subgroup and verify if these species can be recognized based on characters of the male terminalia.

## Material and methods

### Fly stocks

The stocks were reared in a cornmeal medium (Marques et al., 1966) at a constant temperature and humidity ( $17 \pm 1^\circ\text{C}$ ; 60% rh). Some individuals were preserved in 70% ethanol, and mounted stubs and slides will be deposited in Fundação Oswaldo Cruz (Fiocruz) Collection. All strains used in this study are listed in Table 1.

### Species recognition

Species were confirmed with the *AcpH*-1 (Acid Phosphatase) electrophoresis protocol (Garcia et al., 2006). Also, DNA sequences were generated and compared with sequences available in *GenBank* – mitochondrial gene fragments *COI* (Cytochrome oxidase I) (deposited by Gleason et al., 1998), *COII* (Cytochrome oxidase II) (deposited by Robe et al., 2010), and nuclear genes fragment *Adh* (Alcohol dehydrogenase) (deposited by Gleason et al., 1998 and Robe et al., 2010). The sequences obtained will be presented in a further study.

### Scanning electron microscopy (SEM) preparation and observation

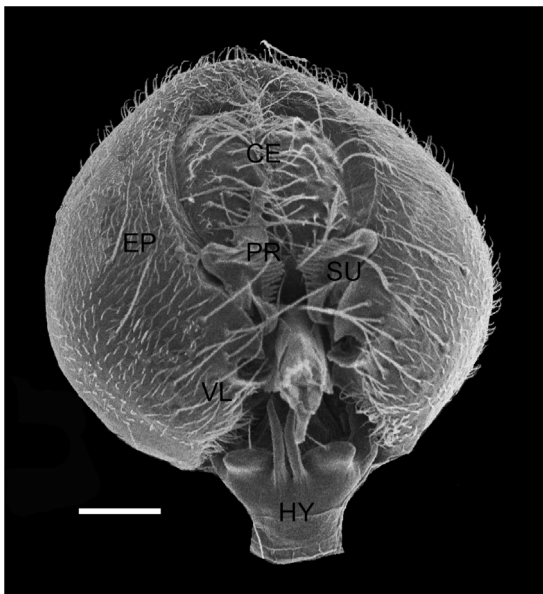
Male terminalia were treated with 10% KOH (Wheeler and Kambyssellis, 1966 modified by Bächli et al., 2004) and dissected in glycerol. Terminalia were dehydrated for 20–30 s with acetone washes in the following concentrations: 30%, 50%, 75% and 100%. The entire terminalia and separated parts were mounted in stubs with carbon tape and metalized with gold in a Balzers SCD050 sputter coater. Visualization and image capture were performed in JSM6060 Scanning Electron Microscope in Centro de Microscopia da Universidade Federal do Rio Grande do Sul. We observed approximately 50 seven-day-old specimens of each species and semispecies.

### Light microscopy preparation and observation

Male terminalia were prepared as previously described. The terminalia, aedeagi and hypandria were mounted in a non-permanent glycerin jelly medium (Klaus et al., 2003). The slides were observed and photographed with a Carl-Zeiss Standard phase contrast microscope. We analyzed the genital structures of 7-day-old males of each species and semispecies (750 individuals).

### Terminology and references

The morphological terminology used in this study follow McAlpine (1981), Grimaldi (1990), Vilela and Bächli (1990), Bächli et al. (2004) and Souza et al. (2014). Figures of external male



**Fig. 3.** General example of external male genitalia of the *D. willistoni* subgroup (picture of *D. paulistorum* Amazonian [5]). Scale bar 50  $\mu$ m. EP, epandrium; CE, cerci; SU, surstylus; PR, prenisetae; AE, aedeagus; VL, ventral lobe; HY, hypandrium.

genitalia of *D. willistoni* subgroup, under light microscopy, are shown in [Supplementary material 1 \(S1\)](#).

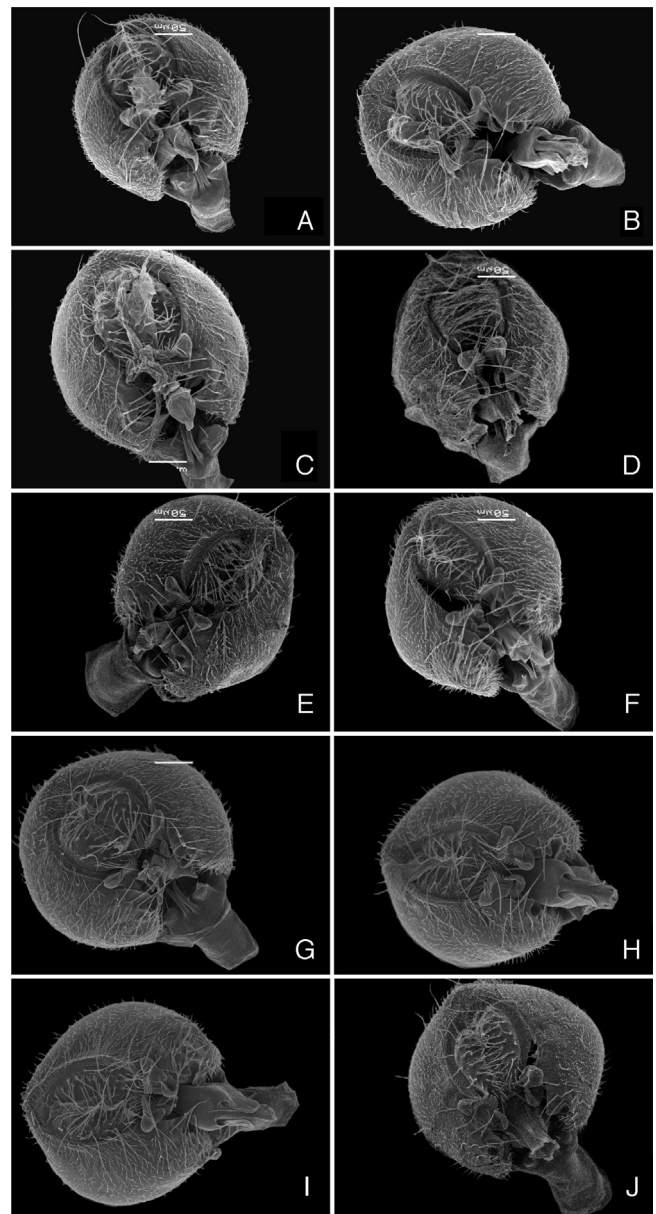
## Results

### *Epandrium, surstylus and prenisetae*

The cerci are not fused to the epandrium (Figs. 3 and 4; Fig. S1) in all the species analyzed. The surstylus is elongated into a hook at the bottom, is not micropubescent, has up to 12 preniseta (also called primary teeth) in *D. paulistorum* Orinocan and *D. paulistorum* Interior, and up to 18 preniseta in *D. equinoxialis* (Fig. 5) in addition to one large prenisetae and one or two setae on the ventral hook.

*Drosophila equinoxialis* presents 18 preniseta, nine smaller and nine larger, and one setae on the ventral hook (Figs. 4A and 5A). *D. insularis* has approximately 17 preniseta of equal size and one setae in the ventral hook (Figs. 4B and 5B). *D. tropiacalis* has 13–14 crescent size preniseta and one setae in the ventral hook (Figs. 4C and 5C). *D. willistoni* also has 13 crescent sized preniseta and one setae in the ventral hook (Figs. 4D and 5D).

Among the *D. paulistorum* semispecies, we found that *Drosophila paulistorum* Amazonian has a surstylus with 15 preniseta, nine longer and six shorter, and one setae in the ventral hook (Figs. 4E and 5E). *D. paulistorum* Andean-Brazilian has a surstylus with 15 preniseta, eight longer and seven shorter, and two setae in the ventral hook (Figs. 4F and 5F). *D. paulistorum* Centroamerican has a surstylus with 15 preniseta, eight larger and seven smaller, and two setae in the ventral hook (Figs. 4G and 5G). *D. paulistorum* Interior has a surstylus with 12 crescent size prenisetae and two setae in the ventral hook (Figs. 4H and 5H). *D. paulistorum* Orinocan has a surstylus with 12 preniseta of approximately the same size and two setae in the ventral hook (Figs. 4I and 5I). *D. paulistorum* Transitional has a surstylus with 12 prenisetae of approximately the same size, with one larger prenisetae in the middle and two setae in the ventral hook (Figs. 4J and 5J). The distance between the sides of surstylus and hooks could be an artifact of the SEM preparation and is not considered a diagnostic character for species identification.

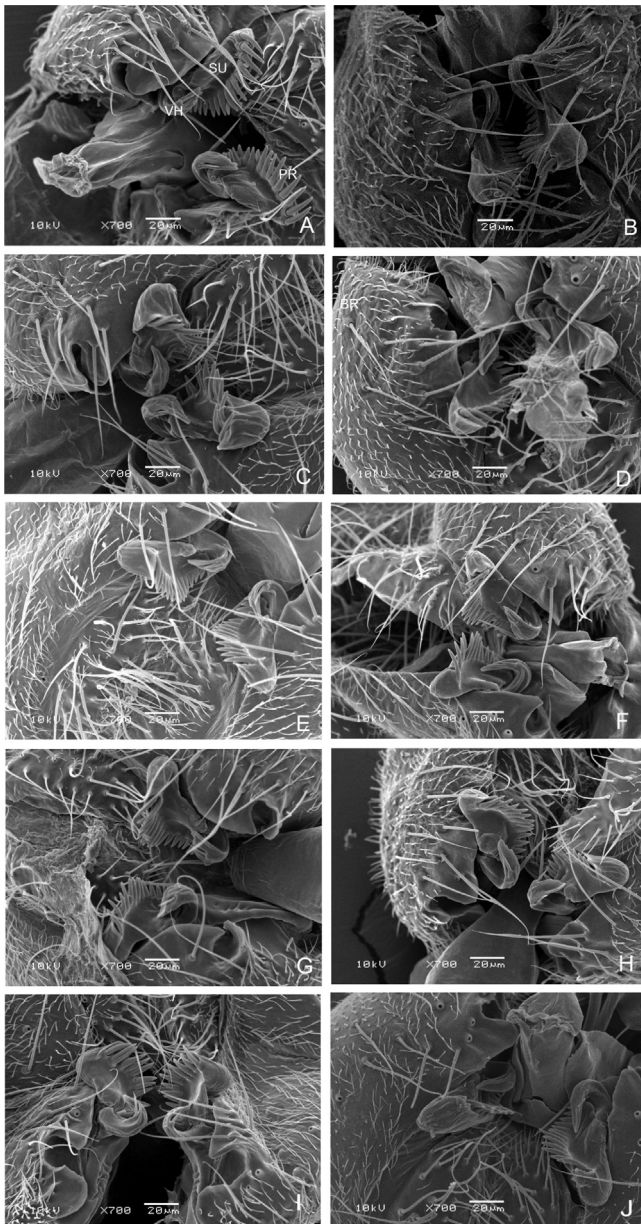


**Fig. 4.** Scanning electron microscopy of *D. willistoni* species subgroup external male genitalia. Scale bar 50  $\mu$ m. The strains are in brackets and listed in [Table 1](#). (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropiacalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brazilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

### *Hypandrium*

The hypandrium is smaller than the epandrium; it is approximately 1/4 to 1/3 of the size of the epandrium. The hypandria in all of the species analyzed have, in the apical region, one pair of heavily sclerotized median teeth, lobes with paramedian seta on the apex and lateral extensions (Figs. 6 and 7). The relative size and thickness of the median teeth, as well as the size and shape of the lobes, vary within the species (Figs. 6 and 7).

In *D. equinoxialis*, the hypandrium is triangular, with well-developed trapezoidal lobes and lobe seta convergent to teeth. The teeth are large and thick, twice the height of the lobes, aligned with the lobes, and do not touch each other. The lateral extensions are very prominent toward the top (Figs. 6A and 7A). *D. insularis* has



**Fig. 5.** Scanning electron microscopy of *D. willistoni* species subgroup *surstyli* and *prensisetae*. Scale bar 20 μm. The strains are in brackets and listed in Table 1. SU, Surstylus; PR, Prensisetae; VH, Ventral hook. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brazilian [6]; (G) *D. paulistorum* Centroamericana [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

a triangular hypandrium, with almost absent lobes. This species presents one or two paramedian seta in the lobes and presents the smaller and more separated teeth that occur in the *willistoni* subgroup (Figs. 6B and 7B). *D. tropicalis* presents a triangular hypandrium, with very large round lobes and seta convergent to teeth and almost touching them. It also has large thick teeth, twice the height of the lobes, inserted slightly below the lobe line. The lateral extensions are located under the lobes (Figs. 6C and 7C). *D. willistoni* also has a triangular hypandrium, with subtle round lobes. Its very close large, thick teeth are thrice the height of the lobes and are inserted far below the lobe line. The lateral extensions are similar to those of *D. tropicalis*, but are adjacent to the lobes (Figs. 6D and 7D).

In the *D. paulistorum* complex, *D. paulistorum* Amazonian presents a square-shaped hypandrium, dome-shaped lobes, and

slightly convergent seta. The lobes almost touch each other and the teeth are large and slightly separated, inserted in the lobe line. The lateral extensions are prominent toward the top, but less than in *D. equinoxialis* (Figs. 6E and 7E). *D. paulistorum* Andean-Brazilian presents a square-shaped hypandrium, slightly square-shaped lobes, convergent seta and very close, medium-sized, thin teeth that are twice the height of the lobes and inserted in the lobe line. There is a visible gap between the lobes and teeth. The lateral extensions are almost continuous with the lobes (Figs. 6F and 7F). *D. paulistorum* Centroamericana presents a rectangular hypandrium, which is the most elongated in *D. willistoni* subgroup, irregular-shaped lobes, convergent seta and very close, medium-sized, thin teeth that are twice the height of the lobes and inserted below the lobe line. The lateral extensions are similar to *D. willistoni* but nearer to the lobes (Figs. 6G and 7G). *D. paulistorum* Interior is very similar to *D. paulistorum* Andean-Brazilian, but the lateral extensions are not continuous with the lobes and there is no gap between the teeth and lobes (Figs. 6H and 7H). *D. paulistorum* Orinocan hypandrium is the smallest of the subgroup, is square-shaped with small round lobes, convergent seta and medium-sized, thin teeth that are thrice the height of the lobes and inserted a little below the lobe line. Lateral extensions are expanded to external sides of the lobes (Figs. 6I and 7I). *D. paulistorum* Transitional presents a square-shaped hypandrium, small round lobes very close to the teeth, convergent seta and large thick teeth that are almost thrice the height of the lobes and inserted in the lobe line. Lateral extensions are prominent toward the top, higher than the lobes (Figs. 6J and 7J).

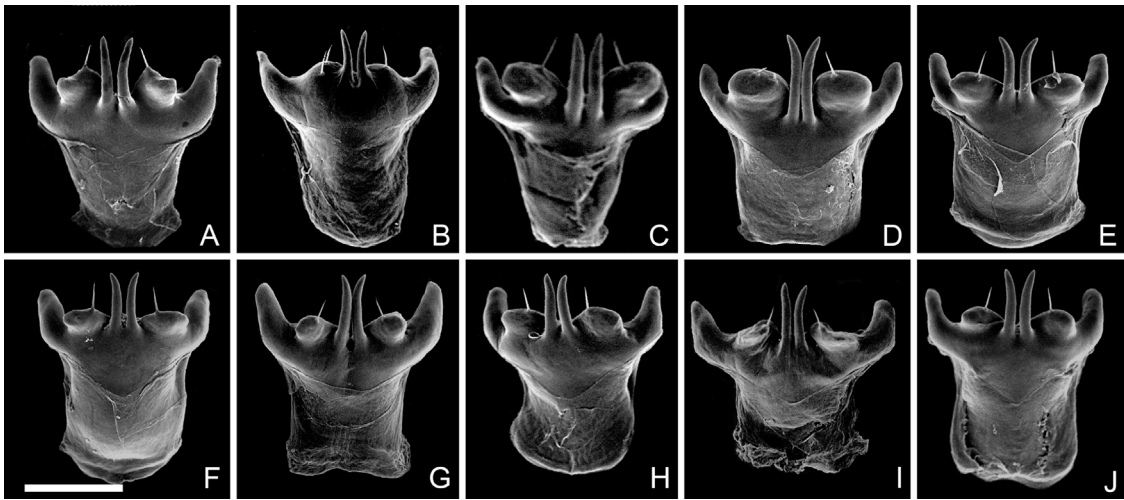
#### Aedeagus, aedeagal apodeme, paramere and lateral projections

In all of the species of the *D. willistoni* subgroup that have been analyzed, the aedeagus is dorsally membranous and ventrally directed downwards, as a bird beak-like protusion at the distal end (distiphallus), with two lateral projections at the anterior half, covered with some tiny spines that are not always visible in preparations. The aedeagal apodeme is as long as the aedeagus, is bar shaped and is linked to the aedeagus by a membranous tissue. The parameres are smooth and are also linked to the apodeme by a membranous tissue. The paramere anchors the aedeagus to the hypandrium through the lateral expansions of hypandrium.

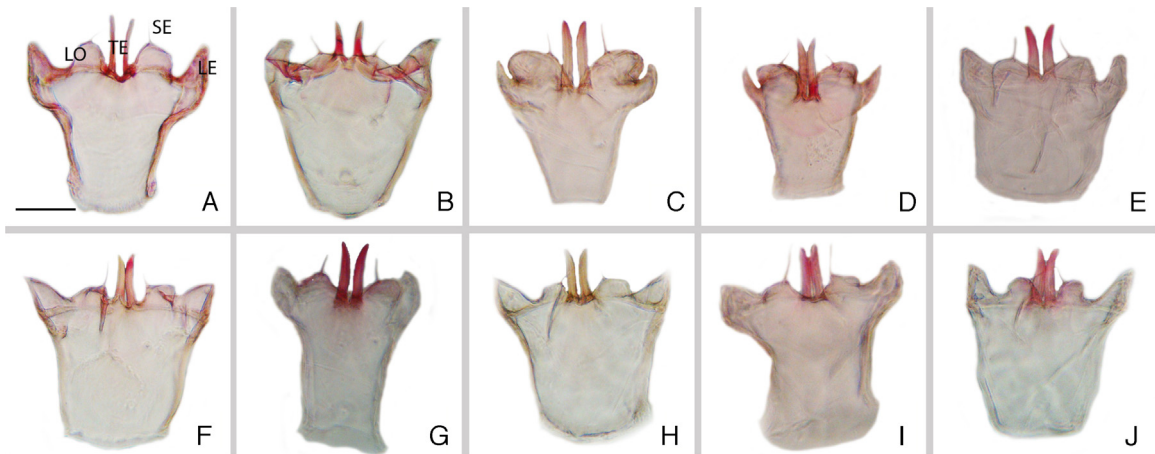
The most noticeable difference within the *willistoni* subgroup is the distal portion of the aedeagus (distiphallus). This is very prominent and curved in *Drosophila tropicalis* (Figs. 8C, 9C and 10C), long and straight in *D. willistoni* (Figs. 8D, 9D and 10D), and straight and shorter than *D. willistoni* in *D. equinoxialis* (Figs. 8A, 9A and 10A). In *D. insularis*, this structure is the shortest among the siblings (Figs. 8B, 9B and 10B). There are small variations in size within the *D. paulistorum* semispecies, but the shape of the distiphallus is unique in each incipient species (Figs. 8E–J, 9E–J and 10E–J).

The lateral projections exhibit some differences within the subgroup, which are more notable in *D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. insularis*. In *D. equinoxialis* and *D. paulistorum* Orinocan the distal portion of lateral expansions is rounded; in *D. insularis*, *D. tropicalis* and *D. willistoni* it is pointy; and in the remaining *D. paulistorum* semispecies, it is slightly pointy.

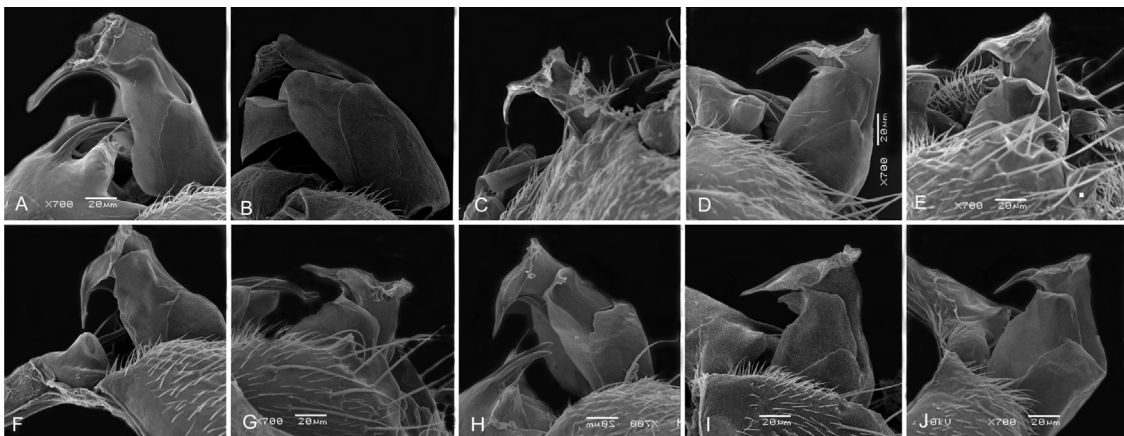
The aedeagal apodeme also shows variation; however, it is not species specific. In *D. equinoxialis*, *D. insularis*, *D. tropicalis* and *D. willistoni*, the aedeagal apodeme is rod shaped, without ornamentation in the distal portion (Figs. 9A–D and 10A–D). In the Amazonian, Andean-Brazilian, Interior and Transitional semispecies, the aedeagal apodeme is also rod shaped but with a small rounded expansion in the distal portion (Figs. 9E,F,H,J and 10E,F,H,J), and in the Centroamerican and Orinocan semispecies, there is a fan-like expansion in the distal area (Figs. 9G,I and 10G,I).



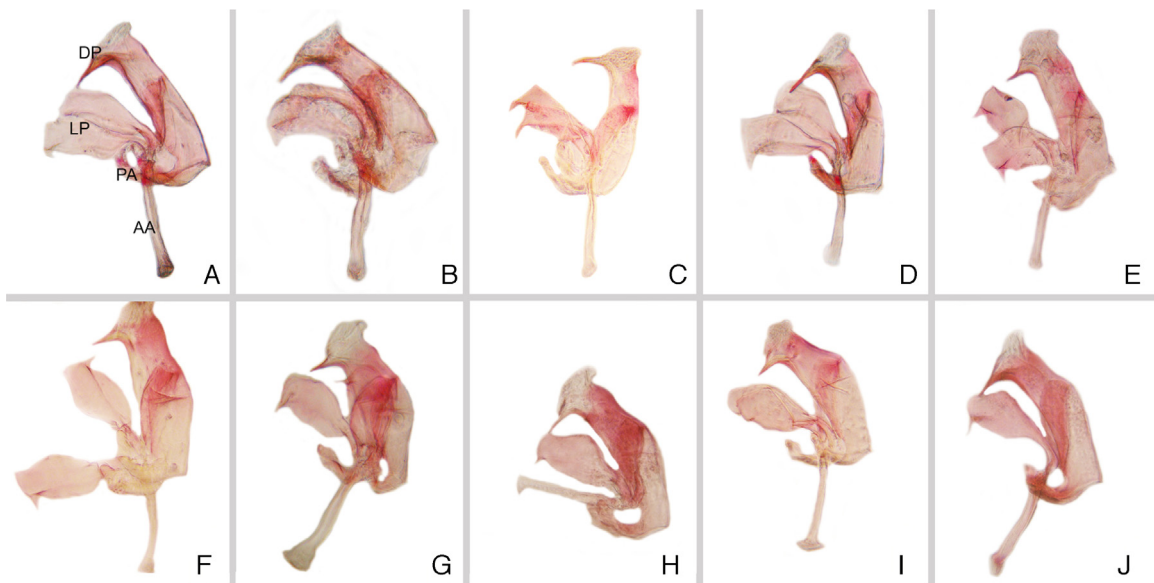
**Fig. 6.** Scanning Electron Microscopy of the *D. willistoni* species subgroup hypandria. Scale bar 50  $\mu$ m. The strains are in brackets and listed in Table 1. LO, lobes; TE, teeth; LE, lateral extensions; SE, seta. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].



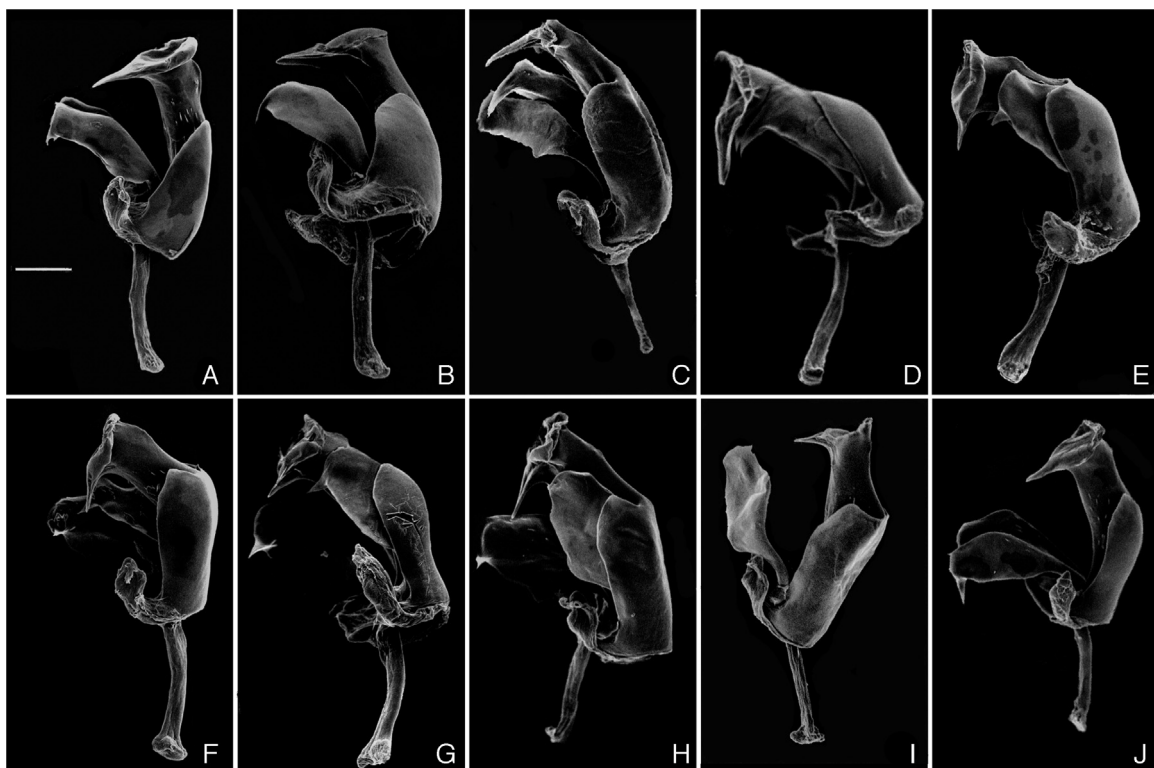
**Fig. 7.** Hypandria of *D. willistoni* species group. Scale bar 0.1 mm. The strains are in brackets and listed in Table 1. LO, lobes; TE, teeth; LE, lateral extensions; SE, seta. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].



**Fig. 8.** Scanning electron microscopy of *D. willistoni* species subgroup aedeagi. Scale bar 20  $\mu$ m. The strains are in brackets and listed in Table 1. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Brasilian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].



**Fig. 9.** Aedeagi, aedeagal apodeme and parameres of the *D. willistoni* species subgroup; lateral view. Scale bar 0.1 mm. The strains are in brackets and listed in Table 1. AA, Aedeagal apodeme; DP, Distiphallus; LP, Lateral projections; PA, Paramere. (A) *D. equinoxialis* [1]; (B) *D. insularis* [2]; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Braslian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].



**Fig. 10.** SEM of aedeagi, aedeagal apodeme and parameres of the *D. willistoni* species subgroup; lateral view. Scale bar 50  $\mu$ m. The strains are in brackets and listed in Table 1. AA, Aedeagal apodeme; DP, Distiphallus; LP, Lateral projections; PA, Paramere. (A) *D. equinoxialis*; (B) *D. insularis*; (C) *D. tropicalis* [3]; (D) *D. willistoni* [4]; (E) *D. paulistorum* Amazonian [5]; (F) *D. paulistorum* Andean-Braslian [6]; (G) *D. paulistorum* Centroamerican [7]; (H) *D. paulistorum* Interior [8]; (I) *D. paulistorum* Orinocan [9] and (J) *D. paulistorum* Transitional [10].

## Discussion

This study sheds new light on the identification of the *D. willistoni* sibling species subgroup, especially regarding the *D. paulistorum* complex. We presented here for the first time images of the male terminalia of the six semispecies of the *D. paulistorum*. Now, the male identification of the cryptic species of this subgroup

could be easier and quicker than enzymatic, molecular and chromosomal approaches.

We found major differences especially in *D. willistoni*, *D. tropicalis*, *D. equinoxialis* and *D. insularis*. While there is a strong sexual isolation within these species, it has been reported that they occasionally interbreed (Dobzhansky et al., 1957; Winge and Cordeiro, 1963; Winge, 1965; Cordeiro and Winge, 1995), and several degrees

of reproductive affinity are present between the sibling species group (reviewed in [Cordeiro and Winge, 1995](#)).

Our findings are consistent with the results presented in [Spassky \(1957\)](#) regarding *D. equinoxialis*, *D. insularis*, *D. tropicalis*, *D. willistoni* and *D. paulistorum* Andean-Brazilian. However, a notable aspect of this comparison is that intraspecific variation was not observed in our results. We analyzed the male genitalia of *D. equinoxialis*, *D. insularis*, *D. willistoni* and *D. paulistorum* Andean-Brazilian from several localities ([Table 1](#)), including some recently collected strains, and no remarkable character variation was found (data not shown). This fact does not imply that there are no intraspecific variation in these species and in other species and semispecies of the *willistoni* subgroup. Also, laboratory strains may have less character variation than found in nature. [Burla et al. \(1949\)](#), however, concluded that “the variability is great enough to make identification of single individuals hazardous”.

[Burla et al. \(1949\)](#) found differences in the hypandria of four of the sibling species – *D. willistoni*, *D. paulistorum*, *D. tropicalis* and *D. equinoxialis* – and described the *D. tropicalis* hypandrium as similar to that of *D. paulistorum* in shape, although larger. These authors also stated that the *D. willistoni* hypandrium is the most distinctive. In contrast with the findings of [Burla et al. \(1949\)](#) we observed that the hypandria of *D. insularis* and *D. tropicalis* are the most distinctive with respect to the remaining species. *D. willistoni* seems to be more similar to the *D. paulistorum* semispecies than to the other species. Some features could only be observed in SEM: *D. willistoni* and *D. insularis* presented two seta in the apex of the lobes, only in one side ([Fig. 6B](#)); Despite the low frequency of this modification (1:50 in *D. willistoni* and 2:50 in *D. insularis*), it is an interesting feature. The specimens with this characteristic did not present any other peculiarity.

[Pasteur \(1970\)](#) considered the teeth of the claspers (the prenisetae in the surstylus) to be the single character of the male genitalia that differentiates the *D. paulistorum* incipient species. In our results ([Fig. 5](#)), the number of prenisetae is consistent with the range of values previously observed by [Pasteur \(1970\)](#). We observed two groups regarding the number of prenisetae – the semispecies Amazonian, Andean-Brazilian and Centroamerican with 15 prenisetae each and Centroamerican, Interior and Transitional semispecies with 12 prenisetae each, although the size and arrangement of the prenisetae are not the same for each one. [Pasteur \(1970\)](#) found variation in the number of prenisetae in each semispecies from different localities. In this study, we observed *D. paulistorum* Andean-Brazilian from several localities and the number of prenisetae was constant, even in the recently collected strains.

In the present report, we show that there are several diagnostic characters of the male genitalia useful for differentiating the species and even the semispecies of the *D. willistoni* species subgroup. However, the aedeagus is not the most important trait for identifying the subgroup's cryptic species, as is common in other *Drosophila* species groups. In the studied species, the hypandrium seems to be the main character for species identification. In addition to this, we suggest the visualization of the aedeagus under light microscopy for identification purposes, since this is a membranous structure and may be deformed in SEM.

Based on visual observations of hypandrium *D. insularis* seems to be the most distinctive species, related to the other sibling species. Within the *D. paulistorum* semispecies, the most dissimilar is the transitional, especially with respect to the hypandrium shape, surstylus and prenisetae. Such variation is in accord with the assumptions of [Spassky et al. \(1971\)](#) and [Dobzhansky \(1970\)](#), stating that the diversification of the semispecies is apparently still in progress.

Concerning the evolutionary relationship among the *willistoni* subgroup, an attempt to phylogenetic reconstruction was made

using the characters of the male terminalia observed and described in this study. The reconstruction, however, were inconclusive, since the generated tree presented some polytomies and low support values for the characters (data not shown). Despite of the differences observed, it is possible that these characters did not accumulated enough differences to represent the evolutionary history of the subgroup. Nevertheless, these characters could be useful in a combined phylogeny and will be presented in a further study.

Although complete reproductive isolation is not present within the species and semispecies of the *D. willistoni* subgroup (reviews in [Ehrmann and Powell \(1982\)](#) and [Cordeiro and Winge \(1995\)](#)), the number of differences among the male genitalia found in our observations is relevant, especially between the *D. paulistorum* semispecies. In insects, the rapid divergence in male genitalia is so pronounced that even recently diverged sibling species show a high degree of variation in the male genitalia ([Richards, 1927](#); [Liu et al., 1996](#); [Song, 2009](#)), as we have observed in the *D. willistoni* species subgroup. Finally, in response to our own question, sibling species of the *D. willistoni* subgroup can be recognized through combined microscopy techniques.

### Conflicts of interest

The authors declare no conflicts of interest.

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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.rbe.2015.09.006](https://doi.org/10.1016/j.rbe.2015.09.006).

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