GMR

The Original by FUNPEC-RP

Development and transferability of microsatellite markers for species of the *saltans* group of *Drosophila* (Diptera: Drosophilidae)

B.E. Roman, B.M. Trava and L. Madi-Ravazzi

Departamento de Biologia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências, Letras e Ciências Exatas, São José do Rio Preto, SP, Brasil

Corresponding author: L. Madi-Ravazzi
E-mail: lilian.madi@unesp.br

Genet. Mol. Res. 19 (4): gmr18700
Received August 14, 2020
Accepted September 21, 2020
Published October 30, 2020
DOI http://dx.doi.org/10.4238/gmr18700

ABSTRACT. Microsatellite markers, also known as single sequence repeats (SSRs), are highly polymorphic, fast evolving, and regarded as neutral markers. Due to these traits, they have been widely used in population studies. The development of SSRs for a given species opens possibilities for their application in population studies of other species that are phylogenetically related. We tested 16 primer pairs developed for *Drosophila sturtevanti* and the transferability test of them in 14 species of the *saltans* group of *Drosophila.* The optimal amplification conditions were established using the DNA of 15 *D. sturtevanti* males. Among the primers pairs developed, 13 have successful in the amplification for *D. sturtevanti*. And in the transferability test, the total percentage of transfer was nearly 50%. The species with the highest success rates of heterologous amplification were in the *sturtevanti* subgroup. Two microsatellite markers amplified in all the species, while one would not amplify for any of the *saltans* group species. These data demonstrate the usefulness of testing transferability of genetic markers, which may be used in studies of genetic diversity and population structure of *D. sturtevanti* and other species of the *saltans* group of *Drosophila*.

Key words: Genetic diversity; Genomic library; Molecular markers; Neutral markers; SSR

INTRODUCTION

Microsatellite or single sequence repeat (SSR) markers are species-specific, highly versatile, relatively abundant, codominant, and display high levels of polymorphism (Tautz, 1989; Weber, 1990; Morgante et al., 2002; Turchetto-Zolet et al., 2017); they have been widely used in genetic studies (Akkaya et al., 1992).The high levels of polymorphism observed in microsatellites and the relative ease of detection using PCR combined with a robust and efficient statistical analysis has favored their use in many biological areas, such as: forensics biology, genetic mapping, paternity tests, population genetics and conservation studies (Jarne and Lagoda, 1996; Schuler et al., 1996; Knapik, et al., 1998; Luikart et al., 2003).

The development of new SSR markers is an elaborate, time-consuming and expensive process; consequently, investigating transferability of SSR markers is advantageous (Ferreira and Grattapaglia, 1998; Fantin, 2007). Transferability may occur due to the conservation of microsatellite sites among related species, enabling the realization of site transfer between species belonging to the same genus, or even to different genera, which makes the use of heterologous initiators possible (Fantin, 2007; Kalia et al., 2011). Thus, the pairs of initiators designed with based on the sequences obtained from a specific species may be used to detect SSRs in related species; this method of transferability has been successfully demonstrated in a number of species (Ellis and Burke, 2007; Varshney et al., 2007; Laborda et al., 2009; Tractz et al., 2012; Oliveira et al., 2016). However, transferability is not always guaranteed. For instance, initiators designed for *Drosophila mediopunctata* have been tested in populations of *D. sturtevanti* and were not efficient, probably because are phylogenetically distant species (Trava et al., 2016).

Drosophila sturtevanti belongs to the *saltans* group of *Drosophila* (family: Drosophilidae, subfamily: *Sophophora*) and presents broad geographic distribution within the *sturtevanti* subgroup and, even, within the *saltans* group (Magalhães, 1962). It is present in nearly the entire group distribution area, from Mexico to Southern Brazil and in the Caribbean islands (Magalhães, 1962). This is a generalist species, common in forest fragments and highly abundant throughout different seasons of the year, which bears proper traits to be used as a model organism in population studies (Penariol and Madi-Ravazzi, 2013; Trava, 2018) . The *saltans* group is comprised of 21 species, being subdivided in five subgroups in accordance with the morphological characters, especially those of the male terminalia, which are *cordata* (two species), *elliptica* (four species), *parasaltans* (two species), *sturtevanti* (six species), and *saltans* (seven species) (Sturtevanti, 1942; Magalhães and Björnberg, 1957; Magalhães, 1962; Mourão and Bicudo, 1967).

The study of the population structure of groups of *Drosophila* is important to obtain knowledge of the demographic and evolutionary history of the groups during geological events, such as, for example, through the study of mitochondrial markers; Zorzato (2019) observed that natural populations of *D sturtevanti* were structured, indicating a correlation with geographic and climatic variables. The events that have occurred since the Pleistocene have shaped the geographical distribution of the populations of this species. Studies of this type are important and can be carried out using SSRs. The development of this type of marker is essential for investigating *D. sturtevanti*, and transferability tests would facilitate studies on the evolutionary dynamics of the *Drosophila saltans* group.

Our objective was to design, validate, and optimize pairs of microsatellite initiators for *D. sturtevanti* and assess their to various species of the *saltans* group, with the objective of determining their applicability for population studies of this species group.

MATERIAL AND METHODS

Genomic library of *D. sturtevanti*

The extraction of the genomic DNA from males of the *D. sturtevanti* species (from the region of Matão, São Paulo) was performed through individual maceration of the samples, using the Salting out DNA extraction protocol (Sunnucks et al., 1996; Aljanabi and Martinez, 1997).

The construction of the genomic library of *D. sturtevanti* was carried out in accordance with Billotte et al. (1999). The DNA samples were digested using endonuclease *Afa* I (Invitrogen, Carlsbad, CA, USA) and linked to the double chain of *Afa* I adaptors (5'-CTCTTGCTTACGCGTGGACTA-3') and (5'-TAGTCCACGCGTAAGCAAGAGCACA-3'). Biotin-linked probes and magnetic spheres coated with streptavidin (paramagnetic particles of Promega, Madison, WI, USA) were used to find fragments of the (GT)8 and (CT)8 types. The fragments of DNA captured were amplified by PCR and cloned with the pGEM-T Easy vector (Promega, Madison, WI, USA). The *E. coli* XL1-Blue (Agilent Technologies, Santa Clara, CA, USA) was transformed with recombinant plasmids through the method of electroporation, and cultivated in agar containing ampicillin, X-galactosidase at 2%, and IPTG in its composition. The positive clones were selected at random and sequenced in the automated ABI 3500xL analyzer *(*Applied Biosystems, Foster City, CA, USA) whilst using the T7 and SP6 initiators and a *Big Dye Terminator* 3.1 version with sequencing kit (Applied Biosystems, Foster City, CA, USA).

The identification of the genomic regions containing the microsatellites and the designs of the pairs of initiators that flanked those regions were performed as follows: for the removal of the adaptors, we used *Chromas* software; then we removed the low-quality regions using *Chromatogram Explorer* software, and aligned the sequences with the *BioEdit* software to create consensus. After editing the sequence, the *VecScreen* program was used to check for traces of the vector. For this analysis, 16 sequences that presented a region containing microsatellite repeat were obtained. We used *Primer3Plus* software (Untergasser et al., 2007) to obtain the specific initiators of these sequences with the following criteria: initiator 22 bp in size; fusion temperature (Tm) between 50 and 60°C; amplified product length between 100 and 500 bp; GC concentration between 50 and 60%. After the initiator sequences had been acquired, they were synthesized (Sigma-Aldrich, San Luis, MO, USA).

The amplification of the microsatellite primers was tested using 15 males of *D. sturtevanti* collected from Matão, São Paulo. The PCR was run in a total volume of 25 µL: 0.1 uL of Taq DNA polymerase (1U), 2.5 µL of 10x buffer solution, 2.5 µL of dNTP (0.04 pmol of each dNTP), 2.5 µL of each primer (0.01pmol of each primer), 0.75 µL of MgCL2 (50 mM), 1 µL of DNA, and 13.15 µL of ultrapure water. For all of the primers, we performed touchdown PCR: denaturation cycle at 94ºC for 2 min, 2 times 10 cycles of 94ºC for 1 min, 65^{o}C (-1^{o}C per cycle) for 1 min and, 72ºC for 2 min; and a final 18 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 5 min. Later, as needed, the primers that amplified were standardized for the best annealing temperature (Ta), ranging between 50 and 65ºC, in

the conditions of 94ºC over 2 min; 30 cycles of 94º C for 1 min, Ta for 1 min and 72ºC for 2 min, and 72ºC for 5 min. The amplification products of the PCR were viewed in 6% polyacrylamide gel based on Sanguinetti et al. (1994) and stained with 15% silver nitrate.

Transferability test

For this study, 14 species from the *saltans* group were used, all obtained from our lab cultures; they were identified based on the aedeagus, the main characteristic indicated to identify cryptic species (Vilela and Bächli, 1990). The genomic DNA of three males of each of 14 species of the *saltans* group (Table 1) was extracted by individual maceration of the samples using Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The PCR was performed with the same parameters as for the source species (*D. sturtevanti*) in touchdown PCR. *Drosophila sturtevanti* was used as a positive control. The amplification products were viewed in 6% polyacrylamide gel based on Sanguinetti et al. (1994) and stained with 15% silver nitrate.

Table 1. Subgroups, species, and location of the samples analyzed for the transferability test for the *Drosophila* species.

Subgroups	Species	Localization
sturtevanti	*D. sturtevanti* (control)	Matão, São Paulo, Brazil
	D. dacunhai	Pétionville, Haiti
	D. milleri	El Yunque, Puerto Rico
saltans	*D. prosaltans*	Matão, São Paulo, Brazil
	D. austrosaltans	Nova Granada, São Paulo, Brazil
	D. septentriosaltans	French Guiana
	D. pseudosaltans	Cantareira, São Paulo, Brazil
	D. nigrosaltans	French Guiana
	D. lusaltans	Pétionville, Haiti
	D. saltans	San José, Costa Rica
parasaltans	*D. parasaltans*	Belém, Pará, Brazil
cordata	*D. neocordata*	Campo Grande, Mato Grosso do Sul, Brazil
elliptica	*D. emarginata*	French Guiana
	D. neosaltans	Rio de Janeiro, Rio de Janeiro, Brazil
	D. neoelliptica	Aguaí, Santa Catarina, Brazil

RESULTS AND DISCUSSION

Among the 16 primer pairs tested on *D. sturtevanti*, three did not amplify (Table 2). The Dsturt_B, Dsturt_C, Dsturt_D, Dsturt_I, Dsturt_K, Dsturt_M, and Dsturt_P primers gave good amplification at touchdown; for the others, the best annealing temperatures were standardized (Table 2). The 13 microsatellites that amplified were used in a study of population structure of *D. sturtevanti* originated from nine geographic locations in Brazil, and all of them were polymorphic (Trava, 2018). We detected a moderate genetic differentiation among the populations of this species (Trava, 2018). These primers were also tested for other species of the *saltans* group.

The heterologous amplification resulted in an overall rate of 49% of positive transfers and 86% of these did not require optimization. The species that have achieved greater success of heterologous amplification were those of the *sturtevanti* subgroup (*D.*

sturtevanti, D. dacunhai and *D. milleri)*, with 88% of transferability. From the 13 microsatellites tested in total, 12 amplified in *D. dacunhai*; and 11 in *D. milleri*. The value of transferability for the other subgroups was 48% for the *saltans* subgroup, 44% for the *elliptica* subgroup, 31% for the *parasaltans* subgroup, and 15% for the *cordata* subgroup (Table 3). The second subgroup that showed greatest success of amplification was the *saltans* subgroup; *D. prosaltans* and *D. austrosaltans* presented 9 microsatellite amplifications each (Table 3).

Table 2. Testing of 16 SSR markers synthesized for *Drosophila sturtevanti*, including primer sequences (forward and reverse); repeat motif; sequenced product size (bp) and annealing temperature (Ta). $TD_{65\text{-}55}$ = touchdown PCR with temperatures ranging between 65 and 55º C; + = Amplified; - = Not Amplified.

Locus	Primers sequence (5´- 3´)	Repeat motif	size (bp)	Ta (Cº)	*D. sturtevanti*
Dsturt_A	GTAAGCGCTGACTGGTCAAC CCTTTTCCTCTAGTCGCAGT	$(AC)_{11}$	262	61ºC	+
Dsturt_B	GCTCTTTCGGATTTGCTGTG GCCACTTTCGAAGAGTCAAG	$(GT)_5$	117	$TD_{65\text{-}55}$	+
Dsturt_C	GTGTTCATAAGGTGCCATC CTGACCACCCACAAGGAAA	$(TG)_6$	169	$TD_{65\text{-}55}$	+
Dsturt_D	GACTGAGTCTATCCATGGGC CAAGTCACGTTTGCTGACAC	$(GAT)_7$	141	$TD_{65\text{-}55}$	+
Dsturt_E	ATGATGACTTCCGCTACTCG GTGTAGGTGTGAGTGAGGAG	$(CAA)_5$	236	56ºC	+
Dsturt_F	GTATGCAATCTCGCTCACAC CAAAAACACTTGCTATGCGC	$(GA)_7$	197	$TD_{65\text{-}55}$	-
Dsturt_G	ACAGGGCTTTAGCATCTTGA ATTATCCCAGGCGATTGTGT	$(AC)_{11}$	227	55ºC	+
Dsturt_H	ACAGCTGCATGATAATCCCA GACGATGAGAATGCGAATGG	$(CA)_{10}...(AG)_{18}$	286	$TD_{65\text{-}55}$	-
Dsturt_I	ACAGCTGCATGATAATCCCA CAGTGACGAGTTGAGGAGTA	$(CA)_{10}$	178	$TD_{65\text{-}55}$	+
Dsturt_J	GCTGGCGCTAAAAGAAAGAA GTGTTGAAATGATGTCGGCA	$(ACAT)_8$	225	65ºC	+
Dsturt_K	TTTCCCTGCTTGTTGTCTCT AGTTCTCGGTTCTCGTTGAA	$(GT)_5...(TG)_8$	265	$TD_{65\text{-}55}$	+
Dsturt_L	CGAGCATTTTGTCGGAGTTT ATAGGCGGAAAAGAAGGAGG	$(CA)_9$	205	56ºC	+
Dsturt_M	AACCAGTTGTGTTCTGTTGC TCAGTTGGAGCCAAGTCAAT	$(TG)_{13}$	210	$TD_{65\text{-}55}$	+
Dsturt_N	CCAATTTTTCTAGCCCAGGC GAACTTGGAAACCGACTTGG	$(CA)_{12}$	262	53ºC	+
Dsturt_O	GAACTTGGAAACCGACTTGG AGAGGAATCGAAACGTAGGG	$(CA)_{11}...(CA)_5$	163	57ºC	+
Dsturt_P	ATATGTGGTGAGCTTGGAGG ATGGGAATCATCCTTGGACC	$(AC)_{10}$	195	$TD_{65\text{-}55}$	-

Laborda et al. (2009) tested specific microsatellites of *D. mediopunctanta* in other species and achieved amplification for both distant and close phylogenetically species. Moreover, they observed transferability for *D. sturtevanti*, while that transferability (microsatellites of *D. mediopunctata* for populations of *D. sturtevanti*) was not confirmed by Trava et al. (2016). Prestes et al. (2015) also used microsatellites of *D. mediopunctata* in the species of the *guarani* group (*D. ornatifrons*) and in species of the *guaramunu* group (*D. maculifrons*; *D. grisolineata*) with greater transferability taking place between species of different groups, rather than between species of the same group (Prestes et al., 2015).

Table 3. Amplification data from 13 microsatellites of *D. sturtevanti* in 14 species of *Drosophila* from the *saltans* group. N = Number of amplifications per species. + Amplified; - Not amplified; +- Amplified but required adjustments in the reagents.

Subgroups	Species	Dsturt_ A	Dsturt_ B	Dsturt_ C	Dsturt_ D	Dsturt_ E	Dsturt_ G	Dsturt_ I	Dsturt_ J	Dsturt_ K	Dsturt_ L	Dsturt_ M	Dsturt_ N	Dsturt_ O	N
sturtevanti	*D. sturtevanti* (positive control)	+	+	+	+	+	+	+	+	+	+	+	+	+	
sturtevanti	*D. dacunhai*	-	+	+	+	+	+	+	+	+	+	+	+	+	12
	D. milleri	-	+	+	+	+	+	+	+	+	+	+	+	-	11
saltans	*D. prosaltans*	-	+	+	+	+	+	-	+	-	-	+	+	+	9
	D. austrosaltans	-	+	+	+	+	+	+-	-	+-	-	-	+	+	9
	D. septentriosaltans	-	+	-	+	+	-	-	-	-	-	+	-	-	4
	D. pseudosaltans	-	+	-	+	+	-	+-	+	-	+	+	-	+-	8
	D. nigrosaltans	-	+	-	+	+	+-	-	-	-	-	+	-	-	5
	D. lusaltans	-	+	-	+	-	-	+-	-	-	-	+-	-	+	5
	D. saltans	-	+	-	+	+	-	-	-	-	-	+	-	-	4
parasaltans	*D. parasaltans*	-	+	-	+	-	-	-	+	-	+-	-	-	-	4
cordata	*D. neocordata*	-	+	-	+-	-	-	-	-	-	-	-	-	-	2
elliptica	*D. emarginata*	-	+	-	+	+	-	-	-	-	-	+-	-	-	4
	D. neosaltans	-	+	-	+	+	+-	+-	-	-	-	+	-	-	6
	D. neoelliptica	-	+	-	+	+	+-	-	+	-	-	+	-	+	7

In our study, the development of microsatellites markers for *D. sturtevanti* proved to be useful due to its high degree of specificity for this species. Furthermore, for the transferability test, the specific microsatellites of *D. sturtevanti* were highly efficient for the species included in the *sturtevanti* subgroup and some of them also for species of the other subgroups, especially the *saltans* subgroup. Therefore they could be useful for population and evolutionary studies in the species belonging to the *saltans* group of *Drosophila* and, possibly, for phylogenetically related groups, such as the *willistoni* group.

ACKNOWLEGDMENTS

We thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), for the financial support (Number processes: 2014/14059-0; 2015/17579-7; 2017/05344-0). We also thank the research team of the laboratory of Professor Anete Pereira de Souza at the Universidade Estadual de Campinas - UNICAMP, for aiding in the production of the genomic library.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Akkaya MS, Bhagwat AA and Cregan PB (1992). Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics*. 132(4): 1131-1139.

Aljanabi SM and Martinez I (1997). Universal and rapid salt-extraction of high-quality genomic DNA for PCR-based techniques. *Nucleic. Acids. Res*. 25(22): 4692-4693.

Billotte N, Lagoda PJL, Risterucci AM and Baurens FC (1999). Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. *Fruits*. 54: 277-88.

Ellis JR and Burke JM (2007). EST–SSRs as a resource for population genetic analyses. *Heredity*. 99: 125-132.

Fantin C, Carvalho CF, Hrbek, T, Sites Jr JW, et al. (2007). Microsatellite DNA markers for *Podocnemis unifilis,* the endangered yellow-spotted Amazon River turtle. *Mol. Ecol. Notes.* 7(6): 1235-1238.

Ferreira ME and Grattapaglia D (1998). Introdução ao uso de marcadores moleculares em análise genética. 3rd edn, Embrapa‑Cenargen, Brasília.

Jarne P and Lagoda PJL (1996). Microsatellites, from molecules to populations and back. *Trends. Ecol. Evol.* 11(10): 424-429.

Kalia RJ, Rai MK, Kalia S, Singh R, et al. (2011). Microsatellite markers: an overview of the recent progress in plants. *Euphytica.* 177: 309-334.

Knapik EW, Goodman A, Ekker M, Chevrette M, et al. (1998). A microsatellite genetic linkage map for zebrafish (*Danio rerio*). *Nat. Genet.* 18: 338-343.

Laborda PR, Klaczko LB and Souza AP (2009). *Drosophila mediopunctata* microsatellites II: cross-species amplification in the *tripunctata* group and other *Drosophila* species. *Conserv. Genet. Resour.* 1(1): 281-296.

Luikart G, England PR, Tallmon D, Jordan S, et al. (2003). The power and promise of population genomics: from genotyping to genome typing. *Nat. Rev. Genet.* 4: 981-994.

Magalhães LE (1962). Notes on the taxonomy, morphology, and distribution of *saltans* group of *Drosophila*, with description of four new species. *Studies in Genetics* 2: 135-154.

Magalhães LE and Bjornberg AJS (1957). Estudo da genitália masculina de *Drosophila* do grupo *saltans* (Diptera). *Rev. Bras. Biol.* 17(4): 435-450.

Morgante M, Hanafey H and Powell W (2002). Microsatellites are preferentially associated with non-repetitive DNA in plant genome. *Nat. Genet.* 30: 194-200.

Mourão CA and Bicudo HEMC (1967). Duas novas espécies de *Drosophila* do grupo *saltans* (Drosophilidae, Diptera). *Pap. Avulsos Zool*. 20: 123-134.

Oliveira FA, Cidade FW, Fávero AP, Vigna BBZ, et al. (2016). First microsatellite markers for *Paspalum plicatulum* (Poaceae) characterization and cross-amplification in different *Paspalum* species of the *Plicatula* group. *BMC Res. Notes.* 9(511): 1-13.

Penariol LV and Madi-Ravazzi L (2013). Edge-interior differences in the species richness and abundance of drosophilids in a semideciduous forest fragment. *SpringerPlus*. 2(1): 114.

Prestes JO, Beira AS, Machado LPB and Mateus RP (2015). Microsatellite heterologous amplification in individual samples of *Drosophila griseolineata. Drosoph. Inf. Serv.* 98: 35-37.

Sanguinetti C, Dias Neto E and Simpson AJG (1994). RAPD silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques.* 17(5): 209-214.

Schuler GD, Boguski MS, Stewart EA, Stein LD, et al. (1996). A gene map of the human genome. *Science.* 274(5287): 540-546.

Sturtevanti AH (1942). The classification of the genus *Drosophila*, with description of nine new species. *University of Texas Public.* 4213: 5-51.

Sunnucks P, England PE, Taylor AC and Hales DF (1996). Microsatellite and chromosome evolution of parthenogenetic *Sitobion aphids* in Australia. *Genetics*. 144(2): 747-756.

Tautz D (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids. Res.* 17(16): 6463-6471.

Tractz CC, Salomon GR, Zorzato SV, Machado LPB, et al. (2012). Allele diversity of cross-species microsatellite amplification on populations of *Drosophila guarani* species group from Araucaria Forest in Brazil. *Drosoph. Inf. Serv.* 95: 76-79.

Trava BM (2018). Estrutura populacional de *Drosophila sturtevanti* (subgrupo *sturtevanti*; grupo *saltans*) por meio de microssatélites espécie-específico e biodiversidade de drosofilídeos em domínios da Mata Atlântica. Master's thesis. Universidade Estadual Paulista "Júlio de Mesquita Filho", São José do Rio Preto. Available at [https://repositorio.unesp.br/handle/11449/154052].

Trava BM, Machado LPB, Mateus RP and Madi-Ravazzi L (2016). Transferability of SRR primers developed for *D. mediopunctata* to the species *D. sturtevanti. Drosoph. Inf. Serv.* 99: 16-18.

Turchetto-zolet AC, Turchetto C, Zanella CM and Passeia G (2017). Marcadores moleculares na Era Genômica: Metodologias e Aplicações. Sociedade Brasileira de Genética, Ribeirão Preto.

Untergasser A, Nijveen H, Rao X, Bisseling T, et al. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic. Acids. Res*. 35: 71-74.

Varshney RK, Thudi M, Aggarwal R and Borner A (2007). Genic molecular markers in plants: development and applications. In: Genomics-Assisted Crop Improvement (Varshney RK, Tuberosa R, eds.). Springer, Dordrecht.

Vilela CR and Bächli G (1990). Taxonomic studies on neotropical species of seven genera of Drosophilidae (Diptera). *Mitt. Schweiz. Entomol. Ges.* 63:1-332.

Weber JL (1990). Informativeness of human (dC-dA)n . (dG-dT) n polymorphisms. *Genomics*. 7(4): 524-530.

Zorzato SV (2019). Filogeografia de *Drosophila sturtevanti* (Diptera: Drosophilidae) em biomas Neotropicais. Master's thesis. Universidade Estadual Paulista "Júlio de Mesquita Filho", São José do Rio Preto. Available at [https://repositorio.unesp.br/handle/11449/191371].