# Development and Transferability of Microsatellites Markers for Species of the *Saltans* Group of *Drosophila* (Diptera: Drosophilidae)

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Genet. Mol. Res. 19 (4): gmr16039987
Received: August 03, 2020
Accepted: August 10, 2020
Published: August 17, 2020



**ABSTRACT.** The microsatellite markers (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 specific SSRs for a given species opens possibilities for the application, by means of transferability, in population studies of other species that are phylogenetically related, thereby optimizing time and cost. This study describes, for the first time, 16 primers pairs developed for *Drosophila sturtevanti*, which were tested their transferability for 14 species of the *saltans* group of *Drosophila*. The adequate conditions of amplification were established by using 15 males of *D. sturtevanti*. The total percentage of transfer has been 49.45%. The species with the highest success rates of heterologous amplification have been included in the *sturtevanti* subgroup. Two microsatellite markers have amplified in all the species, whilst one would not amplify in any of the species of the *saltans* group. The data corroborate the use of the technique for the transferability of these oligonucleotides, which may be used in studies of genetic diversity and population structure of *D. sturtevanti* and other species of the *saltans* group of *Drosophila*.

**Keywords:** Genetic diversity; Genomic library; Molecular markers; Neutral markers; SSR

## INTRODUCTION

The microsatellite or Simple Sequence Repeats (SSR) markers are species-specific, highly versatile, relatively abundant, codominant, and display high levels of polymorphisms [1-4] and it have been widely used in genetic studies [5]. The high levels of polymorphisms observed in the microsatellites and the relative ease of detection through PCR combined with a robust and efficient statistical analysis has been favouring the use of the same in many biological areas, such as: forensics biology, genetic mapping, paternity tests, genetics of populations and conservation [6-10].

The development of new SSR markers is an elaborate process which requires high cost and time; so, the method of transferability of SSR markers and advantageous, as it optimizes time and renders cost viable [11,12]. The transferability may take place due to the conservation of microsatellite sites between related species, enabling the realization of site transfer between species belonging to the same genus, or even to different genus, which makes possible the use of heterologous initiators [12,13]. Thus, the pairs of initiators designed with based on the sequences obtained from a species specific may be used to detect SSRs in related species, this method of transferability has been successfully demonstrated in a number of species [14-18]. However, transferability is not always positive. For instance, initiators designed for *Drosophila mediopunctata* have been tested in populations of *D. sturtevanti* and were not efficient in their results, probably because are phylogenetically distant species [19].

*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. It is present in nearly the entire group distribution area, from Mexico to Southern Brazil and in the Caribbean islands [20,21]. 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. The *saltans* group is comprised by 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* (three species), *sturtevanti* (five species), and *saltans* (seven species) [22,23]. This work had the purpose of designing, validating, and optimizing pairs of microsatellite initiators for populations of *D. sturtevanti*, and of assessing the transferability of these oligonucleotides in 14 species of the *saltans* group, with aims at their applicability in population studies of this species group.

## MATERIALS 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, SP) has been performed through the individual maceration of the samples, whilst following the Salting out DNA extraction protocol [24,25]. The construction of the genomic library of *D. sturtevanti* has been carried out in accordance with Billotte et al. [7]. The DNA samples were digested using endonuclease *Afa I (Invitrogen)* 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*) were used to find fragments of the (GT) 8 and (CT) 8 types. The fragments of DNA captured were amplified by PCR and cloned by the pGEM-T Easy vector (*Promega*). The *Escherichia coli* XL1-Blue (*Agilent Technologies*) bacteria 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 analyser (*Applied Biosystems*) whilst using the T7 and SP6 initiators and a *Big Dye Terminator* 3.1 version with sequencing kit (*Applied Biosystems*).

The identification of the genomic regions containing the microsatellites and the designs of the pairs of initiators which flanked those regions were performed whilst using the applications that follow: for the removal of

the adaptors, we have resorted to *Chromas* software; then we removed the low-quality regions through the *Chromatogram Explorer* software, and aligned the sequences in the *BioEdit* software to create consensus. After edited sequence, the *VecScreen* program was used to check for traces of the vector. For this analysis, 16 sequences which presented a region containing microsatellite repeat were obtained. We used the *Primer3Plus* software [26] to obtain the specific initiators of these sequences with the following criteria: initiator with 22 pb in size; fusion temperature (Tm) between 50°C and 60°C; amplified product length between 100 and 500 pb; GC concentration between 50 and 60%. Once the initiator sequences have been acquired, they were synthesized in the Sigma-Aldrich (Darmstandt, Germany, GER).

The amplification of the microsatellites primers was tested in 15 males of *D. sturtevanti* from Matão, SP. The PCR consisted 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 $MgCl_2$ (50 mM), 1 µL of DNA, and 13.15 µL of ultrapure water. For all of the primers, we have performed PCR at touchdown, denaturation cycle at 94ºC for 2 minutes, 2 times 10 cycles of 94ºC for 1 minute, 65oC (-1oC per cycle) for 1 minute and, 72ºC for 2 minutes; and a final of 18 cycles of 94ºC over 1 minute, 55ºC for 1 minute and 72ºC for 5 minutes. Later, as needed, the primers which amplified would be standardized in regard of the best annealing temperature (Ta), ranging between 50 and 65ºC, in the conditions of 94ºC over 2 minutes; 30 cycles of 94º C for 1 minute, Ta for 1 minute and 72ºC for 2 minutes; and 72ºC for 5 minutes. The amplification products of the PCR were viewed in 6% polyacrylamide gel stained with 15% silver nitrate with modifications [24].

**Transferability test**

The genomic DNA of three males from each one of the 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 Polymerase Chain Reaction (PCR) was performed with the same parameters of the source specie (*D. sturtevanti*). *Drosophila sturtevanti* was used as positive control. The amplification products were viewed in polyacrylamide gel at 6% with silver nitrate at 15% [24] with modifications).

| Subgroups | Species | Localization | N |
|---|---|---|---|
| *sturtevanti* | *D. sturtevanti* (control) | Matão, São Paulo, Brazil | 3 |
| | *D. dacunhai* | Pétionville, Haiti | 3 |
| | *D. milleri* | El Yunque, Puerto Rico | 3 |
| *saltans* | *D. prosaltans* | Matão, São Paulo, Brazil | 3 |
| | *D. austrosaltans* | Nova Granada, São Paulo, Brazil | 3 |
| | *D. septentriosaltans* | French Guiana | 3 |
| | *D. pseudosaltans* | Cantareira, São Paulo, Brazil | 3 |
| | *D. nigrosaltans* | French Guiana | 3 |
| | *D. lusaltans* | Pétionville, Haiti | 3 |
| | *D. saltans* | San José, Costa Rica | 3 |
| *parasaltans* | *D. parasaltans* | Belém, Pará, Brazil | 3 |
| *cordata* | *D. neocordata* | Campo Grande, Mato Grosso do Sul, Brazil | 3 |
| *elliptica* | *D. emarginata* | French Guiana | 3 |
| | *D. neosaltans* | Rio de Janeiro, Rio de Janeiro, Brazil | 3 |
| | *D. neoelliptica* | Aguaí, Santa Catarina, Brazil | 3 |
| Total | | | 42 |

**Table 1.** Subgroups, species, localization of the samples, and number (N) of individuals analyzed for the transferability test.

## RESULTS AND DISCUSSION

From the 16 primer pairs tested in *D. sturtevanti*, three of them have not amplified (Dsturt_F, Dsturt_H, and Dsturt_P). The Dsturt_B, Dsturt_C, Dsturt_D, Dsturt_I, Dsturt_K, Dsturt_M, and Dsturt_P primers have had good amplification at touchdown and, for the others, the best annealing temperatures were standardized (Table 2). The 13 microsatellites which have succeeded in the amplification were used in a study of population structure of *D. sturtevanti* originated from nine geographic locations in Brazil, and all of them were polymorphic ([19] unpublished results), and they were also tested for other species of the *saltans* group.

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

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

The heterologous amplification resulted in an overall rate of 49.45% of positive transfers, in which 85.55% the total would not require optimization. The species that have achieved greater success of heterologous amplification were those of the *sturtevanti* subgroup, with 88.46% of transferability. For the *D. dacunhai*, there have been 12 positive amplifications (92.31%); and 11 for the *D. milleri* (84.61%). The value of transferability for the other subgroup has been 48.35% for the *saltans* subgroup, 43.59% for the *elliptica* subgroup, 30.77% for the *parasaltans* subgroup, and 15.38% for the *cordata* subgroup (Table 3).

| **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** | **Nº** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *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 |

**Table 3.** Amplification data from 13 microsatellites of *D. sturtevanti* in 14 species of *Drosophila* from the *saltans* group. Nº = Number of amplifications per species. + Amplified; - Not amplified; +- Amplified but requires adjustments in the reagents.

The microsatellites have presented greater success of amplification for the species belonging to the *sturtevanti* subgroup (*D. sturtevanti, D. dacunhai* and *D. milleri*). *Drosophila prosaltans* and *D. austrosaltans (saltans* subgroup) has also presented a relatively high number of amplification (9 microsatellites –69.23% each one). The work of Laborda et al. [16], testing specific microsatellites of *D. mediopunctanta* in other species achieved positive amplification for both distant and close phylogenetically species. Moreover, in the work of this author, positive transferability was observed for the *D. sturtevanti*, while that transferability (microsatellites of *D. mediopunctata* for populations of *D. sturtevanti*) was not achieved in the work of Trava et al. [19]. Prestes et al. [27] also used the microsatellites de *D. mediopunctata* in the species of the guarani group (*D. ornatifrons*) and in the 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 [27,28].

## CONCLUSION

In this study, the specific microsatellites of *D. sturtevanti* have shown to be highly efficient for the species included in the *sturtevanti* subgroup and some of them also for the species of the other subgroup, especially the *saltans* subgroup. Thereby rendering its use possible in 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.

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