

# Phylogenetic Relationships Among Species Subgroups in the *Drosophila saltans* Group (Diptera: Drosophilidae): Can Morphology Solve a Molecular Conflict?

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**Abstract:** Proper phylogenetic reconstruction is crucial for understanding many evolutionary phenomena. In spite of the great success of molecular phylogenetics, DNA signal still may be limited by some intrinsic constraints such as codon usage bias. The phylogenetic relationships between the five species subgroups of the *Drosophila saltans* group are a good example of conflicting molecular phylogenies drawn from different genes due to an ancestral substitutional shift. Here, forty morphological characters were analyzed using the same set of species used in previous molecular studies, with at least a single representative of each subgroup. The cladistic analysis was in disagreement with most of the previous hypotheses in placing the *sturtevanti* subgroup as an early branch, whereas the four remaining subgroups form a well supported clade that can be further subdivided into two sister clades: one containing the *cordata* and the *elliptica* subgroups, whereas the second includes the *parasaltans* and the *saltans* subgroups. The molecular evolution (codon usage bias) of the *saltans* group were revised in light of the present finding. The analysis highlights the important role of morphology in phylogeny reconstruction and in understanding molecular evolutionary phenomena.

**Key words:** *Sophophora*; Codon usage bias; Neotropical region; Cladistics

## 果蝇 *Drosophila saltans* 种亚组 (双翅目: 果蝇科) 系统发育关系: 形态学能否解决分子冲突?

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**摘要:** 正确的系统发生重建对于理解进化事件至关重要。尽管分子系统学对于解决此类问题取得了极大的成功, 由于一些诸如密码子使用偏性等内在约束, 来源于 DNA 的信息可能仍然存在着局限。因为发生在祖先的替代性转换, 果蝇 *Drosophila saltans* 5 个种亚组由不同基因构建的分子系统树之间存在着冲突 (在以往发表的分子系统学研究中, 这些种组的每一个种亚组至少有一个代表)。本文用 40 个形态学特征重新分析了这些种组。不同于以前发表的大多数假说, 本研究支序分类学的结果表明, 果蝇 *sturtevanti* 种亚组是一个较早的分支, 而剩下的 4 个亚组形成一个支持度较高的类群; 后者又可以再分为两个姐妹群: 一个包含 *cordata* 和 *elliptica* 亚组, 另一个包含 *parasaltans* 和 *saltans* 亚组。本研究结果修正了果蝇 *saltans* 种组的分子进化 (密码子使用偏性), 并强调形态学对于系统发生重建和理解分子进化现象的重要作用。

**关键词:** 水果果蝇亚属; 密码子使用偏性; 新热带区; 支序分类学

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Molecular data, especially DNA sequences, have revolutionized the practice of systematics, starting from taxonomic identification to phylogenetic reconstruction and historical biogeography (Tautz et al, 2003; Volger &

Monaghan, 2006). In phylogenetics, many authors have even argued that morphological characters would no longer have a major role, neither alone nor combined with molecular data, and that morphology at best can

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only be analyzed within a molecular context (Hillis, 1987; Baker & Gatesy, 2002; Scotland et al, 2003; Wortley & Scotland, 2006). Nonetheless, in spite of its great usefulness, molecular phylogenetics still has many limitations. The first important one is low taxon sampling, as most of museum-preserved or extinct taxa are not suitable for DNA analyses. Furthermore, other limitations can arise from the nucleotide landscape itself, including low number of character states leading to higher level of homoplasy, different mutation rates among sites and degrees of genetic hitchhiking, biased gene conversion and/or to codon usage bias (Lynch, 2007). This usually results into the conflict between trees drawn from different genes, which mislead the interpretation of species trees from gene trees.

Species of the *Drosophila saltans* group are a good example to illustrate such molecular conflicts. These are 21 species predominant in the Neotropical region and characterized by their dark color. They form with species of the *willistoni* group the two major groups of the New World radiation of the subgenus *Sophophora*. Sturtevant (1942) divided the *saltans* group into two subgroups based on thoracic ornamentation, but they were later further classified under five subgroups on the basis of male genitalia (Magalhães & Björnberg, 1957, Magalhães, 1962): *cordata* (2 species), *elliptica* (4 spp.), *parasaltans* (2 spp.), *saltans* (7 spp.), and *sturtevanti* (6 spp.). Throckmorton & Magalhães (1962) proposed the first phylogeny of the subgroups on the basis of their external and internal anatomical comparisons published independently in the same bulletin (Magalhães, 1962; Throckmorton, 1962). Their phylogeny, which was not

built upon a cladistic analysis of their data, showed “an orderly progression from the more primitive *cordata* and *elliptica* subgroups, through the *sturtevanti* and *parasaltans* subgroups, to the *saltans* subgroup” (Tab. 1). However, later molecular phylogenetic studies based on different mitochondrial and nuclear genes and using at least one representative species from each subgroup, failed to confirm Throckmorton’s hypothesis (Pélandakis & Solignac, 1993; O’Grady et al, 1998; Rodríguez-Trelles et al, 1999a). Moreover, although all genes highly confirmed the monophyly of the group and the subgroups, different genes gave different topologies concerning the relationships among the subgroups, and even within the most sampled *saltans* subgroup (O’Grady & Kidwell, 2002). Tab. 1 summarizes the phylogenetic hypotheses between the subgroups according to different genes.

The low phylogenetic signal in coding nuclear sequences in the *saltans* group (*Adh* and *Xdh*) and their discrepancy with other mitochondrial (*COI* and *COII*) and with non-coding nuclear gene (*ITS1* and introns of *Xdh*) may be referred to the characteristic shift in codon bias in New World Sophophorans (Anderson et al, 1993; Powell & Moriyama, 1997; Rodríguez-Trelles et al, 1999b, 2000a,b; Tarrío et al, 2000, 2001; Powell et al, 2003; Singh et al, 2006; Vicario et al, 2007). If it turns to be a whole genome phenomenon, which is true for *D. willistoni* (Vicario et al, 2007), even the future addition of more genes may not increase the signal of nuclear data. Because the relationships among the subgroups appear to be deep, mitochondrial and non-coding nuclear sequences might not be equally adequate.

**Tab. 1 Summary of conflicting phylogenetic hypotheses within the *Drosophila saltans* group from previous studies**

Data	Topology	Method	Source
Morphology1	(CO(EL(ST(PA,SA))))	---	Throckmorton & Magalhães (1962)
Morphology2	(ST,SA(PA(CO,EL)))	MP	Magalhães (1962) in O’Grady et al (1998)
28S	(ST(EL(CO,SA)))	MP	Pélandakis & Solignac (1993)
<i>COI</i>	(CO,EL(PA,SA,ST))	MP	O’Grady et al (1998)
<i>COII</i>	(PA(CO,EL,SA,ST))	MP	O’Grady et al (1998)
<i>ITS1</i>	((CO,EL),PA,SA,ST)	MP	O’Grady et al (1998)
<i>Adh</i>	(CO,EL,PA,SA,ST)	MP	O’Grady et al (1998)
	(CO(SA(PA(EL,ST))))	NJ	Setta et al (2007)
Combined	(CO(EL(ST(PA,SA))))	MP	O’Grady et al (1998)
<i>Xdh</i>	(PA(ST(EL(CO,SA))))	ML	Rodríguez-Trelles et al (1999a)
	(PA(ST((EL,CO)SA)))	NJ	Rodríguez-Trelles et al (1999b)
	(PA(ST(EL(CO,SA))))	MP	Rodríguez-Trelles et al (1999b)

Subgroups are abbreviated as follows: CO = *cordata*, EL = *elliptica*, PA = *parasaltans*, SA = *saltans*, ST = *sturtevanti*; phylogenetic reconstruction methods are abbreviated as: --- = overall similarity, NJ = neighbor-joining, MP = maximum parsimony, ML = maximum likelihood. Combined tree was reconstructed from the analysis of COI+COII+ITS1+Adh+morphology2.



7. 6<sup>th</sup> sternite pigmentation in female, yellow coloration: 0 = absent; 1 = present (CI = 1.00, RI = 1.00, RC = 1.00).

*Male genitalia:*

8. Epandrial ventral lobe, shape: 0 = truncate or lobate; 1 = very prominent (CI = 0.33, RI = 0.00, RC = 0.00).
9. Epandrial ventral margin, horn-like process: 0 = absent; 1 = present (CI = 1.00, RI = 1.00, RC = 1.00).
10. Cercus, shape of medio-dorsal margin: 0 = rounded; 1 = U-shaped (CI = 1.00, RI = 1.00, RC = 1.00).
11. Surstylus, shape: 0 = elongate and curved ventrad; 1 = semi-elliptical (CI = 1.00, RI = 1.00, RC = 1.00).
12. Surstylus, number of prensisetae: 0 = less than 20; 1 = more than 20 (CI = 0.50, RI = 0.75, RC = 0.38).
13. Surstylus, sclerized flap at the anterior region of the interno-lateral margin: 0 = absent; 1 = present (CI = 1.00, RI = 1.00, RC = 1.00).
14. Decasternum, shape: 0 = small and thin; 1 = very large and strongly chitinized (CI = 1.00, RI = 1.00, RC = 1.00).
15. Hypandrium, shape: 0 = small; 1 = elongate; 2 = elongate with anterad restriction (CI = 0.67, RI = 0.67, RC = 0.44).
16. Hypandrium, orientation of lateral gonopods (= posterior parameres): 0 = parallel; 1 = slightly divergent; 2 = highly divergent (CI = 1.00, RI = 1.00, RC = 1.00).
17. Hypandrium, size of lateral gonopods: 0 = large; 1 = medium; 2 = small (CI = 1.00, RI = 1.00, RC = 1.00).
18. Hypandrium, shape of apical margin of lateral gonopods: 0 = concave; 1 = pointed (CI = 1.00, RI = 1.00, RC = 1.00).
19. Hypandrium, length of submedian seta: 0 = short; 1 = very long (CI = 1.00, RI = 1.00, RC = 1.00).
20. Aedeagus, shape: 0 = not cylindrical; 1 = cylindrical (CI = 1.00, RI = 1.00, RC = 1.00).
21. Aedeagus, lateral at dorsal margin: 0 = absent; 1 = present (CI = 1.00, RI = 1.00, RC = 1.00).
22. Aedeagus, pincer-like paraphyses (= anterior parameres): 0 = absent; 1 = present (CI = 0.50, RI = 0.67, RC = 0.44).
23. Aedeagus, size of pincer-like paraphyses: 0 = small; 1 = large (CI = 1.00, RI = 0/0, RC = 0/0).
24. Aedeagus, orientation of pincer-like paraphyses: 0 = parallel; 1 = divergent (CI = 1.00, RI = 1.00, RC = 1.00).
25. Aedeagus, disti-ventral process: 0 = absent; 1 = present (CI = 1.00, RI = 0/0, RC = 0/0).

*Male reproductive system:*

26. Paragonia (= accessory gland), coiling: 0 = partial; 1 = complete (CI = 1.00, RI = 1.00, RC = 1.00).
27. Paragonia, size: 0 = small; 1 = medium; 2 = large (CI = 0.67, RI = 0.67, RC = 0.44).
28. Paragonia, internal coil margin: 0 = separated; 1 = adhesive (CI = 0.50, RI = 0.67, RC = 0.33).
29. Paragonia, junction with vas deferens: 0 = separated; 1 = fused (CI = 0.50, RI = 0.67, RC = 0.33).
30. Ejaculatory bulb, anterior end: 0 = thin; 1 = slightly expanded (CI = 0.50, RI = 0.50, RC = 0.25).
31. Ejaculatory bulb, shape: 0 = elliptical; 1 = spherical (CI = 1.00, RI = 1.00, RC = 1.00).
32. Ejaculatory bulb, lateral lobes: 0 = absent; 1 = absent but caecum present; 2 = present with caecum (CI = 1.00, RI = 1.00, RC = 1.00).
33. Ejaculatory bulb, handle of apodeme: 0 = simple blade; 1 = cylindrical, flared apically and with a conical depression at the tip; 2 = triangular, with a slightly flared tip (CI = 1.00, RI = 1.00, RC = 1.00).
34. Testis, number of coils: 0 = 6-9; 1 = 9-12 (CI = 0.50, RI = 0.50, RC = 0.25).

*Female reproductive system:*

35. Spermatheca, base: 0 = telescoped with collar; 1 = with no collar (CI = 1.00, RI = 1.00, RC = 1.00).
36. Spermatheca, apical indentation: 0 = present; 1 = absent (CI = 1.00, RI = 1.00, RC = 1.00).
37. Spermatheca, apical introvert: 0 = present; 1 = absent (CI = 1.00, RI = 1.00, RC = 1.00).
38. Spermatheca, apical inner column: 0 = absent, 1 = present (CI = 0.50, RI = 0.00, RC = 0.00).

*Pupa:*

39. Anterior spiracles, number of branches: 0 = medium; 1 = few; 2 = many (CI = 1.00, RI = 1.00, RC = 1.00).

*Larva:*

40. Skipping behavior: 0 = absent; 1 = present (CI = 1.00, RI = 1.00, RC = 1.00).

## 2.2 Phylogenetic relationships

The analysis of the morphological characters resulted in a consensus tree of a length of 63 steps shown in Fig. 1. The positive skewness in the tree length frequency distribution (not shown) indicated the high phylogenetic signal in the characters used. This has been translated in the low homoplasy of the resulting tree (CI = 0.73, RI = 0.82, RC = 0.61), and the high bootstrap values at internal nodes.

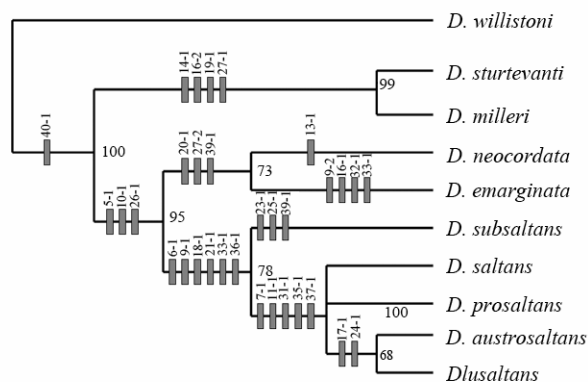


Fig. 1 50%-bootstrap consensus phylogenetic tree of the subgroups of the *Drosophila saltans* group deduced from the cladistic analysis of 40 morphological characters. Numbers besides internal nodes refer to the bootstrap value after 100 iterations. Syn- and autapomorphies are shown as solid bars on internal branches, followed by the character number and state as given in text.

The *sturtevantii* subgroup represents the early branch. The remaining subgroups form two sister clades. One clade includes the *cordata* and the *elliptica* subgroups, whereas the other includes the *parasaltans* and the *saltans* subgroups. Relationships within the *saltans* subgroup are not well resolved, only *D. lusaltans* and *D. austrosaltans* form a well-supported monophyletic clade, that form with the other two species, *D. saltans* and *D. prosaltans* a polytome.

## 3 Discussion

### 3.1 Phylogeny of the *Drosophila saltans* species group

The aim of the present work was to provide a morphological phylogeny of the *Drosophila saltans* group to be compared with its previous conflicting molecular phylogenetic hypotheses. To do so, only species that were used in the previous molecular studies

were used here. Indeed, the phylogeny was in disagreement with previous molecular-based phylogenetic hypotheses (Tab. 1). The cladogram differs from the “tentative phylogeny” proposed by Throckmorton & Magalhães (1962) using the same set of morphological characters in placing *sturtevantii* subgroup as the early offshoot, instead of the *cordata-elliptica* clade, which here appears as a sister to the *parasaltans-saltans* clade. In contrast to the maximum parsimony approach followed in this study, Throckmorton & Magalhães (1962) interpreted their phylogenetic relationships in light of subjective weighting of the characters without conducting a cladistic analysis.

The *saltans* subgroup is a septad of very close species whose branching order has been called “the most difficult systematic issue” by O’Grady et al (1998), as it was completely unresolved using molecular data. This may be attributed to a recent divergence of this subgroup, leading speciation rate to exceed the rate of mutation fixation in different lineages, resulting in a star-like phylogeny (Funk & Omland, 2003). Moreover, with the exception of *D. pseudosaltans*, all species of the subgroup show incomplete sexual isolation (Bicudo, 1973a) and sometimes geographical isolates of the same species (Dobzhansky & Streisinger, 1944; Bicudo, 1978). This may explain why mitochondrial genes tended to cluster sympatric species rather than allopatric populations of some species (O’Grady et al, 1998), indicating a predominant role of introgression and interspecific hybridization in natural populations of this subgroup. Such high gene flow can also explain the transpacific polymorphism of chromosomal inversions (Bicudo, 1973b) and esterase allozymes (Nascimento & Bicudo, 2002, 2006) in the subgroup. In conclusion, multilocus population genetics and comparative morphometrical studies are strongly needed to elucidate the evolutionary relationships within the *saltans* subgroup.

### 3.2 Molecular evolution

Morphological phylogenies were used to understand biochemical evolution in the *saltans* group since the earliest investigation (e.g., the evolution of pteridine accumulation, Throckmorton & Magalhães, 1962), but they have never been used to understand the evolution of DNA sequences in this group. The most striking aspect of molecular evolution within the *saltans* group is the codon usage bias leading to an increase in the (A+T) content. Indeed, such a selective pattern can bias the molecular phylogenies by itself, and render void the

estimation of molecular clocks under neutral models (Cutter, 2008). Recent whole-genome studies in the genus *Drosophila* have shown that codon bias differ between subgenera and even between close species (Singh et al, 2006; Vicario et al, 2007). Powell et al (2003) discussed different evolutionary scenarios, and favored the hypothesis for this pattern to be due to a random shift (“a frozen accident”) in relative abundance of isoaccepting tRNAs. For these authors, this shift was relatively old, prior to the split between the *willistoni* and the *saltans* group about 20 myr ago, and has been stable for a long time. However, they excluded a scenario of relaxation of selection due to small population sizes

and/or bottlenecks due to the old age of the drift. Fig. 2 shows the negative relation between (C+G) and (A+T) contents of the nucleotide landscape of concatenated nuclear genes (*Adh* and *Xdh*) at third codon position in the species studied here. Obviously, one can note the high discrepancy between species in their codon usage bias, a discrepancy that still retains a phylogenetic component. For example, the early branching species of the *sturtevantii* subgroup show the highest (A+T) content, whereas the most derivative species of the *saltans* subgroup show the lowest.

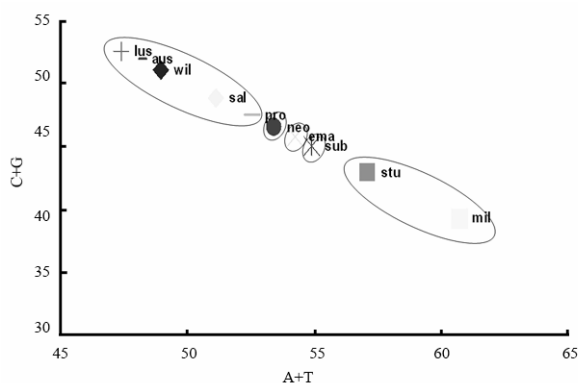
An interesting observation is that, within each subgroup, insular species (*i.e.* *D. milleri* and *D. lusaltans*)

**Tab. 3 Summary of conflicting phylogenetic hypotheses within the *Drosophila saltans* subgroup from previous studies**

Data	Topology	Method	Source
Reproductive isolation	(A(P,S))	---	Bicudo (1973a)
Karyology	(P(S,A))	---	Bicudo (1973b)
Esterases	(P(A,S))	---	Nascimento & Bicudo (2002, 2006)
<i>COI</i>	(L(S(P,A)))	MP	O’Grady et al (1998)
<i>COII</i>	(A(L(P,S)))	MP	O’Grady et al (1998)
<i>ITS1</i>	(L(S(P,A)))	MP	O’Grady et al (1998)
<i>Adh</i>	(A,L,P,S)	MP	O’Grady et al (1998)
	(P(S(A,L)))	NJ	Setta et al (2007)
Combined	(A,L,P,S)	MP	O’Grady et al (1998)

Only species used in the phylogenetic analysis are shown here with the following abbreviations:

A = *austrosaltans*, L = *lusaltans*, P = *prosaltans*, S = *saltans*.



**Fig. 2** Nucleotide content (A+T versus G+C) at third codon position of *Adh* and *Xdh* in species of the *Drosophila saltans* group

Each species is abbreviated to its three first letters. Ellipses include species of the same species subgroup.

reside at the extremity of the whole range, whereas mainland species tend to have more intermediate (A+T) content. If the shift in codon usage was due to random drift as suggested by Powell et al (2003), one would expect the amplitude of the bias (*i.e.* the random fixation of isoaccepting tRNAs) to be higher in species with small population size, whereas in species with large

population size, selection for optimal codon usage would be more effective (Lynch, 2007). The phylogenetic component can be explained assuming that the probability of fixation of a certain bias in a species depends mainly on the nucleotide landscape of the ancestor. Indeed, using a maximum likelihood inference of ancestral codon usage bias, Nielsen et al (2007) showed in the *Drosophila melanogaster* supercomplex that the *D. melanogaster* lineage has experienced a reduction in the selection for optimal codon usage.

### 3.3 Conclusions and perspectives

There are two major conclusions from this study. First, different morphological phylogenetic hypotheses can be obtained when the characters are analyzed cladistically (like here) or arbitrarily (as in Throckmorton & Magalhães, 1962). Second, morphology is still a very important source of phylogenetic information, even in groups like the genus *Drosophila* for which the whole genome sequence of a dozen of species has already been published, and not only for taxa for which DNA can not be obtained. The author does not pretend that the morphological phylogeny presented here is better or more robust than the previous hypotheses based on

molecular data. It is only a contribution to highlight that the evolution of a group of taxa can not wholly be understood without the understanding of each aspect of its evolution, from molecular sequences to geographical distribution, through developmental and anatomical characters. The evolution of morphological characters

has to be analyzed in light of knowledge of molecular evolution and *vice versa*, and both have to be related to the historical biogeography of their taxa. Such integrative approaches can be very promising in solving many systematic conflicts.

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## 杭州师范大学“实验动物科学实验室”简介

杭州师范大学“实验动物科学实验室”由校实验动物中心和医学实验中心优化组合而成，隶属于杭州师范大学实验室与设备管理处；2006年12月被确定为校级重点实验室，2008年12月被确定为杭州市重点实验室建设点；是浙江省首批实验动物公共服务平台成员单位——浙江省大动物实验基地及浙江省唯一的实验动物技术人员培训基地。

实验室拥有动物用房 3 090 m<sup>2</sup>，科研实验用房 6 500 m<sup>2</sup>，万元以上设备总值 600 多万元，既是杭州下沙高教园区动物供应基地和生命科学研究的公共服务平台，也有自己独具特色的科研方向。实验室现有三个研究方向：①吴宝金副教授课题组：人类疾病小鼠模型及遗传发育方向；②孙鹂教授课题组：环境毒物对机体健康的影响及机制；③施心路教授课题组：动物生态及水生动物的实验动物化。实验动物中心注重学术交流，多次承办省内学术会议，邀请国内专家讲座，与世界顶级实验室The Jackson Lab有实质性合作。近 5 年来，本中心研究人员共主持科研课题 30 余项，经费 500 余万元，其中国家级课题 4 项，省部级课题 13 项；共发表学术论文 40 余篇，其中SCI收录 9 篇，有 5 种本中心自主培育的突变系小鼠被国际权威数据库MGI (Mouse Genome Informatics) 收录；获省级奖励 1 项、厅级奖励 1 项，其中“高校实验动物中心构建产学研一体化运作体系的探索与实践”获市教学成果三等奖。

吴宝金

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