

INTERSPECIFIC HYBRIDISATION BETWEEN THE SIX CRYPTIC SPECIES OF *DROSOPHILA WILLISTONI* GROUP

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1. INTRODUCTION

In 1949 Burla *et al.* found that the name of *D. willistoni* Sturtevant was being applied to a cluster of four sibling species. These authors made an interesting comparative study of these species, which included differences in morphological and physiological traits, chromosomes and interspecific sexual isolation. They did not observe any case of *interspecific hybridisation*, but they did observe insemination between various species.

Dobzhansky in 1957 described the fifth species of the cryptic group, *D. insularis* (Dobzhansky, Ehrman and Pavlovsky, 1957) and obtained hybrids with the other sibling species, particularly *D. tropicalis* and *D. willistoni*, in laboratory conditions. Winge and Cordeiro (1960) reported the occurrence of interspecific hybrids between *D. willistoni* and *D. paulistorum* until then considered impossible. Amongst the tested strains of *D. willistoni* and of *D. paulistorum* two were specially interesting, producing *fertile* interspecific hybrids. These strains and their hybrids have been studied in more detail by Winge and Cordeiro (1963). This first hybridisation suggested a more extensive work including all the five sibling species then known. The first results of these crosses were reported by Winge (1961). As a result of these crosses, *D. tropicalis cubana* Townsend was raised to the level of a species by Winge (1962, 1963) and is now *D. cubana* Townsend.

This paper describes the most significant results of the interspecific crosses involving the six cryptic species mentioned.

2. MATERIAL AND METHODS

The following strains were used: from Brazil: *D. willistoni* Vila Atlântica (São Paulo) and São Pedro No. 18 (Rio Grande do Sul); *D. paulistorum*: Florianópolis No. 72 (Santa Catarina), São Pedro No. 12 and São Pedro No. 14 (Rio Grande do Sul); *D. equinoxialis* Tefé (Amazonas); *D. tropicalis* Palma (Goiás); *D. tropicalis* (now *D. cubana*) Trinidad No. 330, and *D. insularis* St. Kitts and Guadalupe Islands.

In order to obtain flies with comparable physiological development, a routine standard culture procedure was adopted. Each strain was cultured in several $\frac{1}{4}$ l. culture bottles with 40 pairs of flies, that were daily transferred to new bottles with fresh food. At the third and sixth days after the transfer these cultures received fresh baker's yeast suspension, in proportion to the amount of larval crowding.

* This work is dedicated to Dr Harry Miller, Jr on the occasion of his seventieth birthday (3rd June 1965).

Each different intra- and interspecific cross was repeated five times, each time with 10 virgin females and 10 males, aged identically but separately for 3 days at about 25° C. The vials used for these crosses were of 30×80 mm. All 90 possible intercrosses amongst the strains were realised, giving 450 cultures which were transferred every third day to new culture vials, until the last female died, which occurred 45 to 60 days after the crossing. All the cultures were maintained at 25±1° C. and carefully examined every third day for larvæ, pupæ or adults of the F₁ hybrids.

Depending upon the number obtained, part of the F₁ hybrids, were transferred every third day to new vials and the remaining flies were backcrossed with both parental species, in order to test the hybrids' fertility. The F₁ of backcrosses, were also transferred to new culture vials to test the fertility *inter se*.

Both F₁ hybrids and F₁ of backcrosses were transferred to new culture vials every third day during about 3 weeks, when they were rapidly etherised and counted, and then again transferred to new culture vials. The flies dead before the counts were also examined and, like all others, kept in a preserving liquid for further studies.

3. RESULTS

The results of the intercrosses were summarised in figs. 1 and 2.

As can be seen in fig. 1 the production of interspecific hybrids is very low. The number at the left side of each square represents the average number of hybrids obtained by each pair crossed, during all its life. In intraspecific crosses each couple produced at least 200 individuals.

D. *willistoni* × D. *paulistorum*

The crosses of *willistoni* males × *paulistorum* females did not produce any hybrids, consistently.

However, in massive crosses made with 100 males and 100 virgin females, some hybrids were obtained from cross of *willistoni* males S. Pedro No. 18 × *paulistorum* females S. Pedro No. 12. These hybrids did not produce any offspring and proved to be sterile in backcrosses with both parental species.

In reciprocal crosses, *paulistorum* males × *willistoni* females, a small number of hybrids were obtained, always less than ten individuals in each type of cross, including all replications of the five repetitions, *i.e.* obtained from fifty pairs of parents, during all their life (see fig. 1). Excepting the crosses of *paulistorum* males S. Pedro No. 12 × *willistoni* females S. Pedro No. 18 which produced fertile hybrids, all other hybrids produced were sterile *inter se*. Massive crosses of the above-named strains showed that only a part of the offspring is fertile; see Winge and Cordeiro (1963) for more details.

The crosses involving *paulistorum* males S. Pedro No. 14, produced fewer hybrids, sterile *inter se* and in backcrosses with both parental species.

The small offspring of each of the three strains of *paulistorum* males with *willistoni* females from Vila Atlântica were only used to test their fertility *inter se*.

It is interesting to note that the two strains of *paulistorum* from São Pedro, in spite of having been collected at the same time and place,

and showing no differences in crosses between them, showed great differences of affinities when test-crossed with *willistoni*.

Dobzhansky and Spassky (1959) reported the existence of sympatric strains of *paulistorum* which presented a similar phenomenon. Strains of the *Transitional group* produce fertile hybrids *inter se* and with at least

♀ \ ♂	w	w	p	p	p	e	t	c	i	i
	V. ATLAN	S. PE-18	FLOR-72	S. PE-12	S. PE-14	TEFE	PALMA	TRINIDAD	ST. KITTS	GUADAL
w V. ATLAN		F	S 0.18	S 0.18	S 0.08	⊙*	S ^{F♂} _{S♀} 0.82	⊙*	⊙	⊙
w S. PE-18	F		S 0.12	F ^{F♂} _{S♀} 0.20	S ^{S♂} _{S♀} 0.02	⊙	S ^{F♂} _{S♀} 1.46	⊙	⊙*	⊙
p FLOR-72	⊙	⊙		F	F	S ^{F♂} _{S♀} 0.10	⊙	⊙	⊙*	⊙
p S. PE-12	⊙	⊙*	F		F	S ^{S♂} 0.04	⊙	⊙	⊙	⊙
p S. PE-14	⊙	⊙	F	F		S ^{S♂} _{S♀} 0.34	⊙	⊙	⊙	⊙
e TEFE	⊙*	⊙	S ^{S♂} _{F♀} 3.64	S ^{F♂} _{S♀} 3.84	S ^{S♂} _{F♀} 9.66		⊙	⊙*	⊙	⊙
t PALMA	⊙	⊙	S 0.08	⊙	⊙*	⊙		S ^{F♂} _{S♀} 9.18	⊙	⊙
c TRINIDAD	⊙	⊙	⊙	⊙	⊙	⊙	⊙		⊙	⊙
i ST. KITTS	S 1.52	S ^{S♂} 1.54	S 0.04	⊙*	⊙	⊙	⊙*	S ^{S♂} 0.32		F
i GUADAL	S ^{S♂} _{S♀} 4.78	S ^{S♂} 1.54	⊙*	S 0.14	S 0.06	?	S ^{S♂} 1.48	S ^{S♂} _{F♀} 0.08	F	

FIG. 1.—Results of intra and interspecific crosses in *willistoni* cryptic group of species; each cross with fifty pairs. *Top left*: ⊙ = no hybrids produced; ⊙ = F₁ died as larvæ; ⊙ = F₁ died as pupæ; S = sterile F₁ hybrids; F = fertile F₁ hybrids; + = improved results were also obtained but very rarely. *Bottom left*: average number of hybrids obtained for each pair crossed. *Right*: fertility of F₁ hybrid females in backcrosses with male (♂) or female (♀) parental species.

one of the other sub-species, forming a “genetic bridge which connects the gene pools of the other groups”.

Cordeiro and Winge (1963) observed differences in chromosome pairing in interspecific hybrids of these two strains of *paulistorum* with *willistoni* São Pedro No. 18. The chromosomes of hybrid F₁s of *paulistorum* males S. Pedro No. 14 × *willistoni* females S. Pedro No. 18, presented completely unpaired strands resembling those that Dobzhansky (1957) observed in hybrids of *tropicalis* × *insularis*. On the

other hand, the hybrids of *paulistorum* S. Pedro No. 12 \times *willistoni* S. Pedro No. 18 showed a variable degree of chromosome pairing which varied from almost unpaired in some individuals to almost completely paired in others.

D. *willistoni* \times D. *equinoxialis*

In both reciprocal crosses, *willistoni* S. Pedro No. 18 did not produce offspring with *equinoxialis* Tefé. Excepting two culture vials which presented some hybrids which died as larvæ and as pupæ respectively, no hybrids were obtained in crosses of *willistoni* males Vila Atlântica \times *equinoxialis* from Tefé. The reciprocal crosses produced hybrids which died at pupal stage. Only a single female with mutated wings (in vertical position) succeeded in attaining the imaginal state. In another series of crosses made for cytological purposes, an adult male, quite abnormal, was also obtained but died soon after emerging.

Dobzhansky (1946) studied the sexual and reproductive isolation between *equinoxialis* from Tefé and *willistoni* from several localities. Although observing interspecific inseminations no interspecific hybrid was observed in these cultures. Notwithstanding the use of two strains derived from the State of São Paulo, the work referred to did not include flies originating from Vila Atlântica, and it is possible that different strains of *willistoni* present different affinities with *equinoxialis*, as well as with *paulistorum*.

D. *willistoni* \times D. *tropicalis*

No hybrids were obtained in crosses of *willistoni* males \times *tropicalis* females.

In the reciprocal crosses F_1 hybrids have been obtained and showed a slight excess of males in relation to females: 23 σ and 18 ϕ when *tropicalis* males from Palma were crossed with *willistoni* females from Vila Atlântica, and 42 σ to 31 ϕ with *willistoni* females from São Pedro No. 18. The F_1 hybrid males are sterile, but the females are fertile in both backcrosses, the offspring of these backcrosses being sterile *inter se*.

D. *willistoni* \times D. *cubana*

Hybrids were not produced in crosses of *willistoni* males \times *cubana* females likely in crosses of *willistoni* males \times *tropicalis* females. However in the reciprocal crosses, *cubana* behaved somewhat differently from *tropicalis*, exhibiting stronger isolation. No hybrids appeared when *willistoni* females came from S. Pedro No. 18; and a single adult hybrid male was observed in the crosses with *willistoni* females from Vila Atlântica. The fertility of this male was not tested.

D. *willistoni* \times D. *insularis*

The results obtained with these species confirmed that of Dobzhansky, Ehrman and Pavlovsky (1957). In crosses of *willistoni* males \times *insularis* females hybrids were obtained with relative facility,

the greatest number (4.28 per pair of P_1) being obtained in crosses of *willistoni* males Vila Atlântica and *insularis* females Guadalupe.

Crosses of *willistoni* males S. Pedro No. 18 with females of both strains of *insularis* produced the same total number of hybrids (1.54 per pair) presenting, however, great differences in the F_1 hybrids sex-ratio. Crosses with females of St. Kitts Islands yielded 31 ♂ : 46 ♀ and crosses with females of Guadalupe Islands: 7 ♂ : 70 ♀. Unfortunately not all backcrosses were performed, yet the ones made showed that males and females are sterile (see fig. 1). The reciprocal crosses did not produce offspring with the exception of *insularis* males St. Kitts × *willistoni* females S. Pedro No. 18 which produced a single and rachitic male.

D. paulistorum × D. equinoxialis

Very interesting and surprising results were obtained with these two species. They produced hybrids in both reciprocal crosses, thus presenting the greatest reproductive affinity in this cryptic group.

The crosses of *paulistorum* males with *equinoxialis* females produced relatively abundant offspring: 3.64 to 9.66 hybrids per couple of P_1 crossed, depending on the strain of *paulistorum* used (see fig. 1). The sex-ratio in these offspring greatly deviated from the normal, showing an excess of females, 12:1, 191:1 and 240.5:1 in crosses of *equinoxialis* females with *paulistorum* males from Florianópolis No. 72, São Pedro No. 12 and São Pedro No. 14 respectively.

The F_1 male hybrids of *paulistorum* males São Pedro No. 14 × *equinoxialis* females had abnormal eyes, wings and bristles as did also all the F_1 males of reciprocal crosses, independently of the *paulistorum* strain used. The eyes were rough and narrowed antero-posteriorly, the wings stretched upward and had notched margins similar to the notch mutation, and some of the dorsocentral and scutellar bristles were absent.

The hybrid male F_1 s of males *paulistorum* Florianópolis No. 72 and *paulistorum* São Pedro No. 12, crossed with *equinoxialis* females showed only abnormal wings (shorter and folded) and abnormal eyes respectively. The F_1 hybrid females were phenotypically normal but these "mutant" characters reappeared segregating in males and females of fertile backcrosses of these F_1 hybrid females with parental species.

Fig. 1 shows that the F_1 male hybrids are sterile and with exception of F_1 females of crosses which involved *paulistorum* males São Pedro No. 12, the F_1 hybrid females only produced offspring (mostly sterile *inter se*) when backcrossed with *equinoxialis* males. The F_1 hybrid females of crosses with São Pedro No. 12 males were fertile in both backcrosses and their offspring were also mostly fertile *inter se*. It is interesting to note that this strain of *paulistorum* is the same which produced fertile hybrids with *willistoni* females São Pedro No. 18 (Winge and Cordeiro, 1963).

The reciprocal crosses: *equinoxialis* males \times *paulistorum* females produced a small number of hybrids, the sex-ratio being almost normal. As was described before, the F_1 males were all "mutants" of eyes, wings and bristles and the females were normal. The hybrid females of crosses with Florianópolis No. 72 females were fertile but only produced offspring with *equinoxialis* males; this offspring being sterile *inter se*. A more detailed study about these species and their hybrids will be published elsewhere.

In 1949 Burla *et al.*, based on several characteristics like the disc patterns of salivary glands chromosomes and external morphology, concluded that these species are the most similar of the cryptic group.

D. paulistorum \times *D. tropicalis*

The results of the intercrosses between males of *paulistorum* and females of *tropicalis* varied according to the strain of *paulistorum* used. As can be seen in fig. 1, the crosses involving males from São Pedro No. 12 did not produce any offspring; those from São Pedro No. 14 produced hybrids which, with exception of a single male that became adult, all died at the pupal stage; and males from Florianópolis No. 72 produced four sterile males.

The reciprocal crosses did not produce adults, but all produced hybrids which died as larvæ (São Pedro No. 12) or as pupæ (São Pedro No. 14 and Florianópolis No. 72).

D. paulistorum \times *D. cubana*

D. paulistorum males did not produce any hybrid with *cubana* females (sub-species from Trinidad). The reciprocal crosses produced only pupæ.

D. paulistorum \times *D. insularis*

Some variability in results of crosses between *paulistorum* males and *insularis* females were observed according to the strains involved. Females of *insularis* St. Kitts did not produce hybrids with males of *paulistorum* São Pedro No. 14; rare pupæ and an adult male were obtained with males *paulistorum* São Pedro No. 12 and, with males from Florianópolis No. 72 a pair of flies, sterile *inter se* and some pupæ were obtained.

In crosses of *insularis* females Guadalupe with *paulistorum* males of São Pedro, some adults of both sexes were obtained; and with *paulistorum* males of Florianópolis only a single female was produced, the hybrids mostly dying at pupal stage. The reciprocal crosses did not produce hybrids, except a single culture of *insulares* males St. Kitts \times *paulistorum* females Florianópolis No. 72 which produced some hybrids which died as pupæ.

D. equinoxialis × *D. tropicalis*

D. equinoxialis males produced no hybrids with *tropicalis* females, but in reciprocal crosses some hybrids were obtained which died as larvæ or as pupæ.

D. equinoxialis × *D. cubana*

The reproductive isolation between these two species is greater than between *equinoxialis* and *tropicalis*. With the exception of a unique hybrid obtained in crosses of *cubana* males × *equinoxialis* females, which died at pupal stage, no hybrids were obtained in these crosses.

D. equinoxialis × *D. insularis*

D. equinoxialis males when crossed to females of *insularis* from St. Kitts produced hybrids which died at pupal stage excepting one adult male fly. The crosses with *insularis* females from Guadalupe were inconclusive—the three females obtained in these cultures proved to be *parthenogenetic*, producing several generations of females only. The reciprocal crosses did not produce any hybrid.

D. tropicalis × *D. cubana*

Townsend (1954) showed that there exist two sub-species, *tropicalis tropicalis* from the Continent, described by Burla and Da Cunha (in Burla *et al.*, 1949), and *tropicalis cubana* from Cuba the new sub-species then described and which inhabits the Greater Antilles. In crosses made in both directions abundant offspring were obtained, the males being sterile but the females fertile in backcrosses with both parental species. Nevertheless, when crosses between *D. tropicalis* from Palma (type locality) and from Trinidad were made during the present experiments, a new situation was disclosed in the cryptic group. In crosses of males from Palma with females of Trinidad no offspring were obtained.

In reciprocal crosses a relatively great number of hybrids were obtained (229 ♂ and 230 ♀) (see fig. 1). To test the F₁ fertility ten crosses of about 10 males and 10 females each were made. They were sterile *inter se*. Backcrosses of flies of both sexes were made with both parental strains. For each type of backcross five replications were made each with 10 males and 10 virgin females. The hybrid males proved to be sterile, and the hybrid females fertile, producing offspring only when backcrossed with males from Trinidad. The offspring of these backcrosses were partially fertile.

The surprising results of the crosses between these strains, suggested the study of other strains of this species. Another series of crossings was made using the two strains above and those from Cuba and from Tefé. The strains from Cuba, Trinidad and Tefé, when tested with each other, produced always, in both directions, a great number of hybrids, all the males being sterile and the females fertile, in both backcrosses. The crosses of any of these strains with flies from Palma

showed similar results to those obtained with Trinidad, *i.e.* hybrids were obtained only when Palma was used as the female parent.

It is interesting to note that males from Tefé produced with Palma females more hybrids than the males from Trinidad, and these more than the ones from Cuba. On the other hand, the fertility of the offspring varied inversely with the number of hybrids produced and also with the geographical distances between Palma and the other places.

The male offspring of the three type of crosses were always sterile in backcrosses to both parental strains. The F_1 females of crosses between Tefé and Palma were also sterile in both backcrosses, that of Trinidad and Palma were partially fertile in backcrosses with the male parental strain (Trinidad) and the F_1 females from Cuba and Palma were fully fertile but only with Cuba males.

The detailed results of sexual isolation, morphological traits, the backcrosses and the offspring of these backcrosses with the pure species, will be published elsewhere. The present results led us to the conclusion that *D. tropicalis cubana* Townsend must be raised to the species level, *i.e.* *D. cubana* Townsend, this new species having at least two subspecies, one in Tefé and another in Trinidad. The geographic distribution of this species and sub-species requires re-study.

D. tropicalis × D. insularis

In crosses of *tropicalis* males with *insularis* females different results were obtained according to the strain of *D. insularis* used. With females of *insularis* from St. Kitts, only one hybrid, which died as larvæ was obtained from the 50 pairs crossed, but with females from Guadalupe a relatively great number of hybrids (33 ♂ and 41 ♀) were obtained, which were sterile *inter se*, and in the backcrosses. In reciprocal crosses no hybrids were obtained.

D. cubana × D. insularis

As was observed with other species, *cubana* and *tropicalis* produced diverse results in crosses with *insularis*. The crosses of *cubana* males with females of St. Kitts produced some sterile hybrids (6 ♂ and 10 ♀), and those with females of Guadalupe produced a smaller number of hybrids (1 ♂ and 3 ♀) one female being a black "mutant". The F_1 male of the last cross was sterile and the backcrosses of hybrid females to *cubana* did not produce offspring.

Surprisingly a F_1 female (the black mutant) when backcrossed with *insularis* males Guadalupe produced a single F_1 male. One might think that this F_1 hybrid female was a parthenogenetic daughter of *insularis* females, but if this were the case the backcrosses with *insularis* males should have produced more offspring and not a single male.

The reciprocal crosses, as with *tropicalis*, did not produce any hybrid.

Parthenogenesis in D. willistoni cryptic group

As we mentioned before, a cross of *equinoxialis* males \times *insularis* from Guadalupe produced three females which proved to be parthenogenetic, producing several generations of females only. To test if *D. insularis* females are parthenogenetic or if this occurs only with the

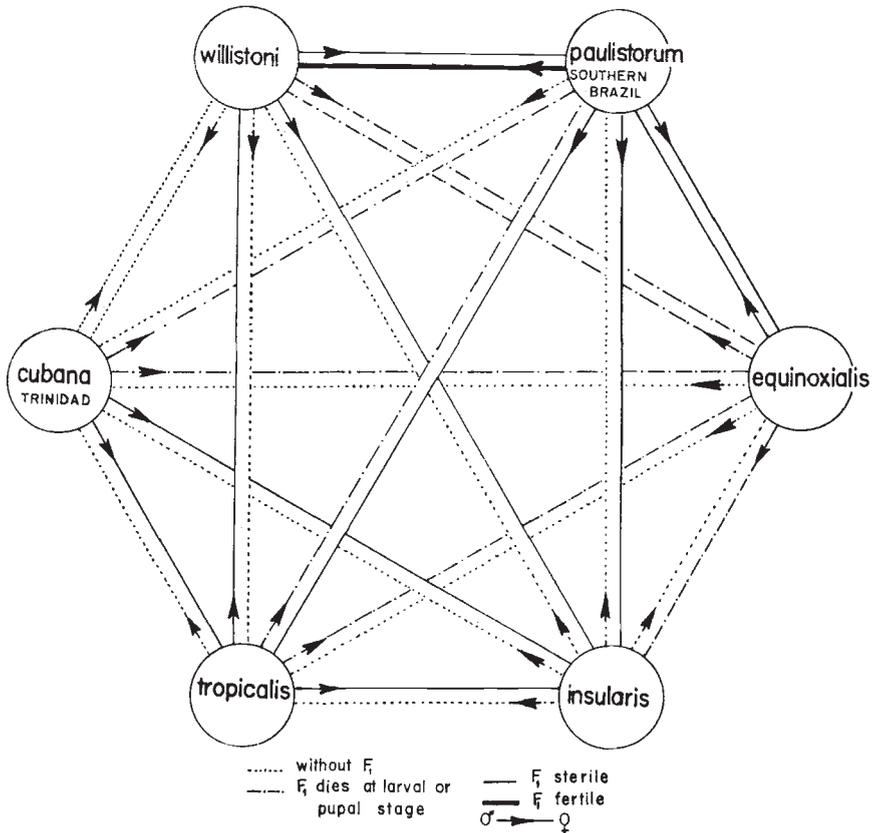


FIG. 2.—Reproductive affinities amongst the six sibling species of *D. willistoni* cryptic group. Maximal affinities were used except with the very rare results of the crosses. See text and fig. 1 for more details.

hybrid females, a few hundred virgin females were transferred every week to new culture media and old vials examined for larvæ, pupæ or adults. Several vials produced larvæ which gave rise to females only. The P_1 females of this offspring were dissected and shown not to be inseminated.

The other cryptic species were tested also. Only *D. insularis* from Guadalupe and *D. paulistorum* São Pedro No. 14 proved to be parthenogenetic, the last producing parthenogenetic offspring very rarely and no parthenogenetic F_2 were obtained. *D. insularis* from St. Kitts and *D. paulistorum* São Pedro No. 12 do not seem to be parthenogenetic.

Unfortunately these tests were made collaterally with the experiments of reproductive isolation, and no counts were made to estimate

the proportion of parthenogenetic eggs. However, it was observed that parthenogenesis is much more frequent in *D. insularis* (Guadalupe) than in *D. paulistorum* (São Pedro No. 14).

Since Carson *et al.*, 1957; Murdy and Carson, 1959; and Carson, 1962 studying *D. mangaberai* Malogolowkin showed that this species, belonging to the *willistoni* group of *Drosophila*, is thelytokous, reproducing by automictic diploid parthenogenesis, it is not surprising that species like *D. insularis* and *D. paulistorum* of the same group are also able to reproduce themselves by parthenogenesis.

4. SUMMARY AND CONCLUSIONS

Ninety different intercrosses involving different strains of the six sibling species of *D. willistoni* cryptic group have been made to study the reproductive affinities among these species (see fig. 1 and 2). The results obtained between two strains apparently belonging to *D. tropicalis* disclosed a complex situation. Crosses involving flies from four different places showed that *D. tropicalis cubana* Townsend needs to be ranked as a species, *D. cubana* Townsend. This new species of the cryptic group consists of at least three sub-species, in Cuba and greater Antilles, in Trinidad and in Tefé (Amazonas), respectively.

The reproductive affinities among the six sibling species are summarised pictorially in fig. 2.

D. paulistorum presents the greatest reproductive affinities with the other five sibling species followed by *D. willistoni* and *D. tropicalis*. The next *D. insularis* produce interspecific hybrids with all other species but almost only as female parental species. *D. equinoxialis* is much more isolated than those cited. *D. cubana* is the most isolated of this cryptic group producing some hybrids only when used as the male parental species.

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