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Postmating Reproductive isolation between strains of *Drosophila willistoni*

Xian B. Mardiros[a], Ronni Park[b], Bryan Clifton[b], Gurman Grewal[a], Amina K. Khizar[a], Therese A. Markow[c], José M. Ranz[b], and Alberto Civetta[a]

[a]Department of Biology, University of Winnipeg, Winnipeg, Canada; [b]Department of Ecology and Evolutionary Biology, University of California Irvine, Irvine, California, USA; [c]Division of Biological Sciences; University of California, San Diego, La Jolla, California, USA

ABSTRACT

Speciation can occur through the presence of reproductive isolation barriers that impede mating, restrict cross-fertilization, or render inviable/sterile hybrid progeny. The *D. willistoni* subgroup is ideally suited for studies of speciation, with examples of both allopatry and sympatry, a range of isolation barriers, and the availability of one species complete genome sequence to facilitate genetic studies of divergence. *D. w. willistoni* has the largest geographic distribution among members of the *Drosophila willistoni* subgroup, spanning from Argentina to the southern United States, including the Caribbean islands. A subspecies of *D. w. willistoni, D. w. quechua*, is geographically separated by the Andes mountain range and has evolved unidirectional sterility, in that only male offspring of *D. w. quechua* females × *D. w. willistoni* males are sterile. Whether *D. w. willistoni* flies residing east of the Andes belong to one or more *D. willistoni* subspecies remains unresolved. Here we perform fecundity assays and show that F1 hybrid males produced from crosses between different strains found in Central America, North America, and northern Caribbean islands are reproductively isolated from South American and southern Caribbean island strains as a result of unidirectional hybrid male sterility. Our results show the existence of a reproductive isolation barrier between the northern and southern strains and suggest a subdivision of the previously identified *D. willistoni willistoni* species into 2 new subspecies.

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Introduction

The biological species concept emphasizes the role of reproductive isolation mechanisms as barriers to gene flow between sexually reproducing organism.[1] Such barriers can manifest themselves as premating, a consequence of differences in sexual behavior or divergence of body parts with a role in copulation, or postmating changes that prevent fertilization or impair the fitness of hybrid progeny.[2] The identification of species "in status nascendi" facilitates tackling several questions related to the problem of speciation. For example, whether there is a particular order in which isolating mechanisms arise relative to one another, what is the degree of genetic differentiation that occurs prior to the appearance of specific (i.e. premating, postmating) reproductive isolation mechanism or whether changes at single major genes can contribute to the appearance of specific isolation mechanisms. It is clear that if these questions are to be rigorously addressed, we must examine different taxa not already designated as separate species. Thus, it is essential to conduct studies that aim at properly identifying partial isolation mechanisms between geographically isolated populations of the same species and or subspecies.

The genus *Drosophila* contains many populations and species that have wide geographic distributions and in some cases evidence of incipient speciation. A few examples are African populations of *D. melanogaster* showing evidence of premating and postmating reproductive isolation from other non-African strains,[3-5] subspecies of *D. p. pseudoobscura* and *D. p. bogotana* that are allopatric and exhibit postzygotic reproductive isolation in the form of unidirectional hybrid male sterility as well as evidence of premating isolation having more recently evolved between the subspecies,[6,7] and a group of subspecies of *D. mojavensis* with niche specializations and different degrees

CONTACT Alberto Civetta a.civetta@uwinnipeg.ca Department of Biology, University of Winnipeg 515 Portage Ave. Winnipeg, Manitoba, Canada. R3B 2E9.
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of sexual isolation.[8] The *Drosophila willistoni* subgroup is also particularly interesting because it is a complex of various taxonomic levels, exhibiting a range of isolation barriers to reproduction.[9,10] These reproductive barriers include different degrees of premating isolation, which have been shown at least partially linked to very distinctive song patterns.[9,11] Nevertheless, detailed studies have shown that in laboratory conditions, the 6 species of the *Drosophila willistoni* subgroup are capable of interbreeding with outcomes ranging from no offspring to fertile hybrid progeny.[10,12]

Early stages of incipient speciation can be identified within the *Drosophila willistoni* subgroup and such cases are often quite informative in dissecting primary mechanisms of isolation and establishing links between genetic changes and speciation. For example, *D. paulistorum* is subdivided into 6 indistinguishable "semispecies" that exhibit strong behavioral isolation but hybridize occasionally, producing unviable or sterile hybrid males when they interbreed.[9,13] Subspecies are geographically isolated and represent an earlier stage of speciation, with lower degrees of behavioral isolation than semispecies and thus capable of interbreeding and producing offspring.[14] Subspecies have been described within *D. tropicalis, D. equinoxialis* and *D. willistoni*. *D. tropicalis* is subdivided into *D. tropicalis tropicalis* and *D. t. cubana*, with the 2 subspecies being only partially sexually isolated in crosses between *D. t. tropicalis* males and *D. t. cubana* females.[15] Regardless of the direction of the intercross, hybrid females are fertile but males are sterile.[15] The 2 other species, *D. willistoni* and *D. equinoxialis*, have subspecies that display patterns of unidirectional hybrid male sterility with hybrid male sterility occurring in only one direction of the interspecies cross. Unidirectional hybrid male sterility between *D. equinoxialis* and *D. equinoxialis caribbensis* depends on the geographic origin of the strains assayed.[16] In *D. willistoni*, hybrid progeny from crosses between *D. w. willistoni* females and *D. w. quechua* males ($F1_{WQ}$) are fertile, but the reciprocal cross between *D. w. quechua* females and *D. w. willistoni* males produce viable progeny ($F1_{QW}$) of fertile females but sterile males.[17-19]

We recently showed that the sterile $F1_{QW}$ males are able both to copulate for as long as the parental species and to produce motile sperm, but are unable to father progeny due to what appears to be a failure to transfer sperm.[20,21] One puzzling aspect is that the site of collection for the *D. w. quechua* strain (Guadeloupe islands) we used does not correspond with the previously reported geographic distribution of *D. w. quechua*. *D. w. quechua* is reportedly found in a very narrow geographic area near Lima, Peru, west of the Andes, while *D. willistoni willistoni* is widely distributed in the American mainland from Argentina to the southern United States of America, also including the Caribbean Islands.[9,17,18] One possibility is an erroneous labeling of the site of collection for the *D. w. quechua* we used, or, alternatively, that the Guadeloupe strain is as yet a different subspecies of *D. willistoni*. The latter possibility is consistent with results obtained by H. Winge, who postulated that a third *D. willistoni* subspecies exists in addition to *D. w. willistoni* and *D. w. quechua*.[10,19] J. de Toledo Cardoso de Mello, however, found conflicting evidence in relation to Winge's claim.[19] To our knowledge, no further inquiries into the possibility of the existence of a third *D. willistoni* subspecies have been conducted since.

Here we ask if there is, in fact, evidence of more than 2 subspecies of *D. willistoni*. We examine the evolutionary relationships among 11 strains of *D. willistoni* with geographically diverse origins. We assess reproductive isolation by testing for progeny production by inter-strain hybrid males and compare the patterns of male sterility to the geographic localities and evolutionary relationships based upon sequences of the Barcode region of the mitochondrial cytochrome oxidase I gene. We find evidence for the existence of 2 subspecies within the currently accepted *D. w. willistoni* subspecies. One subspecies distributed across Central and North America, including Guadeloupe and most other Caribbean islands and the other including populations in the southern Caribbean islands and South America. Our results resolve several contested aspects of the patterns of incipient reproductive isolation among *D. willistoni* strains, which is an unavoidable prerequisite for any future biological characterization of the speciation process associated with *D. willistoni*.

Results

Postmating reproductive isolation

A fecundity assay was performed first to determine the reproduction isolation status of the Guadeloupe strain (14030–0814.10) previously identified as *D. w.*

Table 1. Progeny numbers in crosses between hybrid males and females from 4 different strains.

	Hybrid Males											
	Guadeloupe island ♀ × South American ♂						South American ♀ × Guadeloupe island ♂					
♀\♂	$G_{10}\times G_{24}$	$G_{10}\times E_{11}$	$G_{10}\times U_{16}$	$G_{24}\times G_{10}$	$G_{24}\times E_{11}$	$G_{24}\times U_{16}$	$E_{11}\times G_{24}$	$E_{11}\times G_{10}$	$E_{11}\times U_{16}$	$U_{16}\times G_{24}$	$U_{16}\times G_{10}$	$U_{16}\times E_{11}$
G_{10}	80	0	0	74	0	0	110	69	54	69	67	73
E_{11}	78	0	0	37	0	0	44	40	42	55	42	51
G_{24}	69	0	0	72	0	0	44	50	63	61	74	64
U_{16}	63	0	0	64	0	0	37	82	81	53	57	60

Note. Geographical origin is indicated by a letter (country) and a numeric subscript (last 2 digits of the strain identifier): Ecuador (E_{11}), Guadeloupe (strains G_{10} and G_{24}), and Uruguay (U_{16}). The median progeny number is reported. The nomenclature indicating the identity of the hybrid males follows the maternal strain x the paternal strain (e.g. $G_{24}\times U_{16}$). Hybrid males (top) are grouped from left to right into those with mothers of Guadeloupe origin and those with mothers of South American origin.

quechua. For this purpose, we used: i) the strain from which the genome assembly of *D. willistoni* was obtained,[22] which was collected also in Guadeloupe Island (14030–0811.24), (ii) the only available strain whose collection site is close to the described geographical distribution of *D. w. quechua* (14030–0811.11; Ecuador), and (iii) the strain from Uruguay 14030–0811.16 that we previously found to show unidirectional reproductive isolation from the Guadeloupe strain 14030–0814.10 labeled as *D. w. quechua*.[20] We generated all 12 possible F1 inter-strain hybrid males and crossed them with females from all 4 parental strains.

The results of this fecundity assay show a clear difference in progeny production between sterile and fertile inter-strain hybrid males, with the mating female's strain having no effect on the results (Table 1). The hybrid males resulting from crossing individuals from the 2 different strains from the Guadeloupe Island showed no evidence of reproductive isolation in any direction of the cross. Hybrid males were sterile when the maternal strain was of Guadeloupe origin and the paternal strain of South American origin (Table 1). The median progeny count of paired samples between laboratories was significantly different (Wilcoxon signed rank test; $P < 0.001$) and indicative of some kind of a genotype by environmental interaction affecting male fecundity. Nevertheless, the sterility phenotype (no progeny) was consistent across laboratory locations (Table S1). In no case did we find evidence of progeny inviability.

Importantly, these results suggest that the Ecuador and Uruguay strains are conspecific and reproductively isolated from the 2 Guadeloupe strains (Table 1 and Table S1). The conspecific status of the Uruguay and Ecuador strains is somehow expected, as the site of collection of the Ecuador strain (Tena) is East to the Andes mountain range and thus outside the expected range of distribution of *D. w. quechua*. The fact that 2 strains from Guadeloupe are found to be reproductively isolated from both the Uruguay and Ecuador strains identifies the possibility of an independent episode of reproductive isolation within the *D. w. willistoni* species.

To test whether the Guadeloupe strains represent an isolated speciation event localized to a particular Caribbean island, we set up a second fecundity test. Females from the Guadeloupe (14030–0814.10) and Uruguay (14030–0811.16) strains were allowed to mate with 2 types of inter-strain hybrid sons: (i) those resulting from crosses between Guadeloupe females and males from 5 other Caribbean islands, Panamá, and México (Table 2); and (ii) those resulting from crosses between females from 5 Caribbean islands, Panamá, and México, and males from Uruguay (Table 3). If the speciation event was in fact isolated within the island of Guadeloupe, the only expected sterile hybrid males will be those resulting from crosses whose maternal parent

Table 2. Fecundity of hybrid males produced from crosses between continental or Caribbean island males and Guadeloupe females.

	Hybrid Males						
	Continental males		Caribbean males (north south)				
♀\♂	$G_{10}xP_{YK}$	$G_{10}xM_{YK}$	$G_{10}xPR_{12}$	$G_{10}xGU_{19}$	$G_{10}xSK_{32}$	$G_{10}xSV_{13}$	$G_{10}xGR_{14}$
G_{10}	70	76	124	158	199	0	0
U_{16}	77	77	150	146	165	0	0

Note. The median progeny number is reported. The geographical origins of the Caribbean island strains, other than Guadeloupe (G_{10}), are: GR_{14}= Grenada, SV_{13}= Saint Vincent and the Grenadines, SK_{32}= Saint Kitts, GU_{19}= Guana Island, PR_{12}= Puerto Rico. The two continental strains donated by Dr. Yong-Kyu Kim are identified by the Country of collection and a subscript with the donor's initials (P_{YK}= Panamá; M_{YK}= México). The nomenclature for hybrid males is as in Table 1. Hybrid males (top) are grouped from left to right into those with fathers of Continental *vs*. Caribbean origin. The hybrids with fathers of Caribbean origin are listed left to right following the north to south geographic location of the islands.

Table 3. Fecundity of hybrid males produced from crosses between continental or Caribbean island females and Uruguay males.

	Hybrid Males						
	Continental females		Caribbean females (north south)				
♀\♂	$P_{YK}xU_{16}$	$M_{YK}xU_{16}$	$PR_{12}xU_{16}$	$GU_{19}xU_{16}$	$SK_{32}xU_{16}$	$SV_{13}xU_{16}$	$GR_{14}xU_{16}$
G_{10}	0	0	0	0	0	113	125
U_{16}	0	0	0	0	0	80	110

Note. The median progeny number is reported. The geographical origins of the used strains and the nomenclature for hybrid males are as in Table 2. Hybrid males (top) are grouped from left to right into those with mothers of Continental *vs*. Caribbean origin. The hybrids with mothers of Caribbean origin are listed left to right following the north to south geographic location of the islands.

originated from the island. Alternatively, a more widespread speciation event could be identified by the extent of hybrid male sterility arising from the different crosses performed. The results showed that the Panamá, México, Puerto Rico, Saint Kitts, Guana Island, and the 2 Guadeloupe strains are conspecific and reproductively isolated from the Uruguay, Grenada, and Saint Vincent and the Grenadines strains (Table 2, Table 3 and Table S2). Again, we found no evidence of progeny inviability.

The combined result of the crosses performed suggests the existence of a Northern subspecies whose approximate distribution spans North America, Central America and northern Caribbean Islands and a Southern subspecies whose distribution covers areas of South America and southern Caribbean Islands (Fig. 1).

Genetic differentiation among strains of D. willistoni

The mitochondrial cytochrome oxidase gene (mt*COI*) is a commonly used genetic marker for barcoding of animal species. To assess whether this universally used barcode gene could be useful to genetically fingerprint the Northern and Southern subspecies, we performed a sequence analysis of an approximately 650 bp region of 20 southern and 15 northern strains. Overall, we found limited genetic differentiation between strains, with genetic distance estimates ranging from 0 to 0.009. The Southern subspecies (Segregating site, $S = 21$; $\pi = 0.0045$) is more polymorphic than the Northern subspecies ($S = 8$, $\pi = 0.0018$) and it shows richer haplotype diversity (Hd = 0.984 and 0.571 respectively) (Fig. 2). A haplotype network analyses shows a cluster of 13 strains sharing the same haplotype. This cluster is composed mainly (10) of Northern subspecies strains and all Caribbean strains except for the southernmost island of Grenada. However, *mtCOI* haplotypes do not show an obvious separation of Northern and Southern subspecies (Fig. 3). The intermingling of Northern and Southern haplotypes is reflected by estimates of genetic differentiation. Estimates of genetic differentiation can be sensitive to sample size, haplotype diversity and sequence length. We used Hudson's nearest-neighbor statistic (S_{nn}) to measure genetic differentiation between Northern and Southern subspecies, as this statistic is less sensitive to populations' diversity. We found no evidence of genetic differentiation between subspecies ($S_{nn} = 0.528$; $P = 0.232$).

Discussion

From our inter-strain progeny count results, 2 reproductively isolated subspecies can be identified among *D. w. willistoni* flies. The patterns of unidirectional hybrid male sterility documented here are in good agreement with those proposed by H. Winge in her 1971 doctoral thesis, which were at odds with the results of de Toledo.[10,19] H. Winge recognized a subdivision of *D. willistoni willistoni* into a Northern (found in North and Central America, as well as in all the Caribbean islands except Trinidad) and a Southern (found in most of South America) group. Our results resolve the controversy between Winge and de Toledo and extend the northern boundary of the Southern subspecies to include other southern Caribbean islands nearby Trinidad (Grenada and Saint Vincent and the Grenadines). Winge's observations have been overlooked for many years and so the current main stream knowledge is to consider *D. w. willistoni* as a single subspecies.

An alternative explanation to our results would be a species range shift with *D. w. quechua* spreading north. It is feasible for a species shift in range from the time of the original studies using *D. w. quechua* in the 1970s to the time of collection of the stocks used in our study (1990s and beyond). One aspect that makes such shift unlikely is the Andes mountain range that isolates *D. w. quechua* to the west. Moreover, the geographic distribution of the reproductively isolated strains we used (Fig. 1) is coincidental with the reproductively isolated Northern and Southern "races" reported by Winge in the 1970s. While not formally introducing a new subspecies name, we refer to the Southern subspecies as *D. w. winge*, in honor of whom

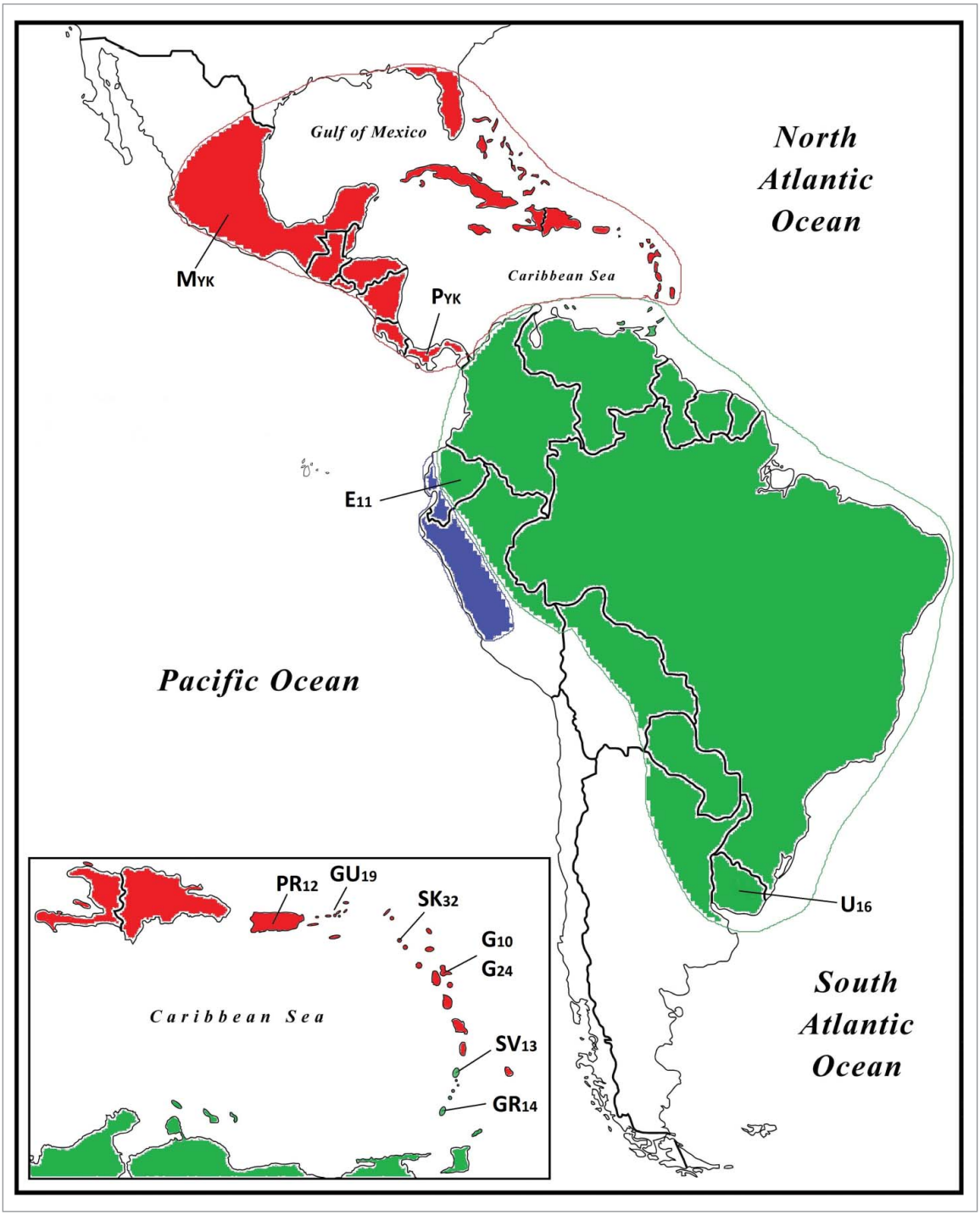

Figure 1. Approximate geographical distribution of the 3 subspecies of *D. willistoni*. Red = *D. willistoni willistoni*; Green = *D. willistoni winge*; Blue = *D. willistoni quechua*. Location sites for strains used in the analysis of reproductive isolation are abbreviated as in tables.

we believe was first in suggesting this subdivision. We keep the *D. w. willistoni* designation for *D. willistoni* flies inhabiting North and Central America, as well as the northern Caribbean islands.

The dichotomy between Northern and Southern subspecies applies to strains well within the range of distribution, but the possible existence of a transitional zone at the limits of the 2 subspecies distribution remains to be tested. The northern boundary within the South American continent is unclear and it is possible that some of the South American subspecies populations may be transitional, with patterns of sterility or fertility that might depend on the specific collection site. This putative transitional area could explain the

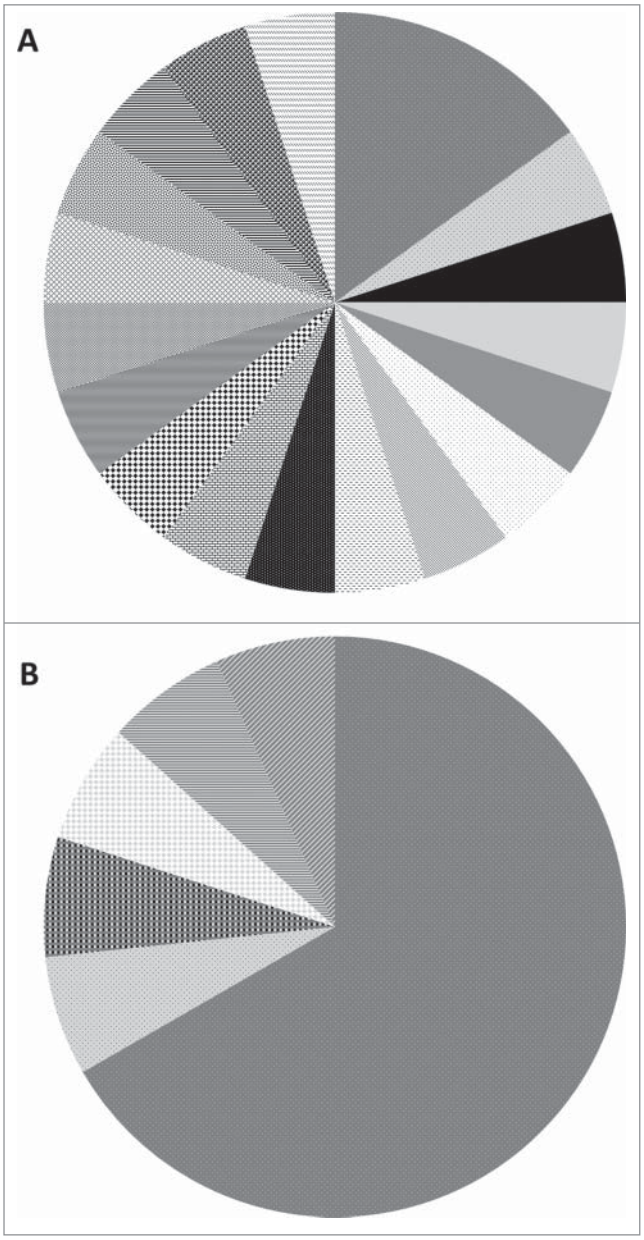

Figure 2. *mtCOI* haplotype diversity for Southern (A) and Northern (B) subspecies. Shared patterns between pies in the chart represent identical haplotypes.

inconsistency between the results of Winge and de Toledo in relation to crosses involving some Northern (Guatemala) females with males collected from different parts of Brazil.[19] Moreover, episodes of hybridization and introgression are known to result in barcoding genes like *mtCOI* being unable to identify species.[23,24] The lack of genetic differentiation between *D. w. willistoni* and *D. w. winge* based on mitochondrial sequence data supports the occurrence of natural hybridization events and possible transitional zones between the 2 subspecies. Partial reproductive isolation, with hybrid fertile females, is expected to result in reticulated patterns of mtDNA haplotype networks due to introgressions. Introgressions are in fact common among species of *Drosophila* in nature. Some examples are nuclear and mitochondrial introgression between species of the *D. simulans* clade,[25,26] post-speciation gene flow between *D. pseudoobscura* and *D. persimilis*,[27,28] and hybridization between *D. yakuba* and *D. santomea* showing more extensive introgressions of mitochondrial than nuclear genome and evidence of the *Drosophila yakuba* mitochondrial genome having replaced the *D. santomea* genome in hybrid zones.[29-31] While the results of our *mtCOI* analysis for the 2 subspecies of *D. willistoni* suggests gene flow events in nature, we cannot fully exclude the possibility that one divergent subspecies may carry mtDNA haplotypes segregating within its populations that reflect historical geographic structure in the ancestor population.

From the perspective of speciation genetics, it is unsurprising that strains showing reproductive isolation might not show genetic differentiation at a single genetic marker. For example, Ayala and Tracey surveyed allelic variation at 30 enzyme coding loci and showed that despite substantial genetic differentiation between *D. w. willistoni* and *D. w. quechua*, the 2 subspecies are still essentially identical at 17 loci.[17] More recently, using both mitochondrial and nuclear gene sequence data, incongruences were detected in the phylogenetic grouping of species of the *D. willistoni* subgroup, especially concerning the mitochondrial sequences. While the nuclear data helped refine the *D. willistoni* subgroup phylogeny, it was unable to consistently separate *D. w. willistoni* and *D. w. quechua*.[32] Further, populations of *D. willistoni* are highly polymorphic for inversions, with an analysis of 30 different populations identifying an interesting north to south latitudinal cline.[33] The unidirectional pattern of hybrid male sterility we report among the Northern and Southern subspecies suggests a major X chromosome effect. While an interesting observation, it remains premature to speculate whether and how clinal differences in inversion frequencies and geographic differences in fixed rearrangements, particularly for X chromosome inversions, may have contributed to the establishment of reproductive isolation barriers between Northern and Southern subspecies of *D. willistoni*.

In summary, our results resolve a previously suggested but controversial subdivision of *D. w. willistoni*

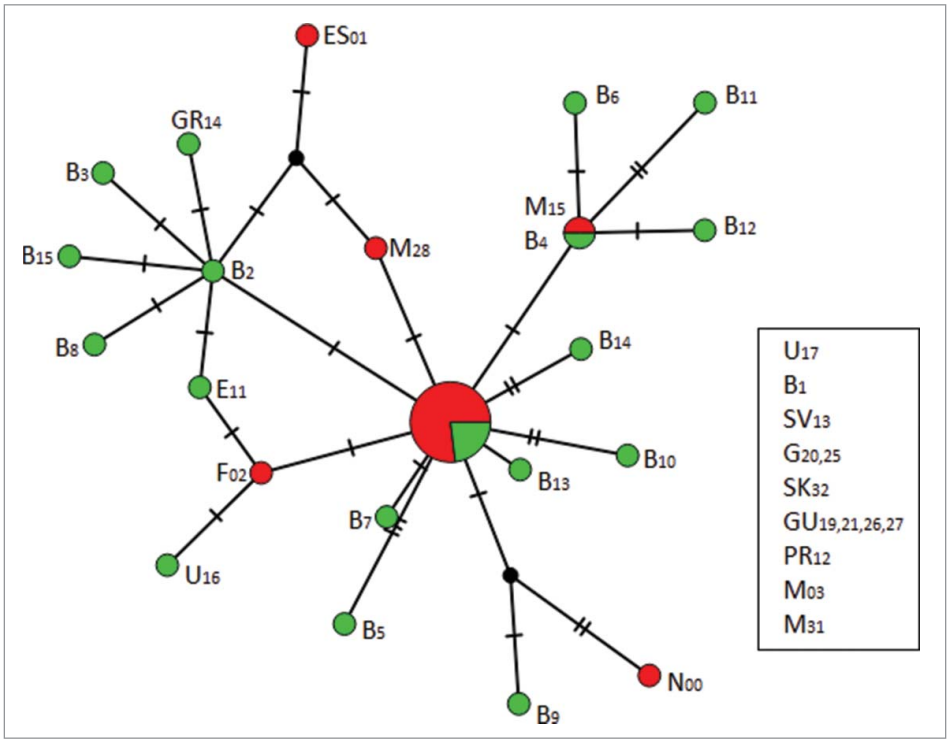

Figure 3. Haplotype network of *D. willistoni* stocks from multiple localities. The identity of the strains as Northern *vs*. Southern subspecies is color coded as in Figure 1. Mutations are shown as hatch marks along connecting lines. The black circles represent missing intermediate haplotypes. Strains with the same haplotype are grouped together with the size of the circle being proportional to the number of strains belonging to a haplotype. The identity of the different strains sequenced is abbreviated as in Figure 1. *Drosophila* strains sequenced but not phenotypically assayed are identified as follows: Brazil (B_1 to B_{15} = haplotypes 1 to 15);[38] Nicaragua (N_{00}); El Salvador (ES_{01}); Florida (F_{02}); México (M_{03}, M_{15}, M_{28}, M_{31}); Guadeloupe (G_{20}, G_{25}); Guana (GU_{21}, GU_{26}, GU_{27}); Uruguay (U_{17}). The names of the strains sharing the most common haplotype are boxed.

populations into 2 subspecies which we now refer to as *D. w. willistoni* and *D. w. winge*. Thus, the *D. willistoni* species should be considered as divided into 3 subspecies with different geographic distribution (Fig. 1). Interestingly, our *mtCOI* barcode gene sequence analysis (Fig. 3) shows a reticulated pattern consistent with introgressions. Identifying and describing patterns of partial reproductive isolation are crucial to our future understanding of mechanisms and the genetic basis of the speciation process. Perfect examples are the use of partially isolated species in genetic screens that have led to the identification of major speciation genes.[34,35] Our results highlight the special suitability of the *D. willistoni* species group, which includes multiple species in *status nascendi*,[9] for studies of speciation related, but not limited, to the identification of major speciation genes, the adaptive/disruptive role of partial genomic introgressions between divergent populations and subspecies, and the mechanical characterization and evolution of premating and postmating barriers between species.

Materials and methods

Drosophila stocks and fly husbandry

We used 11 strains of *Drosophila willistoni* collected from various geographical sites, 9 were obtained from stocks at the San Diego Drosophila Species Stock Center. These include: 14030–0814.10 (Guadeloupe), 14030–0811.11 (Tena, Ecuador), 14030–0811.12 (Toro Negro, Puerto Rico), 14030–0811.13 (Saint Vincent and the Grenadines), 14030–0811.14 (Grand Etang, Grenada), 14030–0811.16 (Rocha, Uruguay), 14030–0811.19 (Guana Island), 14030–0811.24 (Guadeloupe), 14030–0811.32 (Monkey Hill, Saint Kitts). The other 2 strains were YK-17 (Apazapán, México) and YK-18 (Barro Colorado Island, Panamá), kindly donated by Dr. Yong-Kyu Kim (Fig. 1). Flies were reared in bottles containing cornmeal-yeast-agar-molasses (CYAM) medium and kept in a 12:12 light–dark cycle at 24°C. Adult flies used to produce inter-strain hybrids or in fecundity assays were collected as newly emerged every 4 hours to ensure virginity.

Virgin females and naïve males were separated, maintained at a density of 20 flies per vial, and aged to 3–5 d post-eclosion before being used in crosses.

Fecundity assays

Two fecundity assays were performed. The first included the strains: 14030–0814.10 (Guadeloupe), 14030–0811.24 (Guadeloupe), 14030–0811.11 (Tena, Ecuador), and 14030–0811.16 (Rocha, Uruguay). F1 inter-strain hybrid males generated were crossed with females from all 4 parental strains. At least 2 vials were set up for each cross. In each vial, 5 males and 5 females were placed together for 48 h. Males were removed after 48 h. Five days after the initial set-up, females were moved to a new vial for an additional 5 d before being discarded. Any vial that produced no offspring was checked for collapsed eggs (hatching) and the presence of dead larvae that might indicate hybrid progeny inviability. The number of offspring (adult flies) produced by each vial was counted 23 d after the initial set-up and the median number calculated. Most crosses were replicated over 2 different laboratories (AC and JMR). For each cross, the median progeny number was calculated separately for each laboratory and then combining the results from both locations.

Based on results from the first fecundity test, a second assay was set up as described above, but using females from the strains 14030–0814.10 (Guadeloupe) and 14030–0811.16 (Rocha, Uruguay). Females were allowed to mate with inter-strain hybrid sons of females from the strain 14030–0814.10 (Guadeloupe) and males from 5 other Caribbean islands, Panamá, and México. Females were also allowed to mate with inter-strain sons of females from the same 7 Caribbean, Central and North American localities and males from the strain 14030–0811.16 (Rocha, Uruguay).

Haplotype network construction

A 654 bp piece of the cytochrome oxidase subunit I (*mtCOI*), the Barcode region, was sequenced from 20 *D. willistoni* strains that cover the species wide distribution. The sequenced strains, available at the Drosophila Species Stock Center, were: 14030–0811.00 (Santa Maria de Ostuma, Nicaragua), 14030–0811.01 (San Salvador, El Salvador), 14030–0811.02 (Royal Palm Park, Florida), 14030–0811.03 (Puebla, México), 14030–0811.11 (Tena, Ecuador), 14030–0811.12 (Toro Negro, Puerto Rico), 14030–0811.13 (Saint Vincent and the Grenadines), 14030–0811.14 (Grand Etang, Grenada), 14030–0811.15 (Veracruz, México), 14030–0811.16 (Rocha, Uruguay), 14030–0811.17 (Uruguay), 14030–0811.19 (Guana Island), 14030–0811.20 (Guadeloupe), 14030–0811.21 (Guana Island), 14030–0811.25 (Guadeloupe), 14030–0811.26 (Guana Island), 14030–0811.27 (Guana Island), 14030–0811.28 (Jalisco, México), 14030–0811.31 (Oaxaca, México), and 14030–0811.32 (Saint Monkey Hill, Kitts). Primer pairs LCO1490 (59-GGTCAACAAATCATAAAGATATTGG-39) and HCO2198 (59-TAAACTTCAGGGTGACCAAAAAATCA-39) were used to amplify the *mtCOI* gene fragment.[36,37] Sequence data are deposited in GenBank under accession numbers (KX232863 to KX232882). In addition, we incorporated *mtCOI* sequenced data available from Brazilian populations of *D. willistoni* (Accession numbers: JN705920 to JN705934).[38] Sequence data was aligned using MUSCLE within MEGA and the alignments used to calculate genetic distances and differentiation between strains.[39,40] The phylogenetic relationships among different haploid genotypes was reconstructed by using the method of Templeton, Candrall and Sing (TCS) to build a network of interconnected haplotypes.[41] The network was built using popart v1.7.[42]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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