



## ORIGINAL ARTICLE

# Long-term coexistence of a hybridization-derived population of *Drosophila parapallidosa* with closely related *Drosophila ananassae* (Diptera: Drosophilidae)

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## Abstract

A 2012–13 survey on Penang Island, Malaysia, revealed the existence of both *Drosophila ananassae* and *Drosophila parapallidosa*, the latter of which carries chromosomes Y and 4 from *D. ananassae* and thus is of hybrid origin. We collected the flies again from the same location in 2018. The hybrid population remained present, which suggests that the *D. parapallidosa* of hybrid origin does not represent a mere transient population but is stable. Why do these two species coexist irrespective of gene flow? We realized that body size is generally larger in *D. ananassae* than in *D. parapallidosa*, which constitutes a new character with which to discriminate these species; previously the number of sex comb teeth was the only diagnostic trait. Character displacement was not detected, however, for those traits. We crossed these two species, which resulted in offspring that had an altered genomic constitution. The body size of *D. ananassae* was dominant, and the presence of chromosomes Y and 4 did not have a significant effect on body size. By contrast, the presence of chromosome 4 from *D. ananassae* significantly affected the number of sex comb teeth. Even flies having a genomic constitution similar to that of the Penang *D. parapallidosa* exhibited a number of sex comb teeth that was intermediate between the two species. We propose that the *D. parapallidosa* sex comb character underwent selection during evolution of the Penang Island population. Reproductive interference between the species, presumably caused by signal jamming, was detected.

**Key words:** character displacement, *Drosophila parapallidosa*, reproductive interference, speciation.

## INTRODUCTION

Speciation is the process by which one population separates into two or more reproductively isolated species (Dobzhansky 1937; Mayr 1942; Coyne & Orr 2004). In allopatric speciation, mutations accumulate independently in each isolated population, which leads to reproductive isolation. Yet, speciation with gene flow is also possible and may have a substantive impact on sympatric or parapatric speciation or may ultimately

affect geographically isolated populations that experience a secondary contact (Nosil 2008; Feder *et al.* 2012; Abbott *et al.* 2013; Taylor & Larson 2019; Wang *et al.* 2020). In the present study, we examined two species of *Drosophila* (Diptera: Drosophilidae) on which gene flow has taken place.

The ananassae species complex belongs to the ananassae group (formerly the ananassae subgroup under the melanogaster group) of the subgenus *Sophophora* and consists of five described and a few undescribed species (Bock & Wheeler 1972; Lemeunier *et al.* 1986; Tobar 1993; Da Lage *et al.* 2007; Matsuda *et al.* 2009; McEvey & Schiffer 2015). *Drosophila ananassae* Doleschall, 1858 and *Drosophila parapallidosa* Tobar, 2009 are the target species of the present report. There is strong sexual isolation between

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*D. ananassae* females and *D. parapallidosa* males (less than 10% success), and the cross produces fertile hybrid females and sterile hybrid males. The reciprocal cross is easier (~50%) and usually produces fertile hybrid females and males (Matsuda *et al.* 2009). The geographical distribution of *D. parapallidosa* is restricted (Malaysia, Indonesia, Taiwan, and Japan), but that of *D. ananassae* is wider (tropical and subtropical areas worldwide); therefore, *D. parapallidosa* is always sympatric with *D. ananassae* (Tobari 1993; Tomimura *et al.* 1993; Matsuda *et al.* 2009). The sample from Penang Island, Malaysia, which was collected in 2012–13, included *D. ananassae* and *D. parapallidosa*, and the latter had chromosomes Y and 4 from *D. ananassae* (Sawamura *et al.* 2016; Table 1). Hence, these two species seem to be sympatric on this island, and *D. parapallidosa* from Penang is apparently of hybrid origin.

It is possible that the *D. parapallidosa* flies carrying chromosomes Y and 4 from *D. ananassae* represent a mere transient population. If the population is stable, how can it coexist with *D. ananassae* despite gene flow? Has natural selection operated on the system? We conducted several analyses to answer these questions. First, we collected the *Drosophila* species again in 2018 at the same location on Penang Island as the previous collection and assessed whether the hybrid population remained. Second, to understand the impact of character displacement (Brown & Wilson 1956; Grant 1972; Stre *et al.* 1997) on the coexistence of the two species, we compared morphology among strains of sympatric and allopatric populations of the two species. Third, we extended the analysis to laboratory populations that had been established from crosses of the two species, with the goal of replicating the natural population of Penang. Finally, because premating isolation is asymmetrical and postmating isolation is incomplete, we examined if reproductive interference (i.e., the interaction between individuals of different species during mate acquisition that leads to a reduction of fitness

in one or more species; Mallet 2005; Gröning & Hochkirch 2008; Burdfield-Steel & Shuker 2011) has occurred between these two *Drosophila* species.

## MATERIALS AND METHODS

### Wild flies

We used 16 isofemale lines established from flies collected from rotten noni (*Morinda citrifolia* L. (Rubiaceae)) fruit with a sweeping net at Jalan Sungai Dua, Penang Island, Malaysia, in January 2013 (PN13-1) and March 2013 (PN13-3) (Sawamura *et al.* 2016). Additional specimens (PN18-4) were collected in April 2018 at the exact same location as in the previous collection. We also used isofemale lines established from flies collected outside the island. Those included 11 *D. parapallidosa* lines and 14 *D. ananassae* lines, and eight lines of *D. ananassae* were sympatric with *D. parapallidosa* and six lines of *D. ananassae* were allopatric with *D. parapallidosa* (and any other species of the *ananassae* complex). Strain AABBg1 of *D. ananassae* (A) and strain T184 of *D. parapallidosa* (P) were used as the standard strains (for details, see Sawamura *et al.* 2016).

### Morphological characters

The species *D. ananassae* and *D. parapallidosa* are morphologically similar, including the male genitalia, and the only diagnostic trait that discriminates the species is the number of sex comb teeth on the male foreleg metatarsus (Matsuda *et al.* 2009). We also inspected body size between the species; the length of the foreleg tibia was used as the index of body size. The right foreleg was removed from each fly ( $N = 10$  for each group unless specifically indicated), mounted on a slide glass, and observed under a light microscope. The length of the foreleg tibia was measured by using an optic micrometer, and for males the number of sex

**Table 1** Survey of the natural population of flies on Penang Island

Year	Males		Females			
	<i>Drosophila ananassae</i>	<i>Drosophila parapallidosa</i>	<i>D. ananassae</i> or <i>D. parapallidosa</i>		Others	
	Y <sub>ana</sub> <sup>*</sup>	Y <sub>ana</sub>	no Y			
	4 <sub>ana</sub> /4 <sub>ana</sub> <sup>*</sup>	4 <sub>ana</sub> /4 <sub>ana</sub>	4 <sub>ana</sub> /4 <sub>para</sub> <sup>*</sup>	4 <sub>ana</sub> /4 <sub>ana</sub>	4 <sub>ana</sub> /4 <sub>para</sub>	NA <sup>*</sup>
2012-13 <sup>†</sup>	12	202	7	201	3	28
2018	0	20	1	19	1	1

<sup>\*</sup>Y<sub>ana</sub>, chromosome Y from *D. ananassae*; 4<sub>ana</sub>, chromosome 4 from *D. ananassae*; 4<sub>para</sub>, chromosome 4 from *D. parapallidosa*; NA, could not be amplified.

<sup>†</sup>Data compiled from Table S1 of Sawamura *et al.* (2016).

comb teeth in each row on the metatarsus was recorded.

### Flies derived from artificial crosses

AP refers to the  $F_1$  generation from the cross between *D. ananassae* (A) females and *D. parapallidosa* (P) males, whereas PA refers to that from the reciprocal cross. PA females and males were allowed to reproduce, and ten hybrid swarm (H) lines were independently produced via sib matings for ten generations; the genetic constitution of them has been roughly determined by using species-specific inversions (see Table 5 of Sawamura *et al.* 2016). An almost pure *D. parapallidosa* (BC) line was produced by nine repeated backcrosses during which hybrid males were crossed with P females in each generation. The established lines must carry chromosome Y from *D. ananassae* (for details, see Sawamura *et al.* 2016).

The *D. parapallidosa* population in Penang Island carries not only chromosome Y from *D. ananassae* but also chromosome 4 (Muller element F that is presumably no-recombining) from *D. ananassae* at high frequency (Sawamura *et al.* 2016). To replicate the natural population, we further replaced chromosome 4 of the BC line, which was accomplished by utilizing a recessive, visible, eye-character marker for chromosome 4 of *D. ananassae*: *spa*<sup>82</sup> (Moriwaki & Tobar 1993; Sawamura *et al.* 2008). Unexpectedly, the cross between T184 females and *spa*<sup>82</sup> males yielded hybrid males that were sterile. Therefore, we first crossed *spa*<sup>82</sup> females with AABBg1 males, and then the *spa*<sup>82</sup>/+ sons were crossed with T184 females ( $G_0$ : generation 0). Flies that freely mated in  $G_1$  produced some *spa*<sup>82</sup> homozygotes in  $G_2$ , and the *spa*<sup>82</sup>/*spa*<sup>82</sup>  $G_2$  males were backcrossed with T184 females. We repeated ten such cycles, and in the final generation females and males that were homozygous for *spa*<sup>82</sup> were crossed to fix chromosome 4 from *D. ananassae*. This strain (BC-4) also carried chromosome Y from *D. ananassae* in the genomic background of almost pure *D. parapallidosa*. The presence of chromosomes Y and 4 from *D. ananassae* was confirmed with molecular markers.

### Molecular markers

Genomic DNA was extracted from individual flies using reagents of the Qiagen DNeasy Blood & Tissue kit. The *kl-5* sequences of chromosome Y and the  $\psi$ COI sequences of chromosome 4 were amplified from fly extracts using TaKaRa Ex Taq (Takara) and the respective primers reported by Sawamura *et al.* (2016) and Sawamura *et al.* (2008); the PCR conditions are

also described in those reports. The PCR products were digested with *Hae*III for *kl-5* and *Ssp*I for  $\psi$ COI, and the digest products were subjected to agarose gel electrophoresis to confirm the integrity of the fragments.

### Reproductive success

To detect reproductive interference between *D. ananassae* and *D. parapallidosa*, fecundity was measured under eight different experimental conditions by using *D. ananassae* strain AABBg1 (A) and *D. parapallidosa* strain T184 (P). Within 8 h after emergence, flies were sexed under hypothermic anesthesia and kept separated for 3 days. On day 4, females and males were placed in a vial, and on day 6 the flies were discarded. The next generation flies that appeared in the vial were counted. Ten A females were confined with males of: (i) ten A; (ii) five A; (iii) five A and five P; (iv) ten P. Ten P females were confined with males of: (i) ten P; (ii) five P; (iii) five A and five P; (iv) ten A. Groups i and ii served as positive controls (conspecific), and group iv served as the negative control (heterospecific). Group iii was the test for reproductive interference between the species; sons were examined for their sex comb character to determine if they were pure species or a hybrid. Ten replicates were made for each group.

### Statistical tests

We used R ver.3.6.1 (R Core Team 2019) for statistical tests. To analyze the number of sex comb teeth, generalized linear models (GLMs) were fitted using the “glm” function using a Poisson error structure and a log link function. For comparisons with nested subgroups (multiple lines and strains), generalized linear mixed models (GLMMs) were fitted using the “glmer ()” function in the “lme4” package (Bates 2005), by incorporating those as a random factor. The models incorporating experimental category were compared with respective null models using a likelihood ratio test.

To analyze the offspring number in the experiment of reproductive success, GLMs were fitted using the “glm.nb()” function in the “MASS” package (Ripley *et al.* 2013). We adopted a negative binomial error structure and a log link function to resolve the problem of over-dispersion.

For comparison of the body size between two groups, the Student’s *t*-test or Welch’s *t*-test was applied when the two populations had equal or unequal variances, respectively, which was first determined by the *F*-test. A nested analysis of variance

(nested ANOVA) was applied to compare mean values among three or more species, strains, or lines. Significance thresholds in multiple comparisons were corrected using the false discovery rate (FDR; Benjamini & Hochberg 1995) in each comparison category (Tables S1-S4).

## RESULTS

### Collection of flies in 2018

In 2018, we collected 43 flies (22 males and 21 females; Table 1). Based on the sex comb character, 21 of the males were determined to be *D. parapallidosa*; one male was not the species of the *ananassae* species complex. The *D. parapallidosa* males had chromosomes Y and 4 from *D. ananassae*. The males were homozygous for chromosome 4 except one that is heterozygous for chromosomes 4 from *D. ananassae* and from *D. parapallidosa*. The  $\psi$ COI marker could not be amplified in one female, presumably because it was the distinct, yet similar-looking species *Drosophila bipectinata* Duda, 1923 or *Drosophila parabipectinata* Bock, 1971. The remaining females were homozygous for chromosome 4 from *D. ananassae* except one that is heterozygous for chromosomes 4 from *D. ananassae* and from *D. parapallidosa*.

### Morphological analysis of Penang populations

As reported previously (Matsuda *et al.* 2009; Sawamura *et al.* 2016), the number of sex comb teeth is greater in *D. ananassae* than in *D. parapallidosa*. This was confirmed in the PN13 lines established in 2013 (mean  $\pm$  SE,  $20.6 \pm 0.8$  for *D. ananassae* vs.  $8.6 \pm 0.1$  for *D. parapallidosa*). All of our PN18 specimens collected in 2018 ( $N = 21$ ;  $7.7 \pm 0.3$ ; range, 6–11) seem to be *D. parapallidosa*, not significantly different from PN13 *D. parapallidosa* but significantly different from PN13 *D. ananassae* lines (Fig. 1A; see Table S1A for statistical analyses).

Although not previously reported, *D. ananassae* has an overall larger body size than *D. parapallidosa*. This was so in the PN13 lines (for males, mean of the length of the foreleg tibia  $\pm$  SE  $\mu$ m,  $54.0 \pm 0.3$  for *D. ananassae* vs.  $48.2 \pm 0.1$  for *D. parapallidosa*) and our PN18 specimens ( $N = 21$ ;  $47.9 \pm 0.3$ ; Fig. 1B; see Table S1B for statistical analyses).

### Morphological analysis of sympatric and allopatric populations

The same trend (*D. ananassae* > *D. parapallidosa*) was observed for the number of sex comb teeth of strains

from outside Penang ( $19.0 \pm 0.2$  for *D. ananassae* vs.  $7.9 \pm 0.1$  for *D. parapallidosa*; Fig. 2A; see Table S2A for statistical analyses). There was not a significant difference between *D. ananassae* lines sympatric with *D. parapallidosa* ( $18.9 \pm 0.3$ ) and those allopatric with *D. parapallidosa* ( $19.1 \pm 0.3$ ).

The same trend (*D. ananassae* > *D. parapallidosa*) was also observed for body size of strains from outside Penang ( $53.4 \pm 0.2$  for *D. ananassae* vs.  $48.7 \pm 0.1$  for *D. parapallidosa*; Fig. 2B; see Table S2B for statistical analyses). There was not a significant difference between *D. ananassae* lines sympatric with *D. parapallidosa* ( $53.7 \pm 0.2$ ) and those allopatric with *D. parapallidosa* ( $53.1 \pm 0.3$ ).

### Morphological analysis of the populations established from artificial crosses

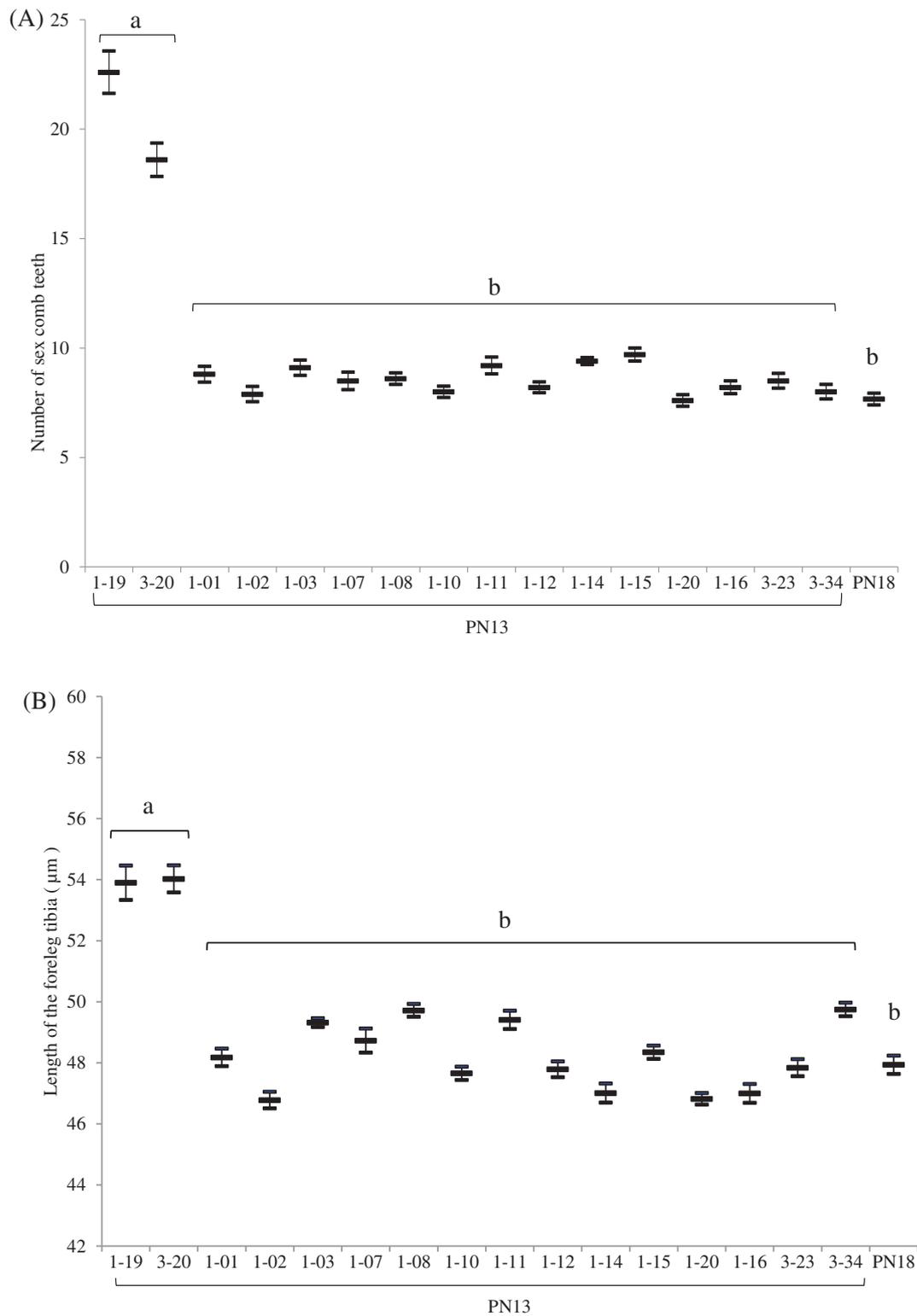
Flies derived from artificial crosses between *D. ananassae* (A;  $18.1 \pm 0.5$ ) and *D. parapallidosa* (P;  $7.3 \pm 0.4$ ) were also examined for the number of sex comb teeth (Fig. 3A). The F<sub>1</sub> hybrids AP ( $13.1 \pm 0.4$ ) and PA ( $12.3 \pm 0.3$ ) and BC-4 which is a backcrossed hybrid to P, but is carrying chromosome 4 from A ( $11.1 \pm 0.3$ ) were intermediate between A and P. Hybrid swarm H lines ( $14.8 \pm 0.3$ ) were close to A but different from P. In contrast, BC which is a backcrossed hybrid to P ( $7.2 \pm 0.4$ ) was close to P but differed from A (see Table S3A for statistical analyses).

Flies derived from artificial crosses between *D. ananassae* (A;  $51.5 \pm 0.5$ ) and *D. parapallidosa* (P;  $47.5 \pm 0.4$ ) were also examined for body size (Fig. 3B). The F<sub>1</sub> hybrids AP ( $52.6 \pm 0.1$ ) and PA ( $51.9 \pm 0.2$ ) and hybrid swarm H ( $51.9 \pm 0.1$ ) were close to A but differed from P. In contrast, BC which is a backcrossed hybrid to P ( $46.7 \pm 0.2$ ) and BC-4 which is a backcrossed hybrid to P, but is carrying chromosome 4 from A ( $47.8 \pm 0.4$ ) were close to P but differed from A (see Table S3B for statistical analyses).

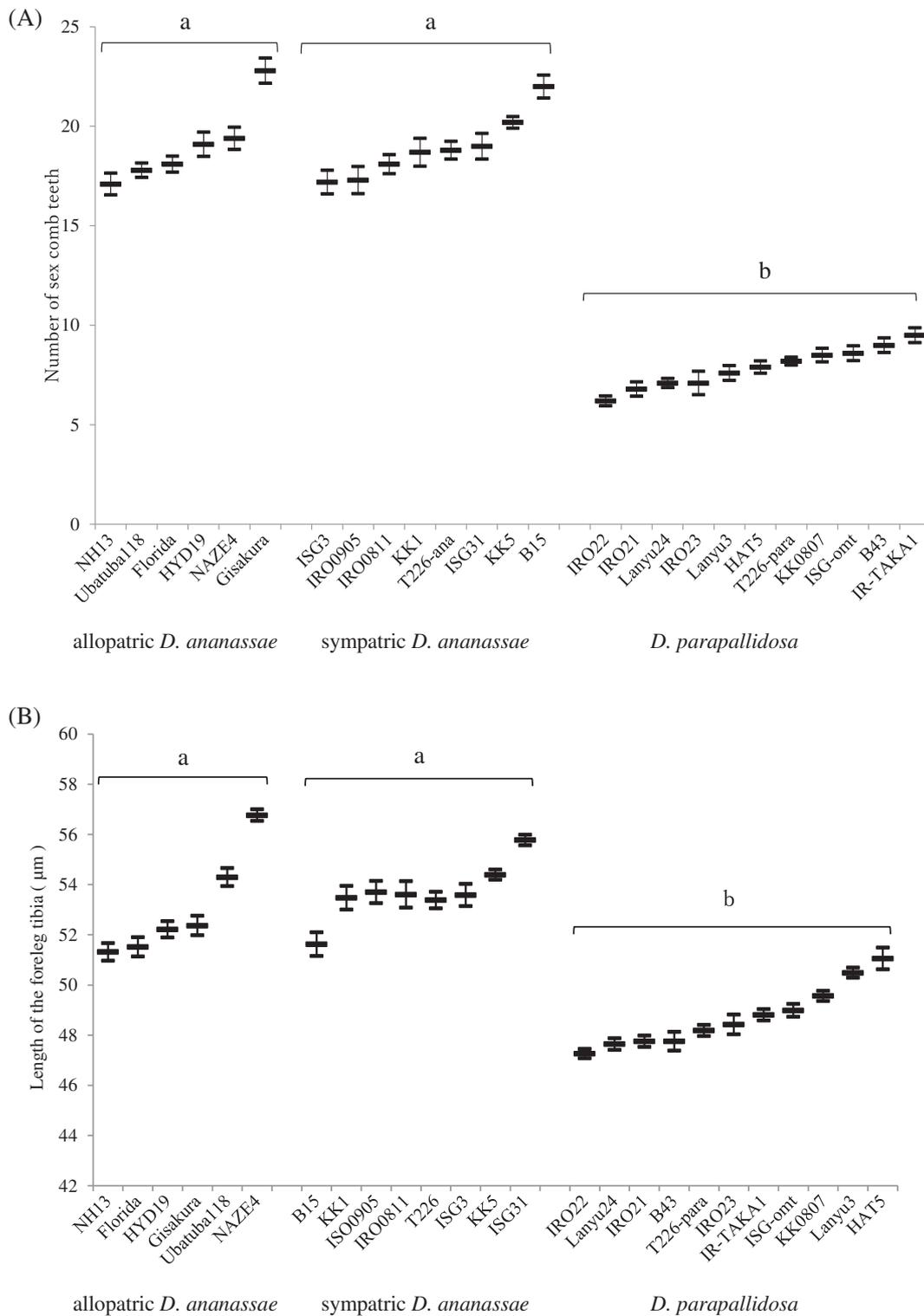
It is interesting that the hybrid swarm (H) lines exhibited variation on those two traits: the number of sex comb teeth and body size. But there was not a correlation between them ( $r = 0.1017$ ,  $P = 0.780$ ,  $N = 10$  lines). This suggests that those traits are genetically independent. Although we presented data from male characters in the above three sections, similar patterns were observed for female body size (Figs. S1, S2, S3; Tables S1C, S2C, S3C).

### Effect of sibling species on reproduction

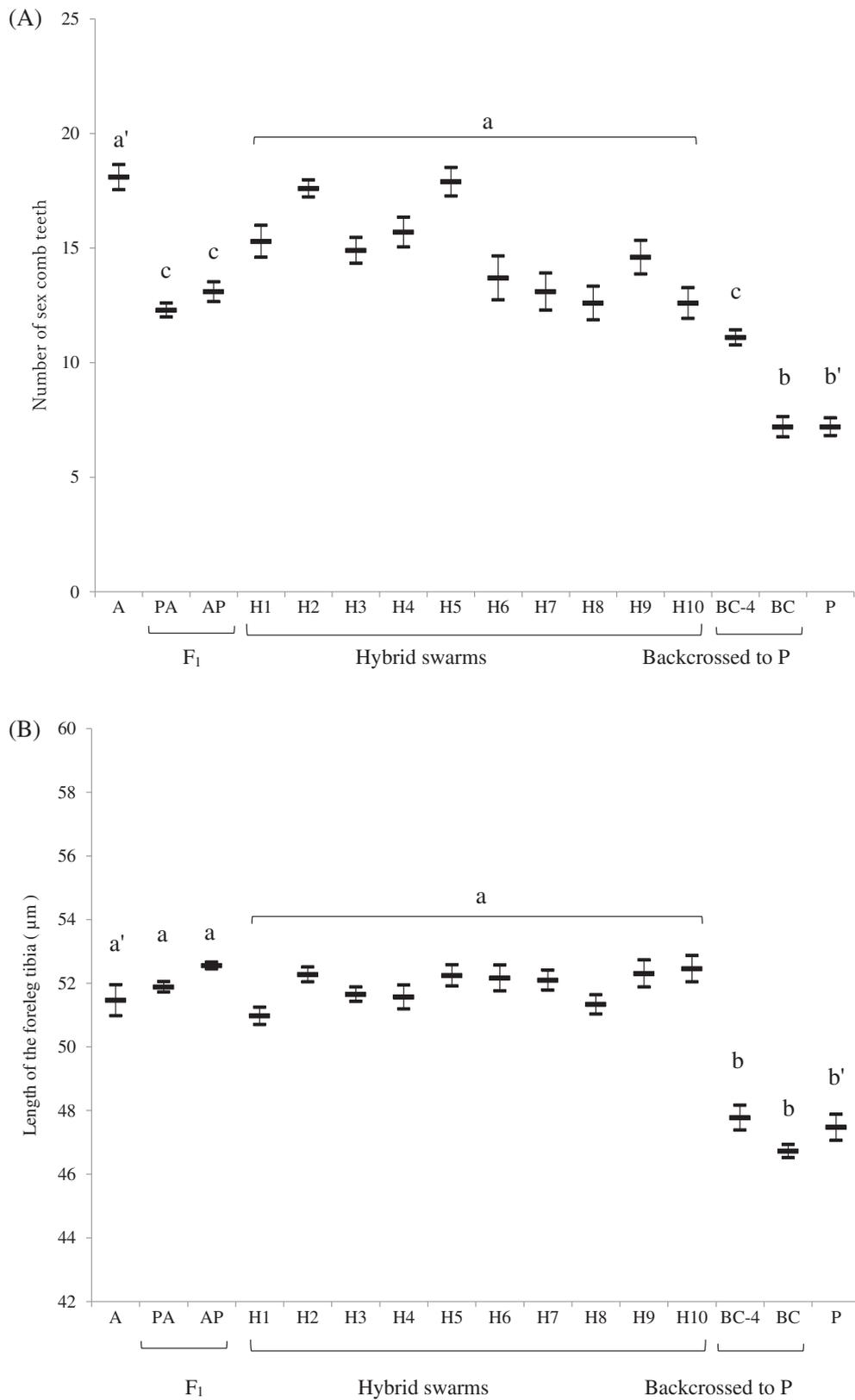
When *D. ananassae* females were confined with conspecific males, they produced numerous offspring



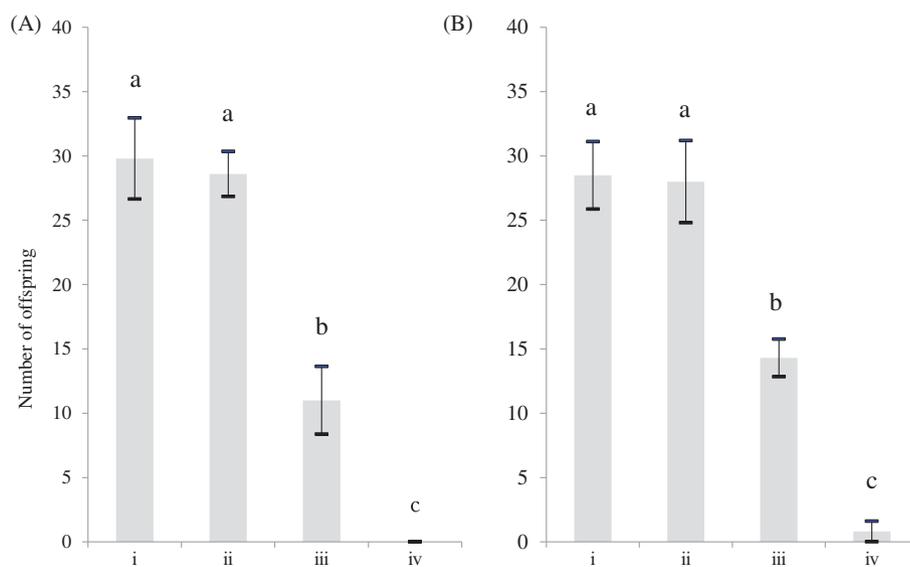
**Figure 1** Morphological analysis of Penang populations. (A) Number of sex comb teeth (mean  $\pm$  SE) of males from Penang Island. (B) Length of the foreleg tibia ( $\mu\text{m}$ , mean  $\pm$  SE) of males from Penang Island. Sixteen isofemale lines collected in 2013 (PN13) and specimens collected in 2018 (PN18) were examined. PN13-1-19 and PN13-3-20 were *Drosophila ananassae*, and the others were *Drosophila parapallidosa*. Bars labelled with different letters are significantly different ( $P < 0.05$  after correction for multiple comparison using FDR; see Table S1AB for statistical analyses).



**Figure 2** Morphological analysis of sympatric and allopatric populations. (A) Number of sex comb teeth (mean  $\pm$  SE) of males from various localities. (B) Length of the foreleg tibia ( $\mu\text{m}$ , mean  $\pm$  SE) of males from various localities. Twenty-five isofemale lines were examined. Six were *Drosophila ananassae* sympatric with *Drosophila parapallidosa*, eight were *D. ananassae* allopatric with *D. parapallidosa*, and 11 were *D. parapallidosa*, which was always sympatric with *D. ananassae*. Bars labelled with different letters are significantly different ( $P < 0.05$  after correction for multiple comparison using FDR; see Table S2AB for statistical analyses).



**Figure 3** Morphological analysis of the populations established from artificial crosses between *Drosophila ananassae* AABBg1 (A) and *Drosophila parapallidosa* T184 (P). (A) Number of sex comb teeth (mean  $\pm$  SE) of males. (B) Length of the foreleg tibia



**Figure 4** Number of offspring (mean ± SE) per female from (A) *Drosophila ananassae* and (B) *Drosophila parapallidosa*. Ten females were confined with males of: (i) 10 conspecifics; (ii) 5 conspecifics; (iii) 5 conspecifics and 5 heterospecifics; (iv) 10 heterospecifics. Bars labelled with different letters are significantly different ( $P < 0.05$  after correction for multiple comparison using FDR; see Table S4AB for statistical analyses).

irrespective of the number of males (mean ± SE per female,  $29.8 \pm 3.1$  for i,  $28.6 \pm 1.8$  for ii; Fig. 4A; see Table S4A for statistical analyses). Confinement of the females with heterospecific males (iv) yielded no offspring. Confinement of the females with both conspecific and heterospecific males resulted in a significant reduction in fecundity ( $11.0 \pm 2.6$  for iii). No hybrid males were produced in experiment iii, which means *D. ananassae* females selectively mated with conspecific males.

When *D. parapallidosa* females were confined with conspecific males, they produced numerous offspring irrespective of the number of males ( $28.5 \pm 2.6$  for i,  $28.0 \pm 3.2$  for ii; Fig. 4B; see Table S4B for statistical analyses). Confinement of the females with heterospecific males yielded essentially no offspring ( $0.8 \pm 0.8$  for iv). Confinement of the females with both conspecific and heterospecific males resulted in a significant reduction in fecundity ( $14.3 \pm 1.5$  for iii). No hybrid males were produced in experiment iii, which means *D. parapallidosa* females selectively mated with conspecific males.

## DISCUSSION

### The population of hybrid origin is stable on Penang Island

Collection of flies during the 2018 survey yielded *D. parapallidosa* males but not *D. ananassae* males. It

was unclear whether *D. ananassae* females were included in the sample because the females of these two species cannot be discriminated morphologically from each other. Because the sample size was small (21 males and 20 females), we cannot rule out the possibility that *D. ananassae* was not collected by chance. In fact, *D. ananassae* was rarer than *D. parapallidosa* in the 2012–13 survey (5.4%; 12 *D. ananassae* in 221 males; Sawamura *et al.* 2016). Similar to the previous survey, chromosomes Y and 4 from *D. ananassae* dominated in the newly collected sample, PN18 (Table 1). Furthermore, for *D. parapallidosa* males, the number of sex comb teeth and body size did not differ significantly between PN18 and PN13 (Figs 1AB and S1). Thus, the population of hybrid origin has been stable on Penang Island for at least 5 years.

### Character displacement was not detected

The number of sex comb teeth is the accepted diagnostic trait that discriminates between *D. ananassae* and *D. parapallidosa* (Matsuda *et al.* 2009; Fig. 1A). In our present study, we also found that the body size of *D. ananassae* is significantly larger than that of *D. parapallidosa* (Figs 1B and S1). We therefore examined if character displacement had taken place for those traits, but this was not the case (Figs 2AB and S2). There is not a significant difference between *D. ananassae* populations sympatric with *D. parapallidosa* and those

( $\mu\text{m}$ , mean ± SE) of males. See the text for the genomic constitution of each of PA, AP, H, BC-4, and BC. a, significantly different from b' but not from a'; b, significantly different from a' but not from b'; c, significantly different from both a' and b' ( $P < 0.05$  after correction for multiple comparison using FDR; see Table S3AB for statistical analyses).

allopatric with *D. parapallidosa*; *D. parapallidosa* is always sympatric with *D. ananassae* and this direction is not informative. We cannot rule out the possibility that character displacement has affected certain other traits.

### The populations established from artificial crosses incompletely mimic the natural population of hybrid origin

#### *Body size*

Body size of the F<sub>1</sub> hybrid (AP and PA) between *D. ananassae* (A) and *D. parapallidosa* (P) was closer to the former (Figs 3B and S3), implying that this morphological characteristic of *D. ananassae* is dominant. Body size of the hybrid swarms (H) was also in the range of *D. ananassae*. As has been suggested by Matute *et al.* (2020), hybrid swarms quickly regress to be phenotypically indistinguishable from one of their parental species. Interestingly, the character of species with wider distribution generally win out over the endemics, and this was also the case in the present species pair. Because *D. ananassae* alleles of genes determining body size are dominant, body size of the hybrid swarms would be *D. ananassa*-type, unless the genes are homozygous for the *D. parapallidosa* alleles.

Then, how could the natural population of hybrid origin acquire the recessive character of *D. parapallidosa*? We found that if the hybrid was repeatedly backcrossed with *D. parapallidosa*, the majority of the genomes reflected the genome of *D. parapallidosa*, and thus the character of them mimicked that of *D. parapallidosa* (Figs 3B and S3). Because the BC and BC-4 lines carried chromosome Y from *D. ananassae* and the latter also carried chromosome 4 from *D. ananassae*, chromosomes Y and 4 do not significantly affect body size. The genomic constitution of BC-4 mimics that of the natural population of Penang Island, i.e., *D. parapallidosa* of hybrid origin. Thus, we replicated such a line in which the *D. parapallidosa* body-size character was evident.

#### *Sex comb character*

The number of sex comb teeth of the populations established from artificial crosses was generally intermediate between the species; the exceptions are the hybrid swarm (H) close to *D. ananassae* and BC close to *D. parapallidosa* (Fig. 3A). This result generally confirmed our previous results (Sawamura *et al.* 2016). The dominance of this character differs from that of body size, in that chromosome Y did not significantly affect the number of sex comb teeth, whereas,

surprisingly, chromosome 4 that carries very small number of genes significantly affected this character.

What was the cause of the discrepancy between the BC-4 line and the natural population? Because chromosome 4 from *D. ananassae* has been maintained for a long time in the *D. parapallidosa* population of Penang, it must have adapted to *D. parapallidosa*. Mutations that produce fewer sex comb teeth might have been selected for by mate discrimination. There is a caveat, however, in that chromosome 4 of BC-4 was not derived from strain AABBg1, the *D. ananassae* reference strain, but rather from an unusual, *spa*<sup>82</sup> marker strain, and this may have skewed the data.

### There is reproductive interference between the species

For both *D. ananassae* and *D. parapallidosa*, the fecundity of females decreased if both heterospecific and conspecific males were present (Fig. 4), suggesting that the presence of heterospecific males suppresses either the mating with conspecifics, sperm transfer, fertilization, or oviposition. Although our results provide strong evidence for premating reproductive interference, we cannot rule out the possibility of post-copulatory mechanisms such as heterospecific mating. Because the courtship song emitted by heterospecific males inhibits copulation in female/male pairs of both *D. ananassae* and its sibling species *Drosophila pallidosa* Bock & Wheeler 1972 (Doi *et al.* 2001; Yamada *et al.* 2002a, 2002b), reproductive interference between *D. ananassae* and *D. parapallidosa* may be the consequence of differences in courtship song, i.e., signal jamming. This possibility remains to be investigated.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Length of the foreleg tibia ( $\mu\text{m}$ , mean  $\pm$  SE) of females from Penang Island.

**Figure S2.** Length of the foreleg tibia ( $\mu\text{m}$ , mean  $\pm$  SE) of females from various localities.

**Figure S3.** Length of the foreleg tibia ( $\mu\text{m}$ , mean  $\pm$  SE) of females from artificial crosses between *Drosophila ananassae* AABBg1 (A) and *D. parapallidosa* T184 (P).

**Table S1.** Statistical tests for flies derived from Penang populations.

**Table S2.** Statistical tests for flies derived from sympatric and allopatric populations.

**Table S3.** Statistical tests for flies derived from artificial crosses between *Drosophila ananassae* and *D. parapallidosa*.

**Table S4.** Statistical tests for reproductive interference.