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A Natural Population Derived from Species Hybridization in the *Drosophila ananassae* Species Complex on Penang Island, Malaysia

Kyoichi Sawamura[1]*, Hajime Sato[2], Chow-Yang Lee[3], Yoshitaka Kamimura[3,4], and Muneo Matsuda[2]

[1]*Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan*
[2]*School of Medicine, Kyorin University, Mitaka, Tokyo 181-8611, Japan*
[3]*Urban Entomology Laboratory, Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Minden 11800, Penang, Malaysia*
[4]*Department of Biology, Keio University, Yokohama, Kanagawa 223-8521, Japan*

We surveyed natural population of the *Drosophila ananassae* species complex on Penang Island, Malaysia. Analyses of phenotypic traits, chromosome arrangements, molecular markers, and reproductive isolation suggest the existence of two species: *D. ananassae* and *D.* cf. *parapallidosa*. Molecular marker analysis indicates that *D.* cf. *parapallidosa* carries chromosome Y and 4 introgressions from *D. ananassae*. Thus, *D.* cf. *parapallidosa* seems to be a hybrid descendant that recently originated from a natural *D. parapallidosa* ♀ × *D. ananassae* ♂ cross. Furthermore, *D.* cf. *parapallidosa* behaves differently from authentic *D. parapallidosa* with respect to its reproductive isolation from *D. ananassae*. Premating isolation is usually seen in only the *D. ananassae* ♀ × *D. parapallidosa* ♂ cross, but we observed it in crosses of both directions between *D. ananassae* and *D.* cf. *parapallidosa*. In addition, hybrid males from the *D. ananassae* ♀ × *D. parapallidosa* ♂ cross are usually sterile, but they were fertile when *D. ananassae* ♀ were mated with *D.* cf. *parapallidosa* ♂. We attempted an artificial reconstruction of the hybrid species to simulate the evolutionary process(es) that produced *D.* cf. *parapallidosa*. This is a rare case of natural hybrid population in *Drosophila* and may be a useful system for elucidating speciation with gene flow.

Key words: *Drosophila ananassae*, *Drosophila parapallidosa*, hybrid speciation, interspecific introgression, Penang Island

INTRODUCTION

Speciation is the process in which different mutations accumulate in separate populations leading to the divergence of the populations (Mayr, 1963; Coyne and Orr, 2004). However, speciation with gene flow also occurs (Nosil, 2008; Feder et al., 2012; Abbott et al., 2013) and has been well documented in a number of diverse organisms, especially in studies investigating hybrid zones and hybrid speciation (Barton and Hewitt, 1985; Rieseberg, 1997; Kubota and Sota, 1998; Seehausen, 2004; Arnold, 2006; Gompert et al., 2006; Mavárez et al., 2006; Mallet, 2007; Harrison and Larson, 2014; Mullen and Shaw, 2014; Schumer et al., 2014; Lamichhaney et al., 2015). In modern human ancestry as well, gene flow from Neanderthals contributed slightly to our genetic character (Green et al., 2010; Prüfer et al., 2014; Sankararaman et al., 2014).

Drosophila is no exception; gene flow has been detected by analyses of molecular markers, chromosome arrangements, and/or phenotypic traits in the following pairs of species: *D. peudoobscura* and *D. persimilis*; *D. subobscura* and *D. madeirensis*; *D. simulans* and *D. mauritiana*; *D. simulans* and *D. sechellia*; and *D. yakuba* and *D. santomea* (Lachaise et al., 2000; Machado and Hey, 2003; Garrigan et al., 2012; Herrig et al., 2014; Matute and Ayroles, 2014; Navascués et al., 2014). However, hybrid zones are rarely known; one exception is São Tomé Island on which both *D. yakuba* and *D. santomea* are found (Llopart et al., 2005a, b; Bachtrog et al., 2006). Hybrid speciation has never been reported for *Drosophila*.

Drosophila ananassae is a human-commensal species distributed in tropical and subtropical areas around the world, and it has several sibling species in Asia-Oceania (Bock and Wheeler, 1972; Lemeunier et al., 1986; Tobari, 1993; Matsuda et al., 2009; McEvey and Schiffer, 2015). The mitochondrial and genomic sequences of these species have not diverged enough to reconstruct well-resolved phylogeny, presumably because of incomplete lineage sorting and/or interspecific gene flow (Schug et al., 2007, 2008; Sawamura et al., 2008a, 2010; Matsuda et al., 2009). A sibling species, *D. parapallidosa*, was first collected with *D. ananassae* in 1971 from Kota Kinabalu, Borneo, Malaysia and recurrently found in the same area (original sympatry;

* Corresponding author. Tel. : +81-29-853-4669;
Fax : +81-29-853-6614;
E-mail: sawamura@biol.tsukuba.ac.jp
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Tobari, 1993; Tomimura et al., 1993). It was not found in other areas until 1998; since then, *D. parapallidosa* has been collected at Ishigaki, Iriomote, and Hateruma, Okinawa, Japan and Lanyu, Taiwan (Matsuda and Tobari, 2009; Matsuda et al., 2009; Supplementary Figure S1 online). This suggests that *D. parapallidosa* has recently migrated north from a tropical to a subtropical area and has become coexisting with *D. ananassae* there (secondary sympatry). These two species are also recorded from Bogor, Java, Indonesia (Kimura and Suwito, 2012). Thus, *D. parapallidosa* is always sympatric with *D. ananassae*, but not vice versa.

We surveyed a natural population of the *D. ananassae* species complex on Penang Island, Malaysia and observed that the population contains two species, *D. ananassae* and *D.* cf. *parapallidosa*; the former seems to be authentic *D. ananassae*, as we show below, and the latter seems to be a hybrid descendant recently originating from *D. ananassae* and *D. parapallidosa*. We examined the pattern of gene flow between these species. Interestingly, unidirectional interspecific introgression was detected, and we therefore explored its evolutionary meaning by artificial reconstruction of the hybrid species.

MATERIALS AND METHODS

Drosophila

Drosophila specimens were collected from rotten noni fruit (*Morinda citriforia*; Rubiaceae) with a sweeping net at Sungai Dua, Penang Island, Malaysia (5°21′04.7″N 100°17′51.1″E) between August 2012 and March 2013. *Drosophila ananassae*-like specimens were selected under a stereoscopic microscope, and some were preserved in 70% (v/v) ethanol. Isofemale lines were established from flies collected between January and March, 2013.

The *D. ananassae* AABBg1 and *D. parapallidosa* T184 strains served as authentic reference strains. AABBg1 (Hawaii, USA, 1945) is the strain of which the whole genome has been sequenced (*Drosophila* 12 Genomes Consortium, 2007), and T184 (Kota Kinabalu, Malaysia, 1979) is the strain from which the holotype was derived (Matsuda and Tobari, 2009).

Isofemale lines established from sympatric (or secondarily contacted) populations outside Penang Island were also used. Those were *D. ananassae* ISG3 (Ishigaki, Japan), IRO0811 (Iriomote, Japan), TWN0701 (Taiwan), and B15, T215, kk1, and kk3 (Kota Kinabalu, Malaysia) and *D. parapallidosa* ISG-omt and ISG02 (Ishigaki, Japan), IRO21, IRO22, and IRO23 (Iriomote, Japan), HAT3 and HAT5 (Hateruma, Japan), Lanyu2, Lanyu3, Lanyu10, Lanyu16, and Lanyu24 (Lanyu, Taiwan), and T226, B43, and kk-k0807 (Kota Kinabalu, Malaysia).

Sex comb teeth numbers

One arbitrarily chosen foreleg was removed from each male, mounted on a slide glass, and observed under a light microscope. The number of sex comb teeth in each row on the foreleg metatarsus was recorded and statistically analyzed. We used R ver.3.2.0 (R Core Team, 2015) for statistical analyses. For comparisons of 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 one-way analysis of variance (ANOVA) or a nested ANOVA was applied to compare the means among three or more species, strains, or lines.

PCR, RFLP, and sequencing

Genomic DNA was extracted from each fly using DNeasy Blood & Tissue Kit reagents (Qiagen). *kl-5* sequences (Koerich et al., 2008) were amplified by Ex Taq (Takara) from extracts using the primers ana_kl5_F1: 5′-CTTGGGAACCGTTTATATTTATAGA-3′ and ana_kl5_R1: 5′-GAACAATTAAACACATAAACCATCAT-3′ (A. B. Carvalho, personal communication). The PCR conditions were 95°C for 4 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension reaction at 72°C for 7 min. The PCR products were digested with *Hae*III (Takara) for restriction fragment length polymorphism (RFLP) analysis.

ΨCOI sequences were also amplified (Sawamura et al., 2008a). PCR was conducted independently three times if a product was not amplified. The PCR products were digested with *Ssp*I, *Mfl*I, or *Xsp*I (all from Takara) for RFLP analysis. To determine if each isofemale line was monomorphic or polymorphic for *ΨCOI*, at least eight flies were examined. The χ^2 test was performed using Excel 2010 (Microsoft).

PCR products were purified from an agarose gel, individually cloned into a pGEM-T Easy vector (Promega), and sequenced using a 3730xl DNA Analyzer (Applied Biosystems) and BigDye Terminator v3.1 Cycle Sequencing Kit reagents (Applied Biosystems). The sequences were edited with Sequencher ver. 4.5 (Gene Codes Corporation) and Genetyx ver. 12 (Genetyx).

Polytene chromosomes

Salivary glands were collected from third instar larvae for each line; at least nine larvae for isofemale lines and at least eight larvae for artificial hybrid lines. Polytene chromosomes were prepared according to Tomimura et al. (2005).

Reproductive isolation

Flies were reared in vials (30-mm diameter × 105-mm height) containing standard *Drosophila* glucose/yeast/cornmeal medium. All crossing experiments were conducted at 25°C with a 14-/10-hr light/dark cycle (light period, 7:00–21:00) (Sawamura et al., 2008b).

Newly emerged flies were collected within 8 hr and separated into unisexual groups of five under quick ice anesthesia. At day 5 post-eclosion, five females and five males from separate vials were tapped into a new vial and allowed to mate for 48 hr, after which they were anesthetized on ice, and the males discarded. Vials containing any dead flies were discarded. The females were kept alive for additional 2–4 days to allow for hardening of their inner organ cuticles (see below), then flash-frozen, and preserved at –20°C. At least two replicates were made for every cross.

To determine whether the preserved females had copulated, they were dissected in phosphate-buffered saline (PBS) and examined under a phase contrast microscope. Because copulation wounds female "pockets" (paired blind invaginations near the female genital orifice; Kamimura, 2007), the presence/absence of melanized patches in the pockets was determined independently by two observers. We applied this method instead of directly observing sperm in the spermathecae and/or seminal receptacle, because it allows verification of copulation at a later time. To determine whether there were significant differences in mating frequencies, the Fisher's exact test was performed using Excel-Tohkei 2015 (Social Survey Research Information).

F_1 females and males produced by the crosses (mass matings were performed if there was strong sexual isolation) were transferred into new vials and their fertility assessed. If no offspring appeared, F_1 females and males were separately tested by mass mating with AABBg1 or T184 of the opposite sex.

Artificial hybrid populations

The *D.* cf. *parapallidosa* population on Penang Island might be a hybrid descendant originating from *D. ananassae* and *D. parapallidosa*. To simulate the possible history of the natural population, we performed a *D. parapallidosa* T184 ♀ × *D. ananassae* AABBg1 ♂ cross. Ten independent H lines were produced from the fertile hybrids via sib matings for 10 generations, and a BC line was made by nine repeated backcrosses during which hybrid males were

crossed to T184 females in each generation.

RESULTS

Morphological analysis

Drosophila ananassae and *D. parapallidosa* are morphologically similar, and the only diagnostic trait that discriminates between the species is the number of sex comb teeth on the male foreleg metatarsus (Matsuda et al., 2009). The number of teeth was significantly different (Welch's *t*-test: $P < 0.001$) between AABBg1 (mean ± SE, 16.60 ± 0.36; range, 13–20; $N = 30$) and T184 (6.53 ± 0.13; 5–8; $N = 32$), confirming the results of the previous report (Fig. 1A). The teeth numbers of the F_1 hybrids were intermediate between the two species (Fig. 1B) and not significantly different between the reciprocal crosses (Student's *t*-test: $P = 0.731$): AABBg1 ♀ × T184 ♂ (12.73 ± 0.26; 11–15; $N = 22$) and T184 ♀ × AABBg1 ♂ (12.60 ± 0.24; 10–16; $N = 35$). However, the F_1 teeth numbers were significantly different from those of AABBg1 (nested ANOVA: $F_{1,1} = 1{,}401$, $P = 0.017$) and T184 ($F_{1,1} = 3{,}487$, $P = 0.011$).

Male specimens from Penang Island exhibited a large range for this trait (8.05 ± 0.16; 4–23; $n = 221$; Fig. 1C). We separated the male flies into two groups in terms of the teeth number (Supplementary Table S1 online). The average number of teeth for one group (15.67 ± 1.01; 12–23; $n = 12$) was not significantly different from AABBg1 (Welch's *t*-test: $P = 0.399$), and was thus assumed to be *D. ananassae*, and the number for the other (7.61 ± 0.09; 4–10; $n = 209$) was closer to that of T184 but still significantly greater than the latter (Welch's *t*-test: $P < 0.001$). Although we cannot rule out the presence of interspecific hybrids in the population, the majority of the flies in the second group seem to be *D.* cf. *parapallidosa*.

In the isofemale lines established from the Penang Island population, the trend was similar, exhibiting a large range of teeth numbers (10.39 ± 0.45; 6–27; $n = 96$), with significant heterogeneity among the lines (ANOVA: $F_{15,80} = 47.52$, $P < 0.001$; Table 1). Based on a post-hoc analysis

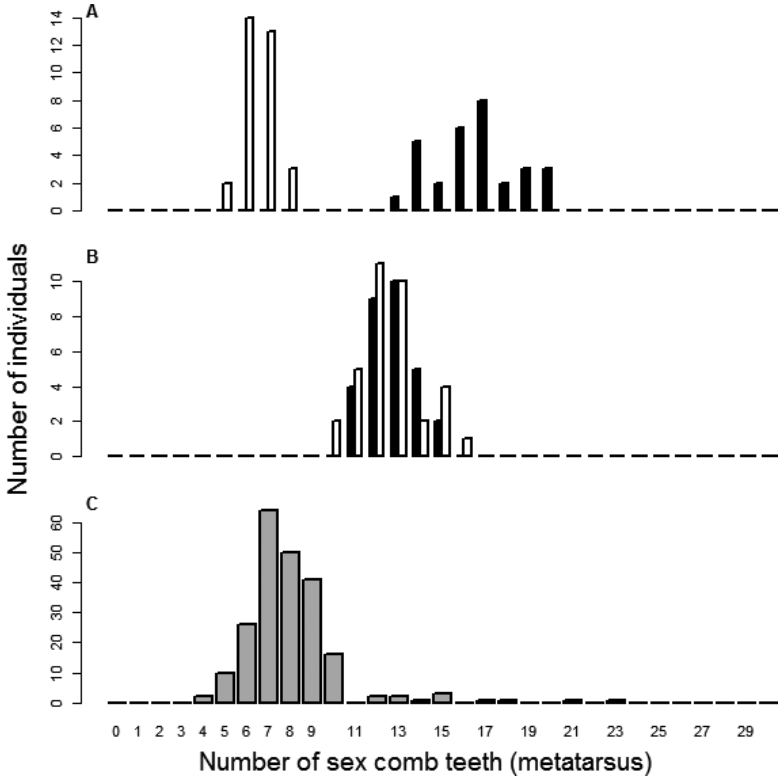

Fig. 1. Number of sex comb teeth on the foreleg metatarsus. **(A)** *D. parapallidosa* T184 (open) and *D. ananassae* AABBg1 (filled); **(B)** AABBg1 ♀ × T184 ♂ (filled) and T184 ♀ × AABBg1 ♂ (open); **(C)** Specimens of the natural population on Penang Island.

Table 1. Characterization of isofemale lines established from the Penang Island population.

Isofemale line name	Species presumed	Number of sex comb teeth mean ± SE (range)	Molecular marker		Chromosome arrangement					
			kl-5	*ΨCOI*	XL	XR	2L	2R	3L	3R
PN13-1-19	*D. ananassae*	22.67 ± 1.33 (19–27)	ana	ana	ST	ST	ST	ST	ST, A	ST, A
PN13-3-20	*D. ananassae*	19.50 ± 0.62 (17–21)	ana	ana	ST	ST	ST, A	ST	ST, A	ST, A
PN13-1-01	*D.* cf. *parapallidosa*	8.00 ± 0.45 (6–9)	ana	ana	A	ST	C + B	A	A, E	ST
PN13-1-02	*D.* cf. *parapallidosa*	7.67 ± 0.42 (6–9)	ana	ana	A	ST	C + B	A	E	ST
PN13-1-03	*D.* cf. *parapallidosa*	9.67 ± 0.56 (8–12)	ana	ana	A	ST	C + B	A	E	ST
PN13-1-07	*D.* cf. *parapallidosa*	8.17 ± 0.65 (7–11)	ana	ana	A	ST	A, C + B	A, P	ST, A, E	ST
PN13-1-08	*D.* cf. *parapallidosa*	9.67 ± 0.67 (7–12)	ana	ana	A	ST	C + B	A	A, E	ST
PN13-1-10	*D.* cf. *parapallidosa*	9.17 ± 0.48 (8–11)	ana	ana	A	ST	ST, C + B	A	E	ST
PN13-1-11	*D.* cf. *parapallidosa*	8.83 ± 0.31 (8–10)	ana	ana	A	ST	C + B	A	A, E	ST
PN13-1-12	*D.* cf. *parapallidosa*	7.17 ± 0.31 (6–8)	ana	ana	A	ST	C + B	A	E	ST
PN13-1-14	*D.* cf. *parapallidosa*	9.50 ± 0.76 (7–12)	ana	ana	A	ST	C + B	A	A, E	ST
PN13-1-15	*D.* cf. *parapallidosa*	10.00 ± 0.63 (8–12)	ana	ana	A	ST	C + B	A	ST, A, E	ST
PN13-1-20	*D.* cf. *parapallidosa*	7.67 ± 0.56 (6–10)	ana	ana	A	ST	C + B	A	E	ST
PN13-1-16	*D.* cf. *parapallidosa*	8.83 ± 0.65 (6–11)	ana	ana/para	A	ST	C + B	A	E	ST
PN13-3-23	*D.* cf. *parapallidosa*	10.50 ± 0.43 (9–12)	ana	ana/para	A	ST	C + B	A	E	ST
PN13-3-34	*D.* cf. *parapallidosa*	9.17 ± 0.48 (8–11)	ana	ana/para	A	ST	C + B	A	E	ST

Number of sex comb teeth: $N = 6$ each. Molecular marker: ana, *D. ananassae* allele; para, *D. parapallidosa* allele; ana/para, polymorphic for these alleles. Chromosome arrangement: ST stans for a standard chromosome, and others are inversions like *In(XL)A*. *In(2R)P* is an inversion (the breakpoints are 51C; 62A) newly discovered on *In(2R)A*.

(pairwise *t*-tests with Bonferroni correction), we separated the lines into two groups. One group (two lines; 21.08 ± 0.85; 17–27; n = 12) had significantly more teeth than the other (14 lines; 8.86 ± 0.17; 6–12; n = 84). We assume that the former is *D. ananassae* and the latter *D.* cf. *parapallidosa*. The teeth numbers of the *D.* cf. *parapallidosa* lines are significantly larger than those of T184 (nested ANOVA: $F_{1,13}$ = 21.18, P < 0.001), significantly smaller than those of AABBg1 ($F_{1,13}$ = 223.9, P < 0.001), but not significantly different than the wild-caught *D.* cf. *parapallidosa* ($F_{1,13}$ = 15.68, P = 0.195). The teeth numbers of the *D. ananassae* lines were not significantly different from those of AABBg1 ($F_{1,1}$ = 5.73, P = 0.252) and the wild-caught *D. ananassae* ($F_{1,1}$ = 5.85, P = 0.250). Unexpectedly, no significant difference was detected between the *D. ananassae* and T184 lines ($F_{1,1}$ = 61.44, P = 0.081), presumably due to the smaller number of *D. ananassae* lines obtained.

Molecular analysis

It is difficult to rigidly discriminate between *D. ananassae* and *D. parapallidosa* using molecular markers, presumably because of incomplete lineage sorting or interspecific gene flow (Sawamura et al., 2010). The diagnostic markers known to date are *ΨCOI* on the smallest and presumably non-recombining chromosome 4 (Sawamura et al., 2008a) and *kl-5* on chromosome Y (Supplementary Figure S2 online). First, we confirmed the presence of these markers by restriction enzyme digestion using DNA from *D. ananassae* and *D. parapallidosa* isofemale lines established from sympatric (or secondary contacted) populations outside Penang Island. The markers showed a perfect match in 7 *D. ananassae* and 14 *D. parapallidosa* lines.

Surprisingly, all male specimens (n = 222) and the isofemale lines (n = 16) of the Penang Island population carried *kl-5* from *D. ananassae* (Tables 1 and 2). Thus, the *D. ananassae* chromosome Y monopolizes the population even though the majority of the population is morphologically similar to *D. parapallidosa*.

ΨCOI was not amplified in 28 of 232 female specimens of the Penang Island population (Table 2). These females may be of other species that are morphologically indistinguishable in females from the *D. ananassae* species complex (Bock and Wheeler, 1972; McEvey and Schiffer, 2015). In fact, some isofemale lines established simultaneously were identified as *D. bipectinata*, *D. parabipectinata*, and *D. atripex*. Among the male and female specimens amplified (n = 426), *ΨCOI* was homozygous for the *D. ananassae* allele in 416 and heterozygous for the *D. ananassae*/*D. parapallidosa* alleles in three females and seven males (Table 2). Among the *D.* cf. *parapallidosa* males, the number of sex comb teeth was not significantly different (Student's *t*-test: P = 0.919) between *ΨCOI* heterozygotes (7.67 ± 0.42; 7–9; n = 6; both forelegs lost in one male) and homozygotes (7.61 ± 0.09; 4–10; n = 203; Supplementary Table S1 online). Furthermore, *ΨCOI* was monomorphic for the *D. ananassae* allele in 13 isofemale (two *D. ananassae* and 11 *D.* cf. *parapallidosa*) lines and polymorphic in three (*D.* cf. *parapallidosa*; Table 1).

The *ΨCOI* alleles were sequenced in two heterozygous specimens (one female and one male). In both specimens one allele was exactly the same as the previously described haplotype #1 of *D. ananassae* (Sawamura et al., 2008a), whereas the other allele had three substitutions (G42A, A51G, and A516G) from the *D. parapallidosa*-specific haplotype #6 (Sawamura et al., 2008a). This new haplotype (#7; DDBJ/EMBL/GenBank databases accession number LC033608) is separated from the basal hypothetical *D. ananassae* haplotype by 20 mutational steps.

These results led us to ask why the *D. parapallidosa* allele of *ΨCOI* is rare (P = 0.014 ; calculated from the population analysis) and no *D. parapallidosa* homozygotes were detected (expected P^2 < 0.001) in Penang Island *D.* cf. *parapallidosa*. To examine segregation and viability of the *ΨCOI* alleles, the locus was characterized in randomly chosen parents and the offspring from the *D.* cf. *parapallidosa* isofemale lines polymorphic for *ΨCOI*. The *ΨCOI* alleles segregated normally in heterozygous females (χ^2 = 2.083, df = 1, P = 0.149) and males (χ^2 = 0.750, df = 1, P = 0.386), and the viability of *D. parapallidosa ΨCOI* homozygotes was normal (χ^2 = 0, df = 2, P = 1.000; Table 3). Therefore, we have to allow for other possibilities than meiotic drive and natural selection.

Polytene chromosome analysis

Chromosome arrangement is different between *D. ananassae* and *D. parapallidosa* (Tomimura et al., 1993; Matsuda et al., 2009). *Drosophila parapallidosa*-specific chromosomes (*In(2L)C* + *In(2L)B*, *In(2R)A*, and *In(3L)E*) were observed in *D.* cf. *parapallidosa* but never in the *D. ananassae* isofemale lines established from the Penang Island population (Table 1). Chromosome arrangements (*2L-ST*, *In(2L)A*, *3L-ST*, and *In(3L)A*) observed in some *D.* cf. *parapallidosa* isofemale lines (Table 1) might be the result of introgression from *D. ananassae*.

Table 2. PCR-RFLP summary for specimens from the Penang Island population.

Sex	Number examined	*kl-5*		*ΨCOI*			
		ana	para	not amplified	ana/ana	ana/para	para/para
Female	232	–	–	28	201	3	0
Male	222	222	0	0	215	7	0

kl-5: ana, *D. ananassae* allele; para, *D. parapallidosa* allele. *ΨCOI*: ana/ana, homozygous for *D. ananassae* allele; para/para, homozygous for *D. parapallidosa* allele; ana/para, heterozygote for these alleles.

Table 3. Inheritance of the *ΨCOI* alleles in the *D.* cf. *parapallidosa* isofemale lines.

Cross	*ΨCOI*			Total	Chi-square test
	ana/ana	ana/para	para/para		
ana/ana ♀ × ana/para ♂	19	29	–	48	n.s. from 1:1
ana/para ♀ × ana/ana ♂	21	27	–	48	n.s. from 1:1
ana/para ♀ × ana/para ♂	12	24	12	48	n.s. from 1:2:1

ΨCOI: ana/ana, homozygous for *D. ananassae* allele; para/para, homozygous for *D. parapallidosa* allele; ana/para, heterozygous for these alleles.

Crossing analysis

There is a strong premating isolation in the *D. ananassae* ♀ × *D. parapallidosa* ♂ cross, and the isolation is weaker in the reciprocal cross (Matsuda et al., 2009). We confirmed these observations using the AABBg1 and T184 strains (Table 4); the mating frequency of AABBg1 ♀ × T184 ♂ (5%) was significantly different (Fisher's exact test: $P < 0.001$ against AABBg1 and T184) from those of the pure species crosses (100%), but the mating frequency of T184 ♀ × AABBg1 ♂ (90%) was not significantly different from the latter ($P = 0.490$ against AABBg1, $P = 0.487$ against T184).

The *D. ananassae* isofemale lines from the Penang Island population behaved in a manner similar to authentic *D. ananassae* (Table 4). The males readily mated with both AABBg1 and T184 females (60–100%), and the females mated with AABBg1 males (89–100%) but not with T184 males (0%). Conversely, the *D.* cf. *parapallidosa* isofemale lines from the Penang Island population (irrespective of their chromosome 4 constitution) were not isolated from *D. parapallidosa*, but were variably isolated from *D. ananassae* in the reciprocal crosses (Table 4). The males frequently mated with T184 females (100%) but less so with AABBg1 females (0–44%), and the females frequently mated with T184 males (80–100%) but less so with AABBg1 males (0–70%). The same pattern was seen in the crosses among the isofemale lines (Supplementary Table S2 online).

The *D. ananassae* ♀ × *D. parapallidosa* ♂ cross produces F_1 fertile females but sterile males, and the reciprocal cross produces F_1 fertile females and males (Matsuda et al., 2009). We confirmed these observations using the AABBg1 and T184 strains (Table 4). The *D. ananassae* isofemale lines from the Penang Island population behaved like authentic *D. ananassae*. F_1 males were sterile when the females were crossed with T184 males (Table 4). Interestingly, the *D.* cf. *parapallidosa* isofemale lines produced fertile F_1 males when the males were crossed with AABBg1 females (Table 4). This might be the consequence of introgressions of *D. ananassae* genes into Penang Island *D.*cf. *parapallidosa*.

Experimental hybridization

As we have suggested, Penang Island *D.* cf. *parapallidosa* seems to carry *D. ananassae* introgressions on a *D.*

Table 4. Reproductive isolation tests for isofemale lines and artificial hybrid populations.

Line name	Species presumed	test ♀ × AABBg1 ♂			test ♀ × T184 ♂			AABBg1 ♀ × test ♂			T184 ♀ × test ♂		
		% mated	(*N*)	F_1 ♂	% mated	(*N*)	F_1 ♂	% mated	(*N*)	F_1 ♂	% mated	(*N*)	F_1 ♂
AABBg1	*D. ananassae*	100	(17)	F	5	(20)	S	100	(17)	F	90	(20)	F
T184	*D. parapallidosa*	90	(20)	F	100	(19)	F	5	(20)	S	100	(19)	F
PN13-1-19	*D. ananassae*	89^{AT}	(9)	F	0^{A}	(10)	S	60^{AT}	(10)	F	90^{AT}	(10)	F
PN13-3-20	*D. ananassae*	100^{AT}	(10)	F	0^{A}	(10)	S	100^{A}	(7)	F	100^{AT}	(7)	F
PN13-1-01	*D.* cf. *parapallidosa*	0	(10)	F	100^{T}	(10)	F	10^{T}	(10)	F	100^{AT}	(10)	F
PN13-1-02	*D.* cf. *parapallidosa*	30	(10)	F	80^{T}	(10)	F	33^{T}	(6)	F	100^{AT}	(7)	F
PN13-1-03	*D.* cf. *parapallidosa*	20	(10)	F	100^{T}	(10)	F	20^{T}	(10)	F	100^{AT}	(6)	F
PN13-1-07	*D.* cf. *parapallidosa*	60^{AT}	(10)	F	100^{T}	(10)	F	10^{T}	(10)	F	100^{AT}	(9)	F
PN13-1-08	*D.* cf. *parapallidosa*	0	(10)	F	100^{T}	(10)	F	10^{T}	(10)	F	100^{AT}	(10)	F
PN13-1-10	*D.* cf. *parapallidosa*	10	(10)	F	100^{T}	(9)	F	11^{T}	(9)	F	100^{AT}	(9)	F
PN13-1-11	*D.* cf. *parapallidosa*	20	(10)	F	80^{T}	(10)	F	20^{T}	(10)	F	100^{AT}	(10)	F
PN13-1-12	*D.* cf. *parapallidosa*	10	(10)	F	100^{T}	(8)	F	0^{T}	(10)	F	100^{AT}	(10)	F
PN13-1-14	*D.* cf. *parapallidosa*	10	(10)	F	100^{T}	(9)	F	33^{T}	(9)	F	100^{AT}	(7)	F
PN13-1-15	*D.* cf. *parapallidosa*	70^{AT}	(10)	F	80^{T}	(10)	F	0^{T}	(7)	F	100^{AT}	(5)	F
PN13-1-20	*D.* cf. *parapallidosa*	10	(10)	F	100^{T}	(10)	F	44^{AT}	(9)	F	100^{AT}	(8)	F
PN13-1-16	*D.* cf. *parapallidosa*	30	(10)	F	100^{T}	(10)	F	0^{T}	(10)	F	100^{AT}	(10)	F
PN13-3-23	*D.* cf. *parapallidosa*	0	(10)	F	100^{T}	(9)	F	33^{T}	(9)	F	100^{AT}	(9)	F
PN13-3-34	*D.* cf. *parapallidosa*	10	(10)	F	100^{T}	(10)	F	10^{T}	(10)	F	100^{AT}	(10)	F
H1	artificial	100^{AT}	(9)	F	17^{A}	(6)	F	100^{A}	(6)	F	86^{AT}	(7)	F
H2	artificial	100^{AT}	(7)	F	44^{A}	(9)	F	70^{A}	(10)	S	100^{AT}	(10)	F
H3	artificial	100^{AT}	(7)	F	10^{A}	(10)	F	100^{A}	(10)	F	100^{AT}	(9)	F
H4	artificial	100^{AT}	(10)	F	0^{A}	(9)	F	100^{A}	(10)	F	100^{AT}	(8)	F
H5	artificial	100^{AT}	(9)	F	0^{A}	(8)	F	100^{A}	(10)	F	90^{AT}	(10)	F
H6	artificial	100^{AT}	(10)	F	0^{A}	(10)	F	100^{A}	(10)	F	100^{AT}	(7)	F
H7	artificial	100^{AT}	(10)	F	30^{A}	(10)	F	100^{A}	(10)	F	88^{AT}	(8)	F
H8	artificial	100^{AT}	(10)	F	10^{A}	(10)	F	100^{A}	(10)	F	100^{AT}	(7)	F
H9	artificial	100^{AT}	(9)	F	11^{A}	(9)	F	90^{A}	(10)	F	100^{AT}	(10)	F
H10	artificial	90^{AT}	(10)	F	70^{T}	(10)	F	100^{A}	(7)	F	100^{AT}	(9)	F
BC	artificial	90^{AT}	(10)	F	100^{T}	(10)	F	0^{T}	(10)	F	100^{AT}	(10)	F

F_1♂ stands for F_1 male fertility (F, fertile; S, sterile). *ΨCOI* is polymorphic for the *D. ananassae* and *D. parapallidosa* alleles in PN13-1-16, PN13-3-23, and PN13-3-34. Shaded columns reproduced from the left. Fisher's exact test with Bonferroni correction: A, not significantly different ($P > 0.05/27$) from AABBg1; T, not significantly different ($P > 0.05/27$) from T184.

parapallidosa genetic background. The direction of the original interspecific cross must have been *D. parapallidosa* ♀ × *D. ananassae* ♂, because Penang Island *D.* cf. *parapallidosa* inherited chromosome Y from *D. ananassae*. This scenario is consistent with the known asymmetry of reproductive isolation: the *D. parapallidosa* ♀ × *D. ananassae* ♂ cross occurs more frequently than the reciprocal cross, and the F_1 males are fertile (Matsuda et al., 2009; confirmed in the present analysis).

We established artificial hybrid populations (H lines) from the *D. parapallidosa* T184 ♀ × *D. ananassae* AABBg1 ♂ cross by sib matings. The numbers of sex comb teeth for the H lines were intermediate between the species (13.64 ± 0.34; 9–20; $n = 59$) and not significantly different among the lines (ANOVA: $F_{9,49} = 0.5621$, $P = 0.821$). However, the numbers of sex comb teeth were significantly different from both parental strains and F_1 (nested ANOVA: $F_{1,9} = 42.46$, $P < 0.001$ against AABBg1, $F_{1,9} = 256.6$, $P < 0.001$ against T184, and $F_{1,10} = 7.75$, $P = 0.019$ against F_1; Table 5).

Chromosome Y of the H lines was from *D. ananassae*, and chromosome 4 seemed to be randomly inherited: the ratio of the *ΨCOI* genotypes was 10:29:11 (ana/ana:ana/para:para/para), which is not significantly different from 1:2:1 ($\chi^2 = 1.32$, df = 2, $P = 0.517$; Table 5). Chromosome arrangement of the H lines was not fixed but was a mix of the *D. ananassae* and *D. parapallidosa* chromosome arrangements (Table 5). Thus, each H line seems to be a hybrid swarm.

Males from the H lines easily mated with AABBg1 females (70–100%) and T184 females (86–100%), whereas females from the lines easily mated with AABBg1 males (90–100%) but not with T184 males (0–70%; Table 4). Thus, the H lines behaved like *D. ananassae* with respect to sexual isolation, although the isolation strength varied among the lines. When the H lines were crossed with AABBg1 or T184, all F_1 flies were fertile, with the exception of sterile males from the AABBg1 ♀ × H2 ♂ cross (Table 4). Thus, H lines (except for H2) behave like Penang Island *D.* cf. *parapallidosa* with respect to hybrid sterility.

We also established an artificial hybrid population (BC line) from the *D. parapallidosa* T184 ♀ × *D. ananassae* AABBg1 ♂ cross by repeatedly backcrossing males to T184 females. The number of sex comb teeth for the BC line (6.71 ± 0.22; 5–8; $n = 14$) was significantly different from AABBg1 (Welch's *t*-test: $P < 0.001$) but not from T184 (Student's *t*-test: $P = 0.468$; Table 5). Chromosome Y of the BC population was from *D. ananassae*, whereas the *D. parapallidosa* chromosome 4 was fixed in this line ($n = 20$; Table 5). Chromosome arrangement in the BC line was exactly the same as in T184 and the line seemed to be pure *D. parapallidosa* except for chromosome Y (Table 5).

The BC line behaved similarly to authentic *D. parapallidosa* with respect to premating isolation. Females from the line easily mated with T184 males (100%) and AABBg1 males (90%), whereas males from the line easily mated with T184 females (100%) but not with AABBg1 females (0%; Table 4). However, when F_1 flies were produced from the cross between the BC line and AABBg1 or T184, they were all fertile (Table 4). Thus, the BC line is closer to Penang Island *D.* cf. *parapallidosa* than the H lines, although the constitution of chromosome 4 differs from that of the natural population.

DISCUSSION

Taken together, our results suggest that the *D. ananassae* species complex population on Penang Island consists of two species, *D. ananassae* and *D.* cf. *parapallidosa*, the latter of which is a hybrid descendant originating from *D. ananassae* and *D. parapallidosa*. Herein, we propose a scenario for the establishment of *D.* cf. *parapallidosa* on the Penang Island (Fig. 2).

We propose that *D. ananassae* colonized Penang Island earlier than *D. parapallidosa*; the latter was endemic to Borneo (perhaps to Java too) until recently (Tobari, 1993; Matsuda and Tobari, 2009; Kimura and Suwito, 2012). If very few individuals of *D. parapallidosa* immigrated to the Penang Island, the founders likely had difficulty in finding conspecific mating partners. Even if *D. parapallidosa* was historically distributed on the Penang Island, a similar scenario is conceivable under the situation of abundant *D. ananassae* and rare *D. parapallidosa*. It has been observed that the less abundant species in a community is more likely to mate heterospecifically (Matute, 2014). Therefore, *D. parapallidosa* females may have been forced to mate with *D.*

Table 5. Characterization of artificial hybrid populations between *D. ananassae* AABBg1 and *D. parapallidosa* T184.

Line name	Number of sex comb teeth mean ± SE (range)	Molecular marker		Chromosome arrangement					
		kl-5	*ΨCOI*	XL	XR	2L	2R	3L	3R
H1	12.83 ± 0.98 (9–15)	ana	ana/para	ST	ST	ST, B, C	ST, A	A, E	ST, B
H2	14.40 ± 1.29 (10–17)	ana	ana/para	ST	ST	ST, B, C	ST, A	ST, A, E	ST, B
H3	12.83 ± 0.60 (11–15)	ana	ana/para	ST	ST	ST, B, C	ST, A	ST, A, E	ST, B
H4	13.33 ± 1.84 (10–20)	ana	ana/para	ST	ST	ST, B, C	ST, A	A, E	ST, B
H5	15.50 ± 1.15 (13–19)	ana	ana/para	ST	ST	ST, B, C	ST, A	ST, A, E	ST, B
H6	14.17 ± 0.48 (13–16)	ana	ana/para	ST	ST	ST, B, C	ST, A	ST, A, E	ST, B
H7	13.00 ± 0.73 (11–15)	ana	ana/para	ST	ST	ST, B, C	ST, A	ST, A, E	ST
H8	13.33 ± 1.33 (10–19)	ana	ana/para	ST	ST	ST, B, C	ST, A	A, E	ST, B
H9	13.67 ± 0.92 (10–16)	ana	ana/para	ST	ST	ST, B, C	ST, A	ST, A, E	ST, B
H10	13.50 ± 1.18 (11–18)	ana	ana/para	ST	ST	ST, B, C	ST	ST, A, E	ST, B
BC	6.71 ± 0.22 (5–8)	ana	para	ST	ST	C + B	A	ST, E	ST, B
AABBg1	16.60 ± 0.36 (13–20)	ana	ana	ST	ST	ST	ST	A	ST
T184	6.53 ± 0.13 (5–8)	para	para	ST	ST	C + B	A	ST, E	ST, B

Number of sex comb teeth: $N = 6$ each; exceptions are H2 (N = 5), BC (N = 14), AABBg1 (N = 30; data from Fig. 1A), and T184 (N = 32; data from Fig. 1A). Molecular marker: ana, *D. ananassae* allele; para, *D. parapallidosa* allele; ana/para, polymorphic for these alleles. Chromosome arrangement: ST stands for a standard chromosome, and others are inversions like *In(2L)B*. For 2L chromosome arrangement, we cannot discriminate between *In(2L)C* + *In(2L)B*/ST and *In(2L)B*/*In(2L)C* individuals.

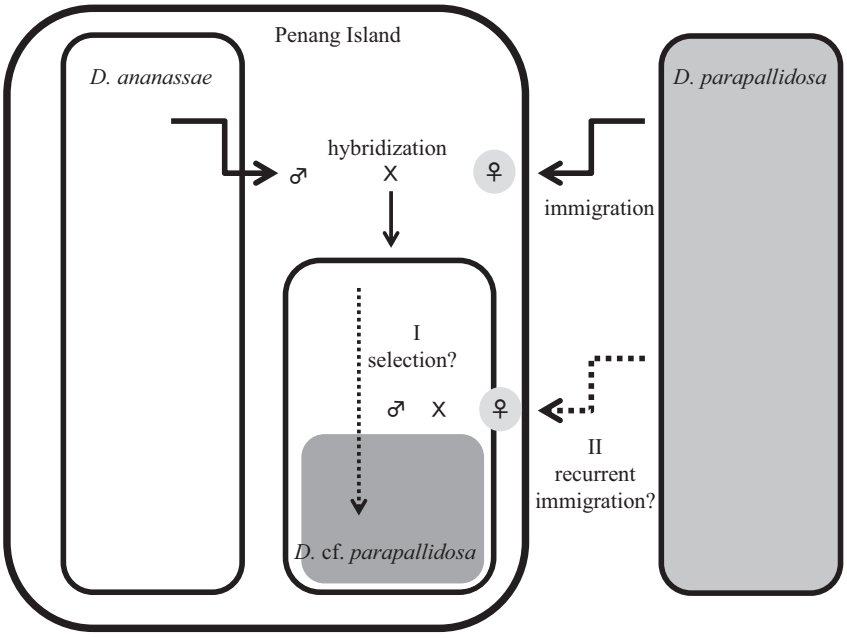

Fig. 2. A scenario for the establishment of the *D. ananassae* complex natural population on Penang Island.

ananassae males, because this direction of the cross is easier than the reciprocal (Matsuda et al., 2009). F_1 females and males from this cross are fertile and can produce offspring by sib mating. This scenario is consistent with the observation that Penang Island *D.* cf. *parapallidosa* has chromosome Y from *D. ananassae*. Unfortunately we do not currently have mitochondrial molecular markers that can distinguish between the two species.

Drosophila cf. *parapallidosa* on the Penang Island is not an intermediate between *D. ananassae* and *D. parapallidosa*, but rather is closer to *D. parapallidosa* in phenotype. This is different from the artificial hybrid lines derived from the single cross of *D. parapallidosa* ♀ × *D. ananassae* ♂ (H lines). *Drosophila ananassae* genes might have been selected against in the natural hybrid population by natural or sexual selection (scenario I in Fig. 2). Alternatively, the phenotype might have arisen via repeated backcrosses of males to *D. parapallidosa* females similarly to the BC line (scenario II in Fig. 2). But scenario II seems unlikely, because we have to postulate abundant *D. parapallidosa* immigration to the Penang Island and we cannot explain why neither the reciprocal backcross nor that to *D. ananassae* occurred. Further investigations are necessary to clarify the later process after the initial hybridization.

The proposed scenario for the direction of the original interspecific cross explains why Penang Island *D.* cf. *parapallidosa* has chromosome Y from *D. ananassae*, but it does not explain why chromosome 4 from *D. ananassae* is also more prominent. Possibilities are selection and meiotic drive, but these are inconsistent with the fact that chromosome 4 inheritance appears to be random in the isofemale lines and the artificial hybrid lines. Prominence of *D. ananassae* chromosome 4 may have been caused by a genetic bottleneck in the ancestor of *D.* cf. *parapallidosa* on the Penang Island.

Interestingly, the pattern of sexual isolation towards *D. ananassae* is different between the authentic *D. parapallidosa* and the Penang Island *D.* cf. *parapallidosa*. Sexual isolation in this system is usually asymmetrical; there is a strong premating isolation in the *D. ananassae* ♀ × *D. parapallidosa* ♂ cross but not in the reciprocal cross (Matsuda et al., 2009). Premating isolation, in contrast, was seen in both directions of crosses between *D. ananassae* and the Penang Island *D.* cf. *parapallidosa*. This may be the consequence of reinforcement in the Penang Island population, if the chance of encounter between interspecific flies increased in the secondary contact area. As has been suggested for other *Drosophila* species pairs (Noor, 1995; Higgie et al., 2000; Matute, 2010), premating isolation might be strengthened in sympatry if hybrids are less fit than the parental species.

Also interestingly, the Penang Island *D.* cf. *parapallidosa* males produced fertile male hybrids when crossed with *D. ananassae* females, in contrast with authentic *D. parapallidosa* males producing sterile male hybrids. The artificial hybrid (H and BC) lines having chromosome Y from *D. ananassae* also produced fertile male hybrids in crosses with *D. ananassae* females, except for H2 line. This indicates that chromosome Y must be involved in the hybrid male fertility. However, H2 showed hybrid male sterility, suggesting that other genomic regions also affect hybrid male fertility. The involvement of chromosome Y in hybrid male sterility is common in *Drosophila* (Coyne, 1985; Johnson et al., 1993; Khadem and Krimbas, 1993; Pantazidis et al., 1993; Sweigart, 2010; Araripe et al., 2016).

Only 5% of the male specimens (12/221) and 12.5% of the isofemale lines (2/16) of the *D. ananassae* species complex from the Penang Island were *D. ananassae*. But we are not sure whether this reflects the relative abundance of the two species on the island. Flies were collected from rotten noni fruits, which most *Drosophila* species avoid (Lachaise and Silvain, 2004). It is possible that *D*. cf. *parapallidosa* is adapted to noni, as has been indicated in the Mayotte population of *D. yakuba* (Yassin et al., 2016). Seasonal *Drosophila* collection at different locations around the island will be helpful in addressing this issue in future studies.

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