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Speciation in progress? A continuum of reproductive isolation in *Drosophila bipectinata*

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Abstract

Incipient species in the early stages of divergence can provide crucial information about the genetic basis of reproductive isolation and the evolutionary forces that promote speciation. In this report, we describe two subspecies of *Drosophila bipectinata* that show a continuum of reproductive isolation. Crosses between strains of the same subspecies produce fully fertile offspring. At the same time, each subspecies harbors extensive variation for the degree of reproductive isolation from the other subspecies. The percentage of fertile hybrid males varies from 0 to 90%, depending on the origin of parental strains, indicating that the genes responsible for hybrid sterility are not fixed within either subspecies, or even within local populations. Reproductive isolation is non-transitive, so that the extent of hybrid sterility depends on the particular combination of strains. The two subspecies show little or no evidence of genetic differentiation at three nuclear loci, suggesting that they diverged very recently or continue to experience significant levels of gene flow. A hybrid zone between the two subspecies may exist in New Guinea and Northeastern Australia.

Introduction

Speciation is, in most cases, a gradual process caused by continual accumulation of developmental, behavioral, or ecological incompatibilities between diverging populations (Mayr, 1963; Coyne & Orr, 2004). Incipient species must pass through a stage where they have few genetic differences, and are only partially reproductively isolated from each other. Reproductive isolation between recently diverged taxa often takes the form of hybrid sterility or inviability (Coyne & Orr, 1989, 2004). Such intrinsic developmental incompatibilities are thought to be caused by epistatic interactions among species-specific alleles of two or more genes (Dobzhansky, 1937; Muller, 1940; Orr & Turelli, 2001). An important implication of this model is that speciation requires either sequential or simultaneous fixation of newly derived alleles at multiple loci.

Our understanding of speciation would be greatly improved if we could observe the early stages of this process. Theoretical studies suggest that developmental incompatibilities between diverging species accumulate at least as fast as the square of genetic divergence (Orr, 1995; Orr & Turelli, 2001). Empirical tests also confirm that the number of genes that cause hybrid sterility is considerably smaller in crosses between recently diverged taxa than between older, well-established species (Naveira & Fondevila, 1991; Davis & Wu, 1996; Wu et al., 1996; Sawamura et al., 2000; Orr & Irving, 2001). With increasing divergence time, it becomes difficult, if not impossible, to determine how many loci were required for the initial origin of reproductive isolation, what these loci were, and which DNA sequence changes in these loci were responsible for the developmental incompatibilities between species. At the same time, selective pressures, geographic ranges, and the levels of

migration and gene flow are likely to change over time, making it increasingly difficult to discern the biogeographic patterns of speciation and the relative contributions of demographic factors and natural or sexual selection to the evolution of reproductive isolation and the separation of the species' gene pools. Thus, whether we seek to understand the developmental-genetic mechanisms or the population genetics of speciation, nascent species in the earliest stages of divergence should provide the most accurate information.

In this report, we describe incipient reproductive isolation in *Drosophila bipectinata*, a species that belongs to the *ananassae* subgroup of the *Drosophila melanogaster* species group (Bock 1971, 1980; Lemeunier et al., 1986). *D. bipectinata* inhabits the area from India and Nepal to Australia and South Pacific islands (Bock, 1971; Okada, 1979; Bock & Parsons, 1984; Lemeunier et al., 1986) (Figure 1), and has been the subject of a number of evolutionary studies (Bock, 1978; Aotsuka & Tobari, 1983; Otsuka et al., 1983; Crossley, 1990; Singh & Chatterjee, 1991; Singh & Banerjee, 1995; Singh & Singh, 2001; Kopp & Barmina, 2004). We find that *D. bipectinata* can be subdivided into two largely allopatric subspecies on the basis of partial reproductive isolation. Crosses between the two subspecies produce completely or partially sterile male hybrids. Most importantly, each subspecies harbors extensive variation for the degree of reproductive isolation from the other subspecies. We find no evidence of genetic differentiation between the two subspecies at any of the three randomly chosen nuclear loci, suggesting that they diverged very recently and/or continue to exchange genes by introgressive hybridization. We conclude that the two subspecies of *D. bipectinata* are currently undergoing the earliest stages of speciation, and that the genes responsible for reproductive isolation are not yet fixed within either subspecies. These features should make *D. bipectinata* an excellent model for investigating the population

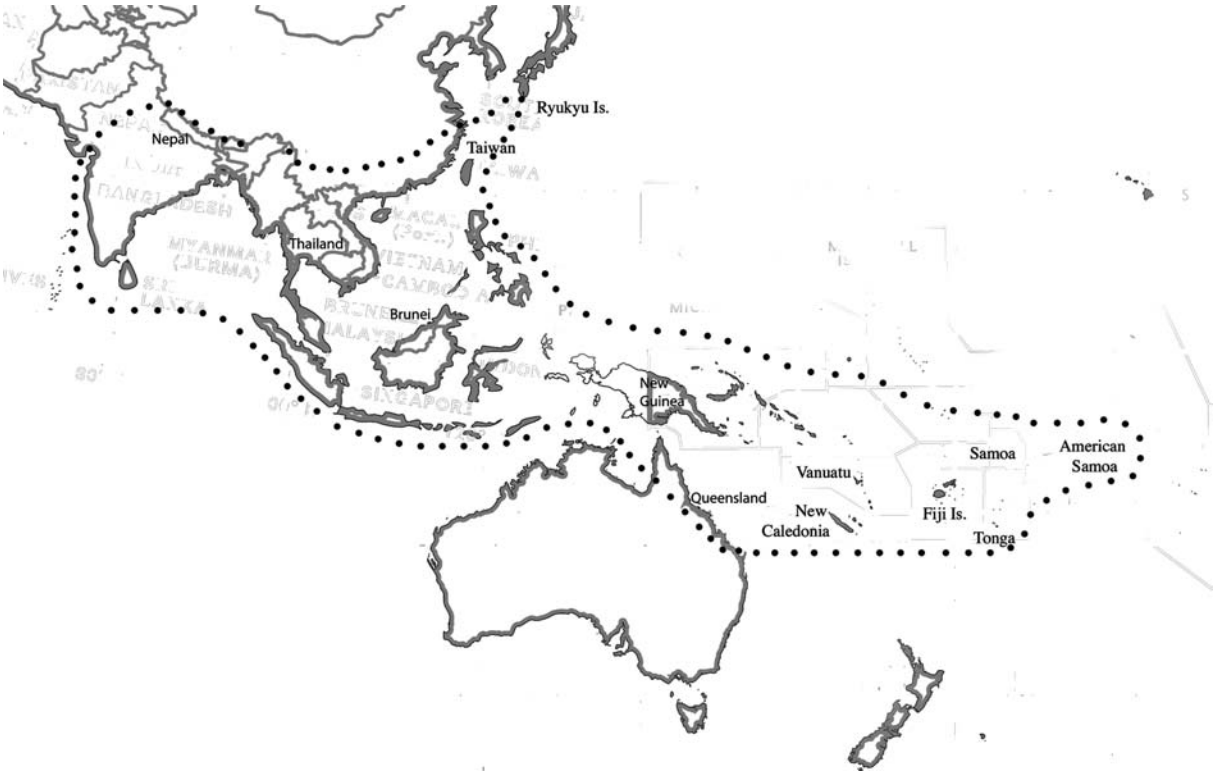

Figure 1. Geographic distribution of *D. bipectinata*. Collection locations are indicated for strains used in hybrid sterility tests. The map is based on published reports (Bock, 1971, 1980; Okada, 1979; Lemeunier et al., 1986), collection catalogs (*Drosophila* Species Stock Center, Univ. of Arizona; Ehime University, Tokyo Metropolitan University, and Tsukuba University *Drosophila* stock collections, and the Bishop Museum and Lund University museum collections), and personal communications from Drs. B. N. Singh, S. McEvey, M. Polak, M. Toda, and S.-C. Tsaur.

genetics of species divergence and the developmental-genetic basis of reproductive isolation.

Materials and methods

Drosophila strains and subspecies designation

Geographic origin and collection dates of all strains used in this work are listed in Table 1. Some of these strains are isofemale, while others were established from multi-female collections (Table 1). For brevity, strains from Australia, New Guinea, and the South Pacific islands are designated as the Pacific subspecies, while the strains from the Indian subcontinent, mainland and insular Southeast Asia, and Japan are referred to as the Asian subspecies. This designation is based solely on the patterns of reproductive isolation. We do not aim for a systematic description

Table 1. Geographic origin of *D. bipectinata* strains

Strains	Type[a]	Origin	Year[b]	Donor
Asian				
381.0*	Multi	Patan, Nepal	1954	TDSSC[d]
381.1	Multi	Cabuyao, Laguna, Luzon	?	TDSSC
381.3*	Multi	Chia-i, Taiwan	1967	TDSSC
381.4	Multi	Samut Songkhram, Thailand	1971	TDSSC
Pune	Multi	Pune, Maharashtra, India	1999	B. N. Singh
211.5	Iso	Nanjenshan, Pingtung County, Taiwan	1989	S.-C. Tsaur
KB*	Iso	Temburong, Brunei, Borneo	2003	our collection
B96*	Iso	Chiang Mai, Thailand	1979	M. Watada
D97	Iso	Coimbatore, India	1979	M. Watada
TKNK2*, TKNK3*	Iso	Tokunoshima, Kagoshima, Japan	2002	M. Watada
AM01-1	Iso	Amamioshima, Kagoshima, Japan	2001	M. Watada
AMM6	Iso	Amamioshima, Kagoshima, Japan	2002	M. Watada
ISG-B19, ISG-C12	Iso	Ishigakijima, Okinawa, Japan	2002	M. Watada
ISG89-5	Iso	Ishigakijima, Okinawa, Japan	1998	M. Watada
MYK98	Iso	Miyakojima, Okinawa, Japan	1998	M. Watada
MYK99-1	Iso	Miyakojima, Okinawa, Japan	1999	M. Watada
IR98-1	Iso	Iriomotejima, Okinawa, Japan	1998	M. Watada
HTM98-1, HTM98-5	Iso	Haterumajima, Okinawa, Japan	1998	M. Watada
YNG98-2	Iso	Yonagunijima, Okinawa, Japan	1998	M. Watada
South Pacific				
Lae321*, Lae327*	Iso	Lae, Papua New Guinea	1981	M. Watada
PPG93*, PPG96*	Iso	Pago-Pago, American Samoa	1981	M. Watada
TBU83*	Iso	Tongatapu, Tonga	1981	M. Watada
CTrib*	Multi	Cape Tribulation, Queensland, Australia	2000	M. Polak
NCal*	Multi	Noumea, New Caledonia	2000	M. Polak
Fiji*	Multi	Viti Levu, Fiji	2001	M. Polak
WSam*	Multi	Apia, Samoa	2003	M. Polak
Vanuatu*	Multi	Port Vila, Efate, Vanuatu	2003	M. Polak
381.2[c]	Multi	Pago-Pago, American Samoa[c]	1967	TDSSC

Strains used in the analysis of hybrid male sterility are indicated by asterisks.
[a] Iso – isofemale strain, Multi- strain established from a multi-female collection.
[b] Collection year.
[c] We cannot rule out that this strain was mislabeled or contaminated prior to arrival in our lab.
[d] Tucson *Drosophila* Species Stock Center.

of the subspecies, which will be provided by Matsuda et al. (this volume).

Fertility tests

For each pair of strains, hybrid males were obtained by mass crosses between 40–50 males and 40–50 virgin females. For some strain combinations, two or three replicate crosses were performed on different dates. 50 F1 hybrid males were usually tested for fertility from the progeny of each cross. Hybrid males 1–3 days of age were collected under CO_2 anesthesia and aged for 7 days. At the end of the aging period, they were again anesthetized, and healthy-looking males with intact wings were selected and crossed individually to two virgin females each in a 90×25 mm vial on standard media. The crosses were kept in a humidified incubator at 25°C under a 12/12 light cycle. After 3 or 4 days, the vials were inspected, and the crosses where either the male or both females were dead were considered "failed" and discarded. The remaining vials were returned to the incubator, and inspected again 7–9 days later. A male was classified as fertile if any larvae or larval tracks were found in the vial, regardless of the number of larvae or of their survival to adult stage. All raw data, including the number of fertile, sterile, and failed crosses, are presented in Supplement table (see website author).

In all crosses involving strain 381.3, half of the hybrid males were crossed to females of the maternal strain, and half to the paternal strain. The identity of females did not significantly influence the hybrid males' fertility in any of the crosses, and the results from both tests were therefore pooled. In all other instances, hybrid males were crossed to virgin females of the standard 381.3 strain.

Hybrid female fertility was tested for several strain combinations by crossing individual virgin females to two males of one of the parental strains. Abundant progeny were produced by all hybrid females, and we did not investigate female fertility further.

Sperm motility assays

Testes were dissected from males 7–10 days of age in insect saline (9.1 g/l NaCl, 0.52 g/l KCl, 1.2 g/l $CaCl_2 \times 2H_20$, 0.8 g/l $MgCl_2 \times 6H_20$). Dissected testes were mounted and gently squashed under a cover slip, and examined under Nomarski optics at 200–400× magnification. A male was considered to produce motile sperm if even a single moving spermatozoon was observed, as proposed by Coyne (Coyne, 1984).

DNA sequencing and sequence analysis

Genomic DNA samples were isolated from single males. For some of the multi-female strains, DNA was isolated and the target fragments amplified separately from three individual males, but in most cases only one individual per strain was used. We amplified and sequenced fragments of 3 nuclear genes (*Gpdh*, *al*, and *ple*) and one mitochondrial gene (*COI*). For each locus, the total length of aligned sequences, the number of non-coding nucleotide positions, the primers used for amplification, and the recommended annealing temperature for PCR are listed in Table 2. PCR fragments were either purified and sequenced directly using the amplification primers, or TA-

Table 2. Sequenced loci and amplification primers

Locus	Total length[a]	Non-coding[b]	Forward primer	Reverse primer	Temp[c]
al	510	432	GCTGGCGATGAAAATTGGATTAAC	TAGGGATTATACGGATGCGACTGG	55°C
Gpdh	764	210	GTGGTGCCCCACCAGTTCAT	GGCTTGAGCTGATTTGTGCA	55°C
ple	751	685	CATCTTCCAGAGCACCCAGTATGTG	GTAGATGGGCTGGTACTCCTGATCC	55°C
COI	553	0	CCAGCTGGAGGAGGAGATCC	CCAGTAAATAATGGGTATCAGTG	55°C

[a] Length of the aligned sequences.
[b] Number of non-coding nucleotide positions.
[c] Recommended annealing temperature for PCR.

cloned and sequenced using vector primers. All sequences have been deposited in Genbank under the following accession numbers: DQ 073837-DQ 073855 and AJ 844757- AJ 844809 (COI); DQ 073856-DQ 073875 and AJ 844670-AJ 844698 (al); DQ 073893-DQ 073911 and AJ 844728-AJ 844756 (Gpdh); DQ 073876. DQ 073892 and AJ 844810-AJ 844838 (ple).

ABI chromatograms were examined and conflicts in base calls were resolved, if needed, using EditView or Contig Express software (from ABI and Invitrogen, respectively). Some individuals were found to be heterozygous at one or more nucleotide positions, which were then represented using IUPAC ambiguity codes. Sequences were aligned using ClustalW (Thompson et al., 1994), and the alignments inspected and edited as needed. Phylogenetic analysis was performed in PAUP (Swofford, 2000) using maximum parsimony and minimum evolution criteria (Swofford et al., 1996). HKY85 distances (Hasegawa et al., 1985) were used for the minimum evolution analysis, but other distance measures were found to produce similar results.

Population genetic analysis was performed using ProSeq software (Filatov, 2002). Nucleotide diversity (π) (Tajima, 1983) and Watterson's estimate of the population mutation rate θ (Watterson, 1975) were calculated for each subspecies and locus (Table 3). The presence of recombination at nuclear loci was detected using the four-gamete test (Hudson & Kaplan, 1985). Recombination was found in *Gpdh* and *ple*, but not in *al* (not shown). Allele frequency spectrum within each subspecies was examined using Tajima's *D* and Fu and Li's *D* and *F* test statistics (Tajima, 1989; Fu & Li, 1993). To obtain mean estimates of sequence variation for each subspecies, the values of π, θ, & Tajima's *D* at each locus were weighted by the length of that locus. Significance of Tajima's *D* values was determined by comparing them to a distribution obtained by coalescent simulations (Hudson, 1990). For Fu and Li tests, nucleotide substitutions were polarized using outgroup sequences from *D. pseudoananassae*, and significance of the test statistics was assessed using critical values listed by Fu and Li (1993). To assess the extent of genetic differentiation between subspecies, Fst values were calculated for each locus, and their significance was determined by permutation tests (Hudson et al., 1992a, b).

Statistical analysis

Data in Figure 2 were analyzed using the χ^2 statistic. Separate analyses were performed for crosses between Pacific females and Asian males, and for crosses between Asian females and Pacific males. Strain Lae321 was excluded from both

Table 3. Polymorphism levels and frequency spectrum

Locus	Subspecies	N	L	S	M	π	θ (W)	Tajima's D	Fu & Li D	Fu & Li F
al	Asian	8	510	17	17	0.0095	0.0129	−1.3369	−2.3764**	−2.5487**
	Pacific	10	510	11	13	0.0068	0.0076	−0.4856	−1.5984	−1.6862
Gpdh	Asian	8	764	33	33	0.0135	0.0167	−1.0203	−1.9430	−2.0634
	Pacific	10	764	13	15	0.0057	0.0060	−0.2594	−1.0212	−1.2042
ple	Asian	8	751	17	17	0.0067	0.0087	−1.2245	−2.0950*	−2.2615*
	Pacific	10	751	7	9	0.0037	0.0033	0.4813	−1.3524	−1.5006
CO1	Asian	26	553	28	29	0.0045	0.0133	−2.4336*	−4.1181**	−4.2351**
	Pacific	12	553	13	13	0.0070	0.0078	−0.4490	−0.0103	−0.2884
Combined nuclear	Asian		2025	67	67	0.0100	0.0128	−1.1758		
	Pacific		2025	31	37	0.0052	0.0054	−0.0417		

N – number of sequences for each subspecies; *L* – number of aligned nucleotide positions; *S* – number of polymorphic sites; *M* – total number of mutations; π – average nucleotide diversity per base pair (Tajima, 1983); θ(W), Watterson (1975) estimate of the population mutation rate per base pair. Tajima's *D* and Fu & Li statistic values (Tajima, 1989; Fu and Li 1993) that are significant under the conservative assumption of no recombination within the locus are indicated by single asterisks ($p < 0.05$) or double asterisks ($p < 0.025$). For each subspecies, average values of π, θ, and Tajima's *D* across nuclear loci were calculated by weighting the value for each locus by the length of that locus (the bottom pair of values).

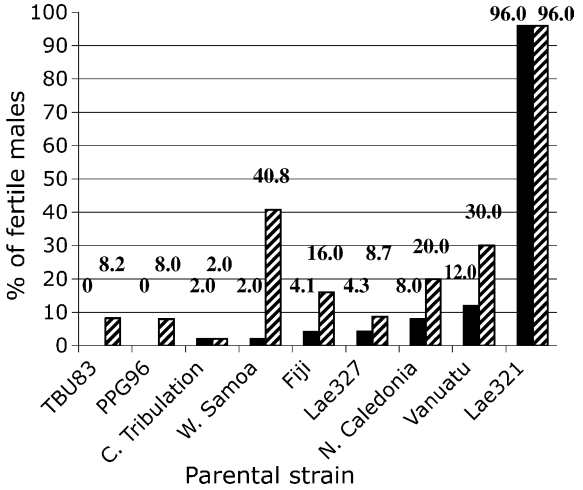

Figure 2. Fertility of F1 hybrid males from crosses between Asian strain 381.3 and 9 Pacific strains. Each bar represents the percentage of fertile hybrid males. Solid black bars represent the progeny of crosses between Pacific females and Asian males; hatched bars represent the progeny of crosses between Asian females and Pacific males. Numbers above each bar are the percentages of fertile hybrid males. The geographic origin of each strain is shown in Table 1, and the raw data in Supplementary table.

analyses. Data in Figures 3 and 4 were analyzed using ANOVA models (procedure GLM in SAS (SAS Institute, 1988)). Data in Figure 4 were fitted to a model $Y_{ik} = \mu + \alpha_i + \pi_{ik}$, where Y_{ik} is the observed percentage of fertile males for line i and replicate k, μ is the overall sample mean, λ_i is the variance attributable to parental line identity, and ϵ is the residual variance. Data in Figure 3 were fitted to a model $Y_{ijk} = \mu + \alpha_i + \pi_j + \alpha\pi_{ij} + \epsilon_{ijk}$, where Y_{ijk} is the observed percentage of fertile males from the cross between Asian line i and Pacific line j, replicate k; μ is the sample mean; α_i is the effect of Asian line identity; π_j is the effect of Pacific line identity; $\alpha\pi_{ij}$ is the effect of line interaction; and ϵ_{ijk} is the residual variance. Separate analyses were performed for crosses between Pacific females and Asian males, and for crosses between Asian females and Pacific males. Two sets of tests were performed. In the first, ϵ_{ijk} was used as the error term for α, π, and $\alpha\pi$. All three effects were highly significant ($p < 0.0001$). In the second analysis, the interaction effect $\alpha\pi$ was included in the error term for α and π. This produced a strong decrease in the significance of α and π effects ($p = 0.0497$–0.4703), indicating that the effect of line interaction was so large that the effects of line identities could not be properly estimated. We therefore report the results of the first type of analysis.

Results

Intraspecific hybrid sterility in D. bipectinata

In the course of our work, we discovered that the cross between *D. bipectinata* strains from Taiwan

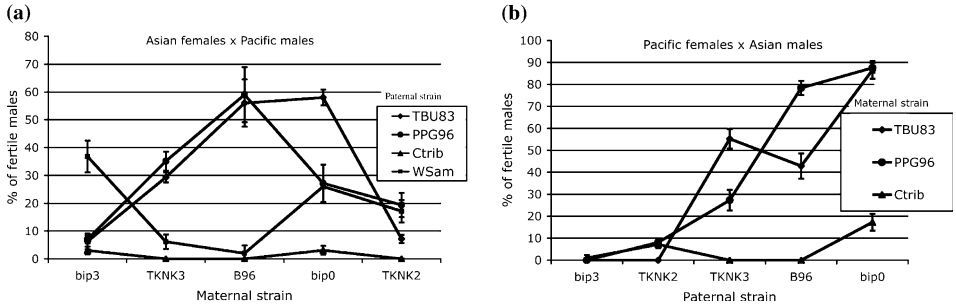

Figure 3. The effect of Asian and Pacific strain identities and strain interactions on hybrid male fertility. (a) F1 male progeny of crosses between Asian females and Pacific males. (b) F1 male progeny of crosses between Pacific females and Asian males. Each point represents the percentage of fertile hybrid males from the corresponding cross. Vertical bars represent ± one standard deviation. The origin of each strain is shown in Table 1. Exact percentages and raw data are in Supplementary table. The results of ANOVA analyses are as follows (see Materials and methods for notation and model definition). Panel a, interaction effect not included in the error term: $F_\alpha = 34.70$, $p_\alpha < 0.0001$; $F_\pi = 114.18$, $p_\pi < 0.0001$; $F_{\alpha\pi} = 36.64$, $p_{\alpha\pi} < 0.0001$. Panel a, interaction effect included in the error term: $F_\alpha = 0.95$, $p_\alpha = 0.4703$; $F_\pi = 3.12$, $p_\pi = 0.0664$. Panel b: interaction effect not included in the error term: $F_\alpha = 499.17$, $p_\alpha < 0.0001$; $F_\pi = 466.12$, $p_\pi < 0.0001$; $F_{\alpha\pi} = 129.78$, $p_{\alpha\pi} < 0.0001$. Panel b, interaction effect included in the error term: $F_\alpha = 3.85$, $p_\alpha = 0.0497$; $F_\pi = 3.59$, $p_\pi = 0.0771$.

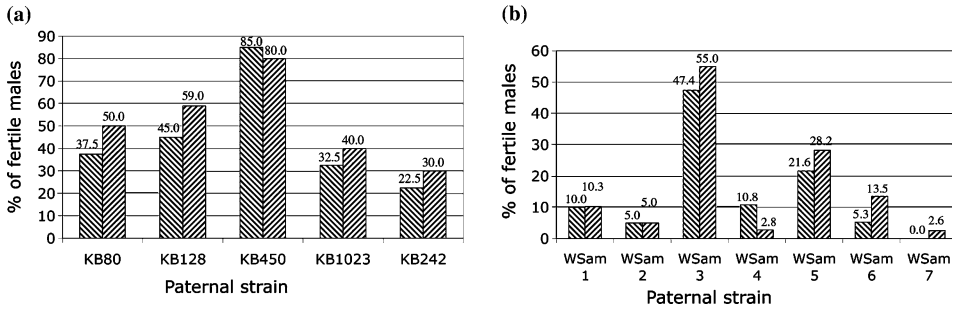

Figure 4. Intra-population variation in the extent of hybrid sterility. (a) F1 male progeny of crosses between females of the Pacific strain TBU83 and males of five isofemale Asian strains from Kuala Belalong, Brunei. (b) F1 male progeny of crosses between females of Asian strain 381.3 and males of seven "pseudo-isofemale" strains from Apia, Samoa. Each bar represents the percentage of fertile hybrid males; two replicates were performed for each cross. Raw data is presented in Supplementary table.

and Tonga produced sterile male hybrids. Consistent with Haldane's rule (Haldane, 1922), hybrid females have apparently normal fertility. A similar observation had been reported earlier by Tobari and colleagues in crosses between Japanese and New Guinean strains of this species (Tobari, 1978; Tobari & Kato, 1983). We therefore decided to investigate intraspecific reproductive isolation in *D. bipectinata* in more detail.

Hundred percent of the hybrid males produced in the cross between TBU83 (Tongan) females and 381.3 (Taiwanese) males were sterile when tested in single crosses (Figure 2). A very small number of F2 progeny were later obtained from mass crosses involving hundreds of hybrid males, indicating that male sterility is not complete. In these and other crosses, hybrid male fertility was not influenced by the geographic origin of the females they were mated to. Thus, male sterility is caused by the genotype of the males themselves, and not by male/female interactions. Numerous, prolonged copulations involving sterile hybrid males were observed. We dissected 20 females that had been mated to the hybrid males, yet failed to produce any progeny. In each case, sperm was found in the females' reproductive tracts. However, the sperm was immotile; only one barely twitching spermatozoon was seen in one of the females.

D. bipectinata males reach sexual maturity in less than 24 h. Testes, accessory glands, and seminal ducts dissected from sterile hybrid males 1–21 days of age were found to have normal size and morphology (Figure 5a). The testes had normal structure and contained numerous pre-meiotic cells as well as apparently mature sperm bundles (Figure 5b, c). Sterile hybrid males appeared to have fewer sperm bundles than males of the parental strains, but we did not attempt to quantify this difference. No motile sperm was observed in any of the 25 dissected males. We conclude that hybrid male sterility is caused by the failure to produce motile sperm due to late spermatogenesis arrest – a phenotype reported in other *Drosophila* hybrids, as well (Perez et al., 1993; Cabot et al., 1994; Palopoli & Wu, 1994; Kulathinal & Singh, 1998). The testis phenotype of the hybrid males produced in the crosses between different populations of *D. bipectinata* is in fact indistinguishable at the level of light microscopy from the phenotype of sterile interspecific hybrids produced by crosses between *D. bipectinata* and its sibling species *D. parabipectinata* or *D. malerkotliana* (data not shown).

To test whether hybrid male sterility was caused by a bacterial endosymbiont, as has been suggested in *D. paulistorum* (Ehrman & Kernaghan, 1971; Perez-Salas & Ehrman, 1971), cultures of the Tongan & Taiwanese strains were maintained on media containing tetracycline for two generations. Hybrid fertility was not restored by tetracycline treatment at the concentration of 0.005%, whereas higher concentrations were lethal to the flies. We also tested these and other strains of *D. bipectinata* for the presence of *Wolbachia* by PCR using conserved primers against 16S RNA and Wolbachia Surface Protein (Zhou et al., 1998). These tests were negative in all cases. Finally, the late spermatogenesis arrest in *D. bipectinata* is clearly different from the testis degeneration phenotype observed in the sterile hybrids of *D. paulistorum* (Kernaghan & Ehrman, 1970; Perez-Salas & Ehrman, 1971). Thus, it appears that hybrid male

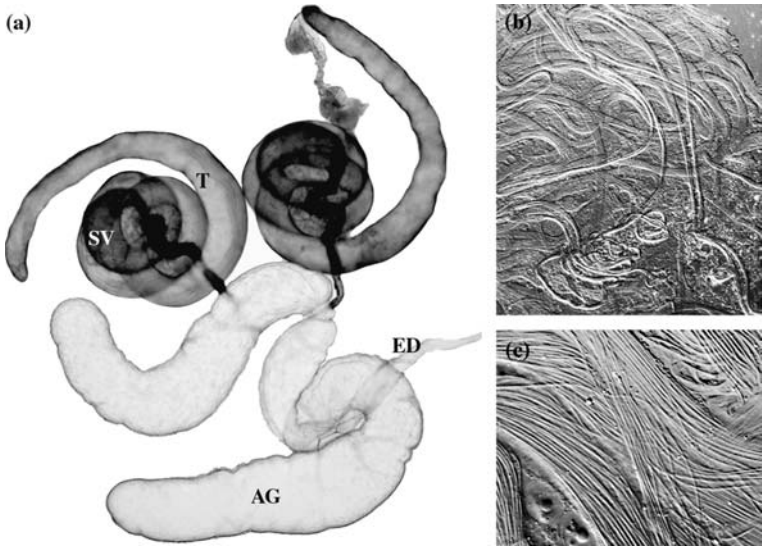

Figure 5. Late spermatogenesis arrest in hybrids between Asian and Pacific strains. (a) The morphology of testes (T), seminal vesicles (SV), accessory glands (AG) and ejaculatory ducts (ED) in sterile hybrid males is apparently normal. (b) Mature sperm bundles in the proximal testis (200×, DIC). (c) Mature sperm bundles under higher magnification (630×, DIC).

sterility in *D. bipectinata* is purely "genetic", i.e. not associated with an endosymbiotic infection.

Extensive variation in the degree of reproductive isolation

The Taiwanese strain 381.3 was crossed to nine strains of *D. bipectinata* from several South Pacific islands, Australia, and New Guinea. Crosses were performed in both directions: Asian females to Pacific males, and Pacific females to Asian males. In most cases, the percentage of fertile males in the F1 progeny varied from 0 to 40.8% (Figure 2). In both directions of crosses, the identity of the Pacific parent had a significant effect on hybrid male fertility ($\chi^2 = 29.65$, $p < 0.005$ for crosses between Pacific females and Asian males; $\chi^2 = 71.63$, $p < 0.005$ for crosses between Asian females and Pacific males). In most cases, crosses between Asian females and Pacific males produced more fertile male progeny than reciprocal crosses (Figure 2).

The only exception to the general pattern was the strain Lae321 from New Guinea, which produced 96% fertile males in crosses with 381.3 in both directions (Figure 2). In subsequent tests, we found that this strain produced 96–100% fertile hybrid males in crosses with four other Asian strains, but only 2–20% fertile males in crosses with four Pacific strains, including one strain that was collected at the same location in New Guinea (Supplement table). Molecular evidence rules out strain contamination or mis-labeling (see below). Thus, Lae321 behaves as an Asian strain, despite its geographic origin.

To further investigate the influence of parental strain origin on hybrid male sterility, we performed crosses between five Asian strains (from Nepal, Thailand, Taiwan, and Japan) and four Pacific strains (from Tonga, Samoa, American Samoa, and Australia) in 35 out of 40 possible combinations. The results are shown in Figure 3. It is clear from this analysis that both Asian and Pacific subspecies are highly variable for the extent of reproductive isolation from the other subspecies. The percentage of fertile F1 males spans the range from 0% to almost 90%, depending on the cross. ANOVA analysis showed that (1) the identity of the Asian parent has a strong effect on the fertility of hybrid males produced in crosses with Pacific strains (F_α = 34.70–499.17, depending on the direction of the cross; $p < 0.001$); (2) the identity of the Pacific parent has a strong effect on the fertility of hybrids produced in crosses with Asian strains (F_π = 114.18–466.12; $p < 0.0001$); and (3) the interaction effect is also highly significant ($F_{\alpha\pi}$ = 36.64–129.78; $p < 0.0001$), indicating that the hybrid sterility phenotype is non-transitive (in other words, the fertility of hybrids between

two strains cannot be predicted from the behavior of these strains in other crosses). There is no obvious correlation between the geographic proximity of two populations (Figure 1) and the fertility of their hybrid progeny.

In general, even the "fertile" hybrid males produce relatively few progeny (2.98 progeny per vial on average in crosses involving 381.3). We examined the testes of hybrid males from seven different crosses, always finding the same phenotype as in the hybrids between 381.3 and TBU83. We also performed a series of control crosses involving pairs of strains of the same subspecies (Supplement table). In 7 crosses among 6 different Asian strains, F1 male fertility varied from 94.3% to 100% (average 96.9%). Similarly, in 6 crosses among 6 Pacific strains, hybrid fertility varied from 88.6% to 100% (average 98.3%). Thus, there is no evidence of reproductive isolation within either subspecies.

Results presented above indicate that the genes responsible for reproductive isolation between the Asian and Pacific subspecies of *D. bipectinata* are not fixed within either subspecies. To test whether sterility genes segregate within local populations, as well as within subspecies as a whole, we first crossed the Pacific strain TBU83 to five isofemale strains of the Asian subspecies collected in Brunei, Borneo, in October 2003. Each cross was performed in duplicate on different dates. Fertility of the F1 hybrid males varied from 26.3% to 82.5% (Figure 4a), and the effect of parental Bornean strain was significant ($F = 18.74$, $p = 0.0033$). We also used the West Samoa strain, established in June 2003 from a multi-female collection, to generate several "pseudo-isofemale" strains by two generations of full-sib crosses. Seven of these derivative strains were crossed to the Asian strain 381.3, in two replicates each. Hybrid male fertility varied from 1.3% to 51.2% (Figure 4b), and the effect of parental Samoan strain was significant ($F = 35.40$, $p = 0.0001$). We conclude that at least some of the local populations of each subspecies harbor extensive variation for the degree of reproductive isolation from the other subspecies.

Genetic differentiation between subspecies

To investigate the degree of genetic differentiation between the Asian and Pacific subspecies, we reconstructed the mitochondrial phylogeny of 26 Asian and 10 Pacific strains. For the most part, the two subspecies carry distinct mitochondrial haplotypes (Figure 6a). Most of the Asian strains carry a group of closely related, geographically widespread haplotypes that are also shared with *D. malerkotliana* and *D. parabipectinata*, and are thought to have been spread across species boundaries by recent introgressive hybridization (Kopp and Barmina, 2005). On the other hand, most Pacific strains carry two highly derived haplotypes that are not found in any other species (Figure 6a). Intriguing exceptions to this pattern are found in the Australian and New Guinean strains. A strain from Northeastern Australia (CTrib) and one strain from New Guinea (Lae327) carry Asian mitochondrial DNA, yet behave as Pacific strains in reproductive isolation tests (Figures 2, 3, and Supplement table). Another New Guinean strain, Lae321, carries the Pacific haplotype, but behaves as an Asian strain (Figure 2 and Supplement table). The two New Guinean strains were collected at the same time in the same location (Table 1), suggesting that secondary hybridization between the Asian and Pacific subspecies is occurring in New Guinea, and possibly in Northeastern Australia.

In contrast to the mitochondrial DNA, nuclear gene trees show a complete lack of differentiation between the two subspecies (Figure 6b–d). Alleles from the Asian and Pacific strains are intermingled in all three genealogies. In the *al* and *ple* gene trees, *bipectinata* alleles are also interspersed with alleles from *D. parabipectinata* and *D. malerkotliana* (Figure 6b, d). This is consistent with the very recent divergence among these three species, which has been estimated at ~283 000–385 000 years (Kopp & Barmina, 2005). The levels of sequence variation in the Asian subspecies are more than two-fold higher than in the Pacific subspecies (Table 3), suggesting that the Asian subspecies has had a larger effective population size.

The difference between mitochondrial and nuclear loci is also evident from the Fst values (Table 4). Fst for the mitochondrial COI locus is highly significant (Fst = 0.611; p (0.001). On the other hand, nuclear Fst values are very low (0.040–0.068), and are not significant after strict Bonferroni correction ($p = 0.022$–0.166). Only one locus, *Gpdh*, shows significant differentiation without the correction for multiple comparisons

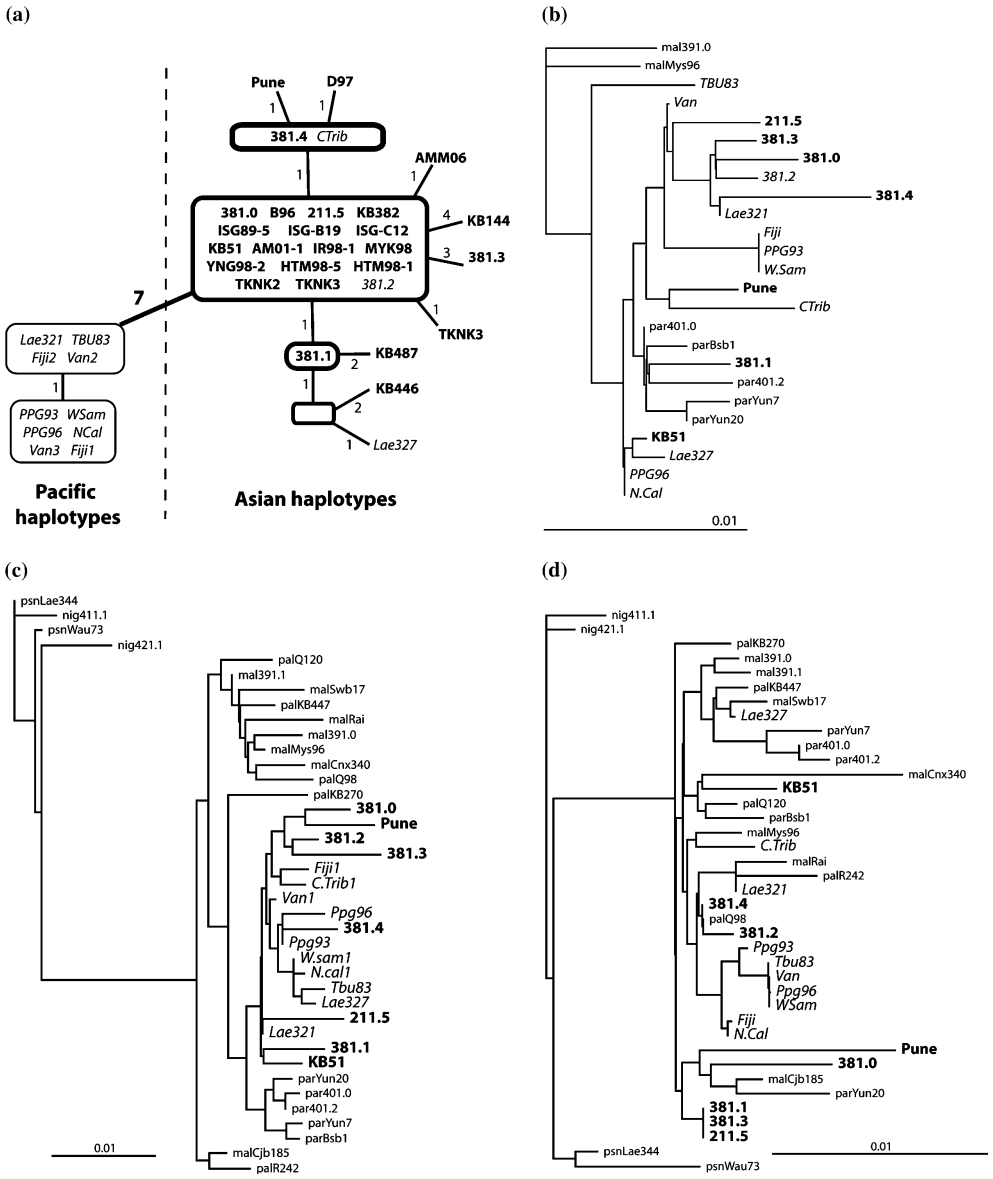

Figure 6. Phylogenetic relationships among Asian and Pacific strains. Asian strains are shown in boldface and Pacific strains in italic font. (a) Mitochondrial haplotype network based on the *COI* locus. Numbers next to each line represent the number of nucleotide substitutions separating the nearest haplotypes. Haplotypes shared between *D. bipectinata* and its sibling species *D. parabipectinata* and *D. malerkotliana* are outlined with thick rectangles; haplotypes exclusive to *D. bipectinata* are outlined with thin lines. Note the deep split between shared Asian and exclusive Pacific haplotypes. (b–d) Minimum evolution trees with HKY85 distances (Hasegawa et al., 1985) for the nuclear *al* (b), *Gpdh* (c), and *ple* (d) loci. Alleles from the Asian and Pacific subspecies are intermingled with each other, and sometimes also with alleles from the sibling species *D. parabipectinata* and *D. malerkotliana* (shown in smaller plain font). The trees were rooted using sequences from *D. malerkotliana* (b) or *D. pseudoananassae* (c, d) (Kopp and Barmina, 2004).

(Fst = 0.068, $p = 0.022$). The weighted average nuclear Fst distinguishing the two subspecies is 0.059, which is far lower than similarly computed measures of genetic differentiation in other recently diverged taxa in which reproductive isolation has been studied. For example, mean nuclear Fst values are 0.182 for *D. bipectinata* and *D. parabipectinata* (Kopp & Barmina, 2005), 0.573 for *D. simulans*

Table 4. Genetic differentiation between the Asian and Pacific subspecies

Locus	S[a]	Sf[b]	Ss[c]	Fst[d]	p[e]
al	24	0	3	0.040	0.166
Gpdh	41	0	4	0.068	0.022
ple	22	0	1	0.064	0.091
CO1	28	0	3	0.611	0.000

[a] Total number of segregating sites in both subspecies.
[b] Number of fixed differences between subspecies.
[c] Number of shared polymorphisms between subspecies.
[d] Calculated as described (Hudson et al., 1992b).
[e] Determined by permutation tests (Hudson et al., 1992a) with 1000 replicates.

and *D. mauritiana*, 0.659 for *D. simulans* and *D. sechellia*, 0.291 for *D. p. pseudoobscura* and *D. p. bogotana*, 0.635 for *D. mojavensis* and *D. arizonae*, and 0.357 for *Anopheles gambiae* and *A. arabiensis* (our calculations based on published multi-locus data sets). We conclude that the two subspecies of *D. bipectinata* have diverged very recently and/or continue to experience significant gene flow.

Discussion

The Asian and Pacific subspecies of *D. bipectinata* appear to be in the earliest stages of speciation. As far as we could determine, each Asian strain is fully inter-fertile with all other Asian strains, and each Pacific strain (with the exception of New Guinea–see below) is fully inter-fertile with all other Pacific strains. In this respect, both subspecies lack any internal differentiation. At the same time, each strain produces either completely or partially sterile male hybrids in crosses with some or all strains of the other subspecies. Our most important finding is that both subspecies harbor tremendous variation in the extent of reproductive isolation from the other subspecies. Depending on the identity of parental strains, the fertility of hybrid males varies from 0% to 90%. Hybrid sterility is non-transitive, so that the degree of reproductive isolation depends on the particular *combination* of strains. Moreover, significant variation in the extent of hybrid sterility is found within at least some local populations of each subspecies. These observations suggest that hybrid sterility is due to a fairly small number of loci, and that the genes responsible for hybrid sterility are not fixed within either subspecies. Thus, *D. bipectinata* may provide a valuable model for the study of genetic changes and developmental processes involved in the early stages of speciation.

Intraspecific variation in the degree of reproductive isolation between recently diverged taxa is not particularly uncommon. In *Drosophila*, the best known example are probably the "races" of *D. paulistorum*, which Th. Dobzhansky named "species *in statu nascendi*" (Dobzhansky & Spassky, 1959). Most pairwise combinations of these races produce sterile male hybrids, but several races produce fertile male offspring in crosses with at least some other races (Dobzhansky et al., 1964, 1969; Dobzhansky & Pavlovsky, 1967). Male sterility in this case appears to be caused by an interaction between the racial genomes and race-specific endosymbionts (Ehrman & Kernaghan, 1971; Perez-Salas & Ehrman, 1971). In *D. mojavensis*, there is extensive intraspecific variation for the degree of hybrid male sterility in crosses with its sibling species *D. arizonae* (Crow, 1942; Ruiz et al., 1990; Reed & Markow, 2004). In this case, sterility appears to be purely genetic, i.e. not associated with an endosymbiont. Patterson & Stone (1952) (Chapter 10) document similar variation in several species of the *Drosophila virilis* group. Variable male sterility has also been observed in hybrid crosses between *D. auraria* & *D. triauraria* (Kimura, 1987), between *D. m. macrospina* & *D. m. limpiensis* (Mainland, 1942), and between *D. e. equionxialis* & *D. e. caribbensis* (Ayala et al., 1974). Outside *Drosophila*, intraspecific variation in the extent of intrinsic post-zygotic isolation has been found in the flour beetle *Tribolium* (Wade et al., 1994) and in the hawksbeard weed *Crepis* (Asteracea) (Hollingshead, 1930). There are many more examples of intra-

specific variation in pre-zygotic isolation (Mather, 1964; Ayala, 1965; Dobzhansky & Pavlovsky, 1967; Miller et al., 1974). In fact, the nature of allopatric speciation and the typically polygenic basis of reproductive isolation imply that most incipient species must go through a stage where the genes responsible for reproductive isolation are not fixed within one or both species. On closer examination, variable reproductive isolation between young taxa may prove to be the rule rather than exception.

In *D. bipectinata*, the species as a whole retains the potential for gene flow throughout its entire geographic range. All hybrid females are fertile, and each strain we tested produces fertile male hybrids with at least one strain of the other subspecies. Although both subspecies carry chromosomal inversions, none of these inversions are fixed between subspecies (Kopp, unpublished). Thus, genes located anywhere in the genome can spread from any population to the entire species by a ''stepping-stone'' mechanism. *D. bipectinata* has a highly fragmented geographic distribution that includes thousands of large and small islands, and the real extent of gene flow within and between subspecies remains to be investigated. The nature of the New Guinean and Australian populations, and their relationships to the Asian and South Pacific populations, are particularly intriguing. On the one hand, it is possible that the Asian and Pacific subspecies arose by a parapatric mechanism with stepping-stone population structure (Barton & Hewitt, 1985; Gavrilets et al., 1998, 2000), and that New Guinea and Northeastern Australia are the zones of permanent range overlap. However, based on the mitochondrial phylogeny, it appears more likely that the two subspecies arose in allopatry, and that New Guinea and Northeastern Australia represent a relatively young hybrid zone. Since the hybrid females are fully fertile, mitochondrial DNA would introgress easily in both directions (Figure 6a), and the same might be true for large portions of the nuclear genome. In this case, the future of the Asian and Pacific subspecies will depend on the balance between gene flow, disruptive natural or sexual selection, and recombination (Barton and Hewitt, 1989; Gavrilets et al., 2000; Wu, 2001; Ortiz-Barrientos et al., 2002). In many different groups of animals, distinct species can apparently be maintained in the face of considerable gene flow (Shaw, 2002; Sota, 2002; Besansky et al., 2003; Grant et al., 2004). However, the impact of gene flow on the *initial* stages of speciation is less well understood, and *D. bipectinata* may provide a useful model for addressing this problem.

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