Evolution of mating isolation between populations of *Drosophila ananassae*

MALCOLM D. SCHUG,* JOHN F. BAINES,† AMANDA KILLON-ATWOOD,* SUJATA MOHANTY,†¶
APARUP DAS,†§ SONJA GRATH,† SHELLY G. SMITH,* SHIVA ZARGHAM,* SHANE F. MCEVEY‡
and WOLFGANG STEPHAN†

Biology Department, University of North Carolina Greensboro, Greensboro, NC 27402, USA, †Section of Evolutionary Biology, Department of Biology, Ludwig-Maximilians University, 82152 Planegg-Martinsried, Germany, ‡The Australian Museum, Sydney, NSW 2010, Australia, §National Institute of Malaria Research (ICMR), 22, Sham Nath Marg, New Delhi 110054, India, ¶Department of Biotechnology, Jaypee Institute of Information Technology University, Noida 201307, India

Abstract

Prezygotic mating isolation has been a major interest of evolutionary biologists during the past several decades because it is likely to represent one of the first stages in the transition from populations to species. Mate discrimination is one of the most commonly measured forms of prezygotic isolation and appears to be relatively common among closely related species. In some cases, it has been used as a measure to distinguish populations from subspecies, races, and sister species, yet the influences of various evolutionary mechanisms that may generate mate discrimination are largely unknown. In this study, we measured the level and pattern of mate discrimination among 18 populations of a cosmopolitan drosophilid species, *Drosophila ananassae*, from throughout its geographical range and its sister species, *Drosophila pallidosa*, which has a restricted geographical distribution in the South Pacific Islands. In addition, we measured genetic differentiation between all 18 populations using mitochondrial DNA polymorphism data. Mate discrimination varies considerably throughout the species range, being higher among populations outside the ancestral Indonesian range, and highest in the South Pacific. Our results suggest that colonization and genetic differentiation may have an influence on the evolutionary origin of mate discrimination. Our phylogeographical approach clarifies the ancestral relationships of several populations from the South Pacific that show particularly strong mate discrimination and suggests that they may be in the early stages of speciation. Furthermore, both the genetic and behavioral results cast doubt on the status of *D. pallidosa* as a good species.

Keywords: *Drosophila ananassae*, *Drosophila pallidosa*, mate discrimination, prezygotic isolation, reproductive isolation, speciation

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Introduction

Sexual isolation before fertilization may be one of the most important isolating mechanisms leading to speciation. Theory predicts that reproductive isolation is most likely to arise in allopatry as a by-product of one or more of three mechanisms: (i) adaptive divergence during exposure of isolated populations to different environments, (ii) gradual divergence and the accumulation of genetic incompatibilities

by drift while populations are separated, or (iii) rapid genetic divergence and accumulation of incompatibilities by genetic drift as a consequence of founding events (Otte & Endler 1989; Howard & Berlocher 1998; Tregenza *et al.* 2000; Coyne & Orr 2004). Perhaps the only systematic attempt to distinguish between these three mechanisms was performed by Tregenza *et al.* (2000) in which a likelihood-based analysis revealed founder events during colonization as the most likely explanation for assortative mating patterns observed between populations of the European meadow grasshopper. They were, however, not able to rule out the additional effects of long-term isolation and sympatry with closely

Correspondence: Malcolm D. Schug, Fax: 336 334 5839; E-mail: mdschug@uncg.edu

related species. Other studies have examined the relationship between genetic divergence and assortative mating in a number of species including salamanders (Tilley *et al.* 1990), *Drosophila willistoni* (Gleason & Ritchie 1998), and brown grasshoppers (Claridge *et al.* 1985, 1988) to test for assortative mating associated with gradual genetic divergence, but have found no relationship. At present, the degree to which population isolation and genetic differentiation among populations influence the evolution of mate discrimination remains largely unclear.

One approach to identifying the evolutionary mechanisms that generate mate discrimination is to assay a species distributed across a broad geographical range that includes continuous and isolated populations, and additionally displays various levels of divergence in mating behaviors. *Drosophila ananassae* is distributed throughout the subtropical and tropical regions of the world, has been studied extensively by geneticists (Tobari 1993), exists in highly structured populations in Asia and the South Pacific (Tomimura *et al.* 1993; Vogl *et al.* 2003; Das *et al.* 2004; Schug *et al.* 2007) and its biogeographical history is well characterized. Ancestral populations are from Indonesia and peripheral populations in Asia and the South Pacific have colonized these geographical regions since the last glaciation and human migration to Oceania (Das *et al.* 2004; Schug *et al.* 2007). The young age of the populations make it unlikely that natural selection has had a substantial affect on DNA sequence variation in most of the populations (Das *et al.* 2004), but evidence for adaptive evolution was inferred from patterns of DNA sequence variation in northern vs. southern populations in Asia (Stephan *et al.* 1998; Chen *et al.* 2000; Kim & Stephan 2000; Baines *et al.* 2004). The strong population structure, potential local adaptation and observations of mate discrimination among populations (Spieth 1952; Spieth 1966) suggest that prezygotic sexual isolation may be a salient feature of geographically local populations.

In this study, we assay levels and patterns of mate discrimination between 18 populations of *D. ananassae* spanning their geographical range and two populations of *Drosophila pallidosa* that occur in sympatry with *D. ananassae* in Fiji and Samoa. Previous studies provide strong evidence that the geographical origin of *D. ananassae* is in Southeast Asia, an area called the Sunda Shelf, and peripheral populations in Asia and the South Pacific represent migration since the time that sea levels rose approximately 20 000 years ago (Das *et al.* 2004). We focus our sampling on populations that represent the ancestral range in Indonesia (Vogl *et al.* 2003; Das *et al.* 2004), the peripheral range extending to Nepal, Japan, Northern Australia, and Brazil, and to island populations in the South Pacific. Our sampling strategy intended to identify patterns of mate discrimination that may be associated with varying levels of population isolation and demography and the presence of a sister species in sympatry. Although the populations included in our survey

are well-characterized by either DNA sequence polymorphism at introns (Das *et al.* 2004), microsatellites (Schug *et al.* 2007), or both, an analysis of mating behavior with respect to genetic differentiation using a common genetic data set would not be possible without the collection of additional data. Furthermore, several populations for which previously only microsatellite data were available display striking patterns of differentiation at these loci, suggesting they may be morphologically indistinguishable nascent species (Schug *et al.* 2007). Thus, to obtain a genetic data set common to all populations and confirm previous observations at other loci, we also obtained mitochondrial DNA (mtDNA) polymorphism data for all populations.

Materials and methods

Population samples

We performed multiple choice mating experiments on 18 populations of *Drosophila ananassae* from throughout their worldwide geographical range (Fig. 1). We chose populations that represented the ancestral range in Indonesia (A), the peripheral range in Asia and northern Australia (P), and island populations in the South Pacific (SP). For the purposes of our analysis, we include two populations from northeast Australia within the 'South Pacific' group (SP). This is justified by our phylogenetic analysis (see below). We also assayed populations of *Drosophila pallidosa*: one from Nadi, Fiji, and another from Malololelei, Samoa (Fig. 1). *Drosophila pallidosa* is recognizable in mixed samples because it is paler than *D. ananassae* and there is a reduced expression of metatarsal sex combs in males (Bock & Wheeler 1972; McEvey *et al.* 1987). Population samples are previously described (Das *et al.* 2004; Schug *et al.* 2007), except for the specimens from Nadi, Fiji, and Moorea, French Polynesia, which were collected in 2004 by M.D.S., A.K-A., S.G.S, and S.F.M.

Multiple choice experiments

We designed our experiments to reduce or eliminate potential effects of using isofemale lines that have been maintained in the laboratory for many generations. For populations from Indonesia, Australasia, and the South Pacific, virgin females and males from three different isolines were tested for mate discrimination for each pairwise combination of populations. These experiments were performed at University of North Carolina Greensboro (UNCG) in North Carolina. Three replicate experiments using three different isofemale lines per population were performed for each comparison, using 25 males and females from each line per replicate. Crosses between *D. ananassae* and *D. pallidosa* were performed with two replicates because of the number of isolines available. We maintained the flies

Fig. 1 Map of *Drosophila ananassae* ancestral (A, closed diamonds), peripheral (P) and South Pacific (SP, closed dots) and *Drosophila pallidosa* (open dots) populations. Ancestral populations are inferred from multilocus intron DNA sequence variation (Vogl *et al*. 2003; Das *et al*. 2004).

for at least 3 days to ensure that females were virgins and both sexes were fully mature. In our experience, males court vigorously and both sexes consistently mate successfully at 3 days of age in *D. ananassae* and *D. pallidosa* and are thus fully mature. We also reared the flies at a constant temperature and humidity, collected flies for each experiment on the same day, and at the same time in the life cycle to reduce any potential effects of environmental conditions on variance in mating behavior among replicates. Replicate experiments between laboratories showed similar results, confirming the low variance among replicates.

Red or green food colouring was added to the food 12–24 h before each experiment to identify the flies after mating. Colour-swaps were performed such that each population received the same type of food colouring twice. The combination of three lines (replicates) per population and reversal of food colouring between replicates eliminates potential line-by-food colouring effects. Males were separated into individual vials 12–24 h before the experiment began to increase courtship vigour. Ultimately, approximately 300 males and virgin females (25 flies per sex per isofemale line × three lines per population), were released into a

$25 \times 37 \times 25$-cm mating cage made of Plexiglass. This is the same mating cage used by Wu *et al*. (1995), kindly provided to us on loan from C. Aquadro. The mating experiments took place soon after the beginning of the light cycle. Flies were observed for up to 2 h but the experiment was terminated earlier if approximately 50% of the flies had mated. Copulating pairs were aspirated from the cage, frozen at –20 °C, and later scored for population origin based on the colour of their abdomens.

For populations from Indonesia, Asia, and Brazil, representative outcrossed populations were created by combining 10 isofemale lines collected from the same location and maintaining them for 10 generations. This method was used to eliminate individual line effects and reduce the workload associated with testing multiple independent lines. These experiments were performed at the University of Munich, Germany. Virgin females from the outcrossed populations were collected and tested for mate discrimination as described above, using food colouring to identify the population origin of individuals in copula.

Ten males and 10 virgin females from each population were introduced into the mating cage (40 flies total) for

each replicate, and five replicates were performed for each pair of populations. Thus, a total of 200 flies were assayed per comparison using mating chambers constructed according to Elens & Wattiaux (1964). Mating experiments took place in the morning and lasted for 1 h.

We replicated these experiments in the UNCG, North Carolina laboratory using the independent line method for a subset of these same population samples including Kathmandu vs. Mandalay, Chennai vs. Mandalay, and Kathmandu vs. Chennai. The results were similar to those obtained in the Munich laboratory using the mass-mating method. We thus believe the two different experimental designs had little or no effect on the results.

Statistical analysis of behavioral data

We used a discrimination index described by Rolan-Alvarez & Caballero (2000) to quantify the relative mating preferences between each cross and overall discrimination for each population pair. This index measures the combined effects of sexual isolation and sexual selection and is described in detail by (Coyne *et al*. 2005). The index is designed to disentangle the effects of mating propensity (sexual selection or asymmetric mating between species) from mate discrimination. The index is as follows: PSI (pair sexual isolation) × PSS (pair sexual selection) = PTI (pair total index). PSS measures the sexual selection differences between observed mating pairs and expected noncopulating pairs for each pair type. PSI measures sexual isolation by dividing the observed mating pairs for every pair type by the expected noncopulating pairs. Expected pair types are calculated assuming random mating. I_{PSI} measures a total isolation index based on discrimination and sexual isolation, and ranges from –1 (complete disassortative mating) to 0 (random mating), to 1 (complete assortative mating; Coyne *et al*. 2005). Although there will always be some effect of mating propensity inherent in the total isolation index, the PTI approach outperforms other isolation indices at eliminating such effects and is thus the best index available for quantifying sexual isolation (Rolan-Alvarez & Caballero 2000).

Because variance among replicates was low, we pooled the replicates and constructed contingency tables to calculate I_{PSI}, the significance of which we determined by 10 000 permutations of each contingency table using the software PTI version 1.0 (Rolan-Alvarez & Caballero 2000; Takada & Rolan-Alvarez 2000). N_{AB} represents a mating pair between population A and B, where the female is from population A and the male from population B, while N_{BA} indicates that a female is from population B and a male from population A. The calculation of I_{PSI} values requires at least one mating in each cell of the contingency table. In eight comparisons between *D. ananassae* and *D. pallidosa*, one cell in the contingency table had a zero value representing no mating

pairs. To calculate I_{PSI} for these comparisons, we added a single mating for each empty cell. The construction of contingency tables to analyze mate discrimination also provides a means to quantify one-way sexual isolation (IA_{PSI}; Rolan-Alvarez 2004) by examining patterns of asymmetry that may result from stronger mate discrimination in one population relative to another. We estimated IA_{PSI} and its statistical significance using JMATING (Carvajal-Rodriguez & Rolan-Alvarez 2006), an updated version of PTI version 1.0 which evaluates statistical significance of asymmetry using bootstrapping.

Molecular analysis

We determined 1315 bp of mtDNA sequence including 645 bp of the cytochrome *b* gene and 670 bp of the control region for 163 individuals from isofemale lines previously assayed for mate discrimination behavior. Polymerase chain reaction (PCR) products were sequenced on an ABI 3730, Amersham MegaBACE, or LI-COR IR2. Sequences were concatenated and aligned using CLUSTAL W in MEGA 4.0 (Tamura *et al*. 2007) and analyzed using DNASP version 4.10.9 (Rozas *et al*. 2003). The evolutionary history of haplotypes was inferred using the minimum evolution (ME) method in MEGA 4. The evolutionary distances were computed using the maximum composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the close-neighbour-interchange (CNI) algorithm at a search level of 3. The neighbour-joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of positions in the final data set. GenBank Accession nos are EU528677–EU528841, and EU601555–EU601719.

Statistical analysis of relationships between behavioural and molecular data

To evaluate the correlation between genetic distance, geographical distance, and I_{PSI} (mate discrimination), we performed permutation analyses. To evaluate the statistical significance of the correlations between genetic distance (F_{ST}) and geographical distance (kilometres), we performed Mantel tests (Mantel 1967) by permuting the matrices 1000 times and plotting the observed *r* value on the distribution of *r* values to arrive at a level of significance. For the relationship between genetic distance (F_{ST}) and mate discrimination (I_{PSI}), the matrix was not complete because we did not perform all of the possible pairwise comparisons of mate discrimination between populations. We re-sampled the I_{PSI} values without replacement 1000 times to obtain the probability of the observed *r* value, ignoring the missing data points in the I_{PSI} matrix, the equivalent of a Mantel test, but with an incomplete matrix.
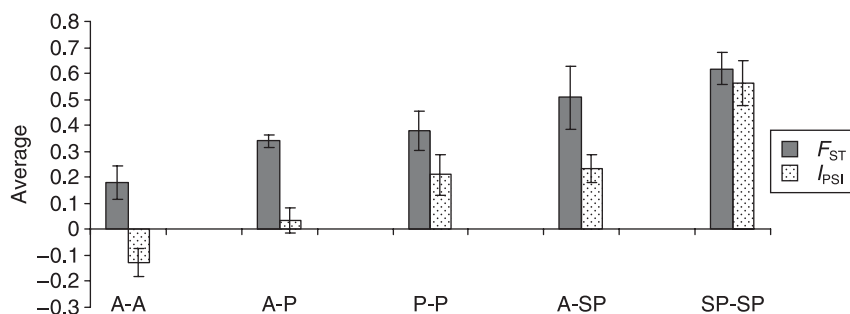
Fig. 2 Mean and standard error of sexual discrimination between ancestral vs. ancestral (A-A), ancestral vs. Asia-peripheral (A-P), Asia-peripheral vs. Asia-peripheral (P-P), ancestral vs. South Pacific (A-SP), and South Pacific vs. South Pacific (SP-SP) populations of *Drosophila ananassae*. I_{PSI} is a measure describing a total isolation index based on discrimination and sexual isolation, and ranges from –1 (complete disassortative mating) to 0 (random mating), to 1 (complete assortative mating). F_{ST} is calculated by pairwise comparisons of mtDNA sequences between populations within each category.

Results

Levels and patterns of mate discrimination

Mate discrimination varied considerably among populations throughout the geographical range. In general, populations in the ancestral and peripheral range showed low and insignificant levels of discrimination (Table 2). In the two cases where mate discrimination was statistically significant after Bonferonni correction, one was between peripheral populations (Kathmandu and Puri) and the other between ancestral and peripheral populations (Bangkok and Chennai; Table 2). In contrast, populations from the South Pacific showed high levels of differentiation (Table 3). Thursday Island, Apia, and Trinity Beach display the highest levels of mate discrimination against all populations tested, including the ancestral Bogor population. Interestingly, populations from Nadi and Moorea show much lower levels of mate discrimination with one another and with the ancestral population from Bogor. Both populations did, however, show statistically significant discrimination with Apia, Trinity Beach, and Thursday Island.

Of the 54 pairwise mate discrimination assays, six showed significant asymmetric discrimination. Two of the six cases were between ancestral and South Pacific populations (Table 3) and the remaining was between peripheral and/or South Pacific populations (Tables 2 and 3). Both cases of asymmetry between South Pacific and ancestral populations were in the opposite direction than predicted by the Kaneshiro hypothesis (Kaneshiro 1980, 1983; see Discussion). Thursday Island and Apia females were choosier than the ancestral Bogor females.

Distinctions between ancestral, Asia-peripheral, and South Pacific populations

A comparison of pairwise genetic differentiation and mate discrimination estimates for the ancestral, Asia-peripheral, and South Pacific populations shows a gradual increase in both estimates from the ancestral to South Pacific range (Fig. 2). While there is a significant difference in the average levels of mate discrimination among the groups (Kruskal–Wallace $H = 18.29$, d.f. = 3, $P = 0.0004$), the means within each category may be biased by multiple comparisons because of the pairwise nature of the design. The results clearly do show, however, that mate discrimination among comparisons in the South Pacific range is substantially higher than those within either the ancestral or Asia-peripheral ranges, and that there appears to be a trend towards higher levels of discrimination between Asia-peripheral populations than with ancestral populations.

Levels and patterns of mtDNA sequence polymorphism

Molecular variation (Table 1) varied considerably among the ancestral, peripheral, and South Pacific populations, consistent with previous studies of intron polymorphism (Vogl *et al*. 2003; Das *et al*. 2004; Schug *et al*. 2007) and microsatellites (Schug *et al*. 2007) that show substantial genetic structure throughout the geographical range we sampled. We found a total of 139 segregating sites among populations, 56 of which were singletons and 83 of which were shared among at least two individuals. There were a total of 111 haplotypes with an average haplotype diversity (H_d) of 0.99 and average nucleotide diversity (π) of 0.0086. Average F_{ST} between all *Drosophila ananassae* populations was 0.52 and ranged from 0 between Mandalay and Puri, and Mandalay and Chennai to 0.94 between Apia and Chiang Mai. The correlation between genetic distance and geographical distance between all *D. ananassae* populations was weak, negative, and not significant (Spearman's $r = –0.031$, $P = 0.56$). When we restricted our analysis of geographical distance and genetic differentiation to the 11 populations in the ancestral region, north, and northwest in Asia, after taking the existence of Sundaland into account

Table 1 Mitochondrial DNA variation within 18 populations of *Drosophila ananassae* and two populations of *Drosophila pallidosa*. Biogeographical location of each population is designated at ancestral (A), peripheral (P), or South Pacific (SP)

Population	N	S	h	H_d	K	π	θ	Tajima's D
Apia, W. Samoa (SP)	10	1	5	0.67	1.2	0.000	0.0014	–1.74
Bangkok, Thailand (A)	9	2	9	1	7.67	0.005	0.0068	–1.28
Bogor, Indonesia (A)	9	2	8	0.97	6.17	0.003	0.0045	–0.87
Chennai, India (P)	12	2	11	0.99	4.18	0.003	0.0038	–0.68
Chiang Mai, Indonesia (P)	3	1	3	1	3.33	0.000	0.0005	n/a
Darwin, Australia (P)	4	1	3	1	3.33	0.005	0.0042	1.68
Kathmandu, Nepal (P)	6	1	6	1	8.27	0.004	0.0041	0.013
Kota Kinabalu, Indonesia (A)	10	2	9	0.98	5.82	0.004	0.006	–1.55
Kuala Lumpur, Indonesia (A)	8	3	8	1	9.11	0.003	0.0036	–0.139
Kumejima, Japan (P)	8	2	6	0.89	4.75	0.001	0.0018	–1.64
Moorea, French Polynesia (SP)	11	3	10	0.98	6.15	0.004	0.0063	–1.63
Mandalay, Myanmar (P)	8	2	8	1	8.57	0.003	0.006	0.0005
Manila, Philippines (A)	5	3	5	1	17.2	0.008	0.0075	1.23
Nadi, Fiji (SP)	12	5	11	0.98	12.68	0.005	0.0081	–1.23
Puri, India (P)	14	5	14	1	11.23	0.004	0.0072	–1.49
Sao Paulo, Brazil (P)	5	1	5	1	6.2	0.003	0.0034	1.12
Trinity Beach, Australia (SP)	10	2	9	0.97	7.93	0.005	0.006	–0.47
Thursday Island, Australia (SP)	10	2	10	1	15.09	0.011	0.0134	–0.73
D. pallidosa (Nadi, Fiji)	4	3	4	1	15.33	0.009	0.0097	–0.17
D. pallidosa (Malololelei, W. Samoa)	8	2	6	1	8.53	0.004	0.0058	–1.15

N, number of individuals; S, segregating sites; h, number of haplotypes; H_d, haplotype diversity; K, average number of differences between individuals; π, average nucleotide diversity; θ, per site nucleotide diversity.

(Bogor, Bangkok, Chennai, Chiang Mai, Kathmandu, Kota Kinabalu, Kuala Lumpur, Kumejima, Mandalay, Manila, Puri), we found a statistically significant pattern of isolation by distance at mtDNA (Spearman's $r = 0.34$, $P = 0.005$), as for X-linked loci (Das *et al*. 2004).

The estimate of F_{ST} reported here for mtDNA is higher than that found for the X-linked loci (Das *et al*. 2004). However, this does not indicate that the mtDNA and the intron data sets are inconsistent. When only those 13 populations are analyzed for which both types of data are available, we find average pairwise estimates of $F_{ST} = 0.22$ and 0.32 for the X-linked and mtDNA data, respectively, while average nucleotide diversity θ is 0.0089 and 0.0046. We currently lack divergence data from an appropriate outgroup to estimate differences in the neutral mutation rate for these loci, but published estimates suggest that mtDNA sequences mutate on average twice as fast as nuclear ones in the subgenus *Sophophora* (Powell 1997). Then, taking into account that the X is hemizygous and that the inheritance of mitochondria is maternal, we find that the ratio of N_e (effective population size estimated from the X-linked data) to that estimated from mtDNA is approximately 4/3. This difference in N_e is qualitatively in agreement with our observation that F_{ST} at the mtDNA loci is larger than that at the X-linked introns. A possible cause for the difference may be a more pronounced effect of purifying selection at the mtDNA loci. This may also partly

explain the observed generally negative Tajima D values (Table 1), which are more strongly negative than at the introns (Das *et al*. 2004). Finally, for our analysis of the mate discrimination data (below), it is important to note that the correlation between the observed values of F_{ST} at the mtDNA and X loci is positive and significant ($r = 0.45$, $P = 0.01$).

In general, populations from the ancestral range in Indonesia and the peripheral region in Asia and Australia showed less genetic differentiation than populations from the South Pacific Islands (Table 2, Table 3, and Fig. 2). Among the populations for which we assayed mate discrimination, Apia and Chiang Mai showed the highest average pairwise F_{ST} values. A phylogenetic tree using mtDNA haplotypes clearly distinguishes Thursday Island, Trinity Beach and Apia from the remaining populations, placing them ancestral to all other populations which themselves were poorly resolved (Fig. 3). A similar pattern is seen in a neighbour-joining tree based on pairwise F_{ST} between populations (Fig. 4). Both populations of *Drosophila pallidosa* (Nadi and Malololelei) showed levels of F_{ST} within the range of the ancestral and peripheral populations and appeared within the poorly resolved area of the haplotype tree (Figs 3, 4).

As a reference to a well-known *Drosophila* species group, Fig. 5 shows a phylogeny of 808 bp of the cytochome *b* gene for which we were able to obtain high-quality sequences

Table 2 Multiple choice experiments between Indonesian and Asian populations of *Drosophila ananassae*. I_{PSI} is the total mate discrimination index. Genetic distance between populations was inferred between populations based the average F_{ST} estimated from mtDNA polymorphism between individuals in each population. Biogeographical location of each population is designated at ancestral (A), peripheral (P)

Population A	Population B	Comparison	N_{AA}	N_{BB}	N_{AB}	N_{BA}	I_{PSI}	s.d.	P	F_{ST}
Bangkok	Chennai	A-P	16	15	11	2	0.516	0.124	< 0.0001	0.393
Bangkok	Darwin	A-P	12	5	20	15	−0.38	0.133	0.004	0.424
Bangkok	Kumejima	A-P	17	2	10	2	0.113	0.312	0.676	0.379
Bangkok	Mandalay	A-P	11	15	22	7	0.018	0.153	0.928	0.405
Bangkok	Sao Paulo	A-P	15	21	17	9	0.173	0.133	0.18	0.175
Bogor	Chiang Mai	A-P	9	15	12	8	0.086	0.165	0.622	0.13
Bogor	Kathmandu	A-P	21	11	3	24	0.282	0.148	0.043 (NS)	0.302
Bogor	Kota Kinabalu	A-A	7	8	19	7	−0.206	0.173	0.242	0.027
Bogor	Kuala Lumpur	A-A	19	14	19	13	0.019	0.126	0.902	0.113
Bogor	Kumejima	A-P	9	9	4	3	0.453	0.192	0.036	0.479
Bogor	Mandalay	A-P	3	14	5	18	−0.195	0.205	0.31	0.52
Bogor	Sao Paulo	A-P	12	27	7	15	0.276	0.141	0.06	0.25
Chennai	Kuala Lumpur	P-A	7	18	27	8	−0.142	0.151	0.332	0.042
Chennai	Chiang Mai	P-P	14	21	14	11	0.163	0.13	0.226	0.639
Chennai	Manila	P-A	12	13	12	5	0.23	0.156	0.144	0.386
Chennai	Sao Paulo	P-P	15	9	5	12	0.202	0.165	0.214	0.379
Darwin	Kota Kinabalu	P-A	12	17	14	8	0.154	0.15	0.288	0.459
Darwin	Kuala Lumpur	P-A	3	24	16	8	−0.142	0.181	0.412	0.432
Darwin	Manila	P-A	14	18	17	7	0.187	0.136	0.176	0.417
Kathmandu	Kota Kinabalu	P-A	7	19	29	8	−0.149	0.156	0.324	0.213
Kathmandu	Kuala Lumpur	P-A	22	1	5	12	−0.227	0.224	0.312	0.168
Kathmandu	Manila	P-A	8	15	23	8	−0.103	0.16	0.484	0.238
Kathmandu	Puri	P-P	14	13	10	4	0.604	0.143	< 0.0001	0.109
Kathmandu	Sao Paulo	P-P	9	20	11	4	0.343*	0.159	0.05 (NS)	0.042
Kota Kinabalu	Kumejima	A-P	16	3	4	13	−0.026	0.226	0.87	0.418
Kota Kinabalu	Mandalay	A-P	19	17	6	26	0.183	0.136	0.172	0.466
Kota Kinabalu	Manila	A-A	10	11	14	17	−0.207	0.137	0.134	0.272
Kota Kinabalu	Sao Paulo	A-P	4	11	4	7	0.106	0.229	0.714	0.174
Kuala Lumpur	Kumejima	A-P	24	10	10	15	0.119	0.146	0.44	0.431
Kuala Lumpur	Mandalay	A-P	24	18	8	15	0.312	0.122	0.01 (NS)	0.049
Kuala Lumpur	Manila	A-A	19	14	19	23	−0.122	0.118	0.302	0.288
Kuala Lumpur	Puri	A-P	19	12	9	18	0.084	0.144	0.57	0.273
Kuala Lumpur	Sao Paulo	A-P	3	7	4	20	−0.335	0.224	0.142	0.017
Kumejima	Mandalay	P-P	15	15	23	13	−0.072	0.132	0.586	0.603
Kumejima	Manila	P-A	4	7	12	6	−0.244	0.204	0.266	0.238
Kumejima	Puri	P-P	16	4	3	4	0.409	0.24	0.104	0.495
Kumejima	Sao Paulo	P-P	11	22	14	13	0.072	0.14	0.598	0.356
Mandalay	Manila	P-A	26	14	17	13	0.121	0.122	0.332	0.395
Mandalay	Puri	P-P	16	2	4	5	0.09	0.305	0.76	0
Manila	Sao Paulo	A-P	8	12	4	20	−0.489	0.183	0.782	0.27

I_{PSI} is the discrimination index (Rolan-Alvarez & Caballero 2000) and the probability, P, is based on 10 000 permutations. *Asymmetry statistically significant; $P < 0.05$. NS, not statistically significant after sequential Bonferroni correction (Rice 1989).

from GenBank for *Drosophila melanogaster* and its sister species and the same 808 bp from *D. ananassae*, *D. pallidosa*, *D. atripex*, and *D. phaeopleura*. The tree confirms the ancestral position of Thursday Island and Trinity Beach and the relatively young age of the lineages relative to members of the *D. melanogaster* species group.

The correlation between pairwise genetic diversity (F_{ST}) and mate discrimination between all populations (Fig. 5) was positive and significant using both the nonparametric Spearman's rank order correlation ($r = 0.54$, $P < 0.0001$) and Pearson's r ($r = 0.47$, $P < 0.0001$). The correlation between genetic distance (F_{ST}) and mate discrimination (I_{PSI}) including

Table 3 Multiple choice experiments between Indonesia, Australia, and Oceania populations of *Drosophila ananassae*. I_{PSI} is the total mate discrimination index. Genetic distance between populations was inferred between populations based the average F_{ST} estimated from mtDNA polymorphism between individuals in each population. Biogeographical location of each population is designated at ancestral (A), or South Pacific (SP)

Population A	Population B	Comparison	Mating pairs				I_{PSI}	s.d.	P	F_{ST}
			N_{AA}	N_{BB}	N_{AB}	N_{BA}				
Apia	Bogor	SP-A	100	55	50	46	0.217*	0.064	< 0.0001	0.079
Apia	Moorea	SP-SP	33	26	7	19	0.433*	0.097	< 0.0001	0.816
Apia	Thursday Island	SP-SP	107	111	8	5	0.889	0.03	< 0.0001	0.405
Apia	Trinity Beach	SP-SP	60	74	24	25	0.465	0.066	< 0.0001	0.131
Bogor	Moorea	A-SP	20	27	12	21	0.19	0.115	0.114	0.786
Bogor	Nadi	A-SP	18	21	14	16	0.134	0.125	0.306	0.522
Bogor	Thursday Island	A-SP	75	69	29	27	0.442	0.064	< 0.0001	0.323
Bogor	Trinity Beach	A-SP	46	92	31	64	0.177*	0.069	0.011 (NS)	0.522
Moorea	Nadi	SP-SP	15	15	14	13	0.054	0.139	0.69	0.859
Moorea	Thursday Island	SP-SP	27	43	3	3	0.846*	0.061	< 0.0001	0.789
Moorea	Trinity Beach	SP-SP	27	25	9	12	0.433	0.107	< 0.0001	0.76
Nadi	Thursday Island	SP-SP	65	34	4	11	0.665	0.078	< 0.0001	0.8
Nadi	Trinity Beach	SP-SP	28	37	5	19	0.517	0.087	< 0.0001	0.168
Thursday Island	Trinity Beach	SP-SP	122	80	21	7	0.768*	0.041	< 0.0001	0.764

I_{PSI} is the discrimination index (Rolan-Alvarez & Caballero 2000) and P is based on 10 000 permutations. *Asymmetry statistically significant; $P < 0.05$. NS, Not statistically significant after sequential Bonferroni correction (Rice 1989).

Table 4 Multiple choice experiments between *Drosophila pallidosa* and *Drosophila ananassae*. I_{PSI} is the total mate discrimination index. Genetic distance between populations was inferred between populations based the average F_{ST} estimated from mtDNA polymorphism between individuals in each population

Population A (*D. pallidosa*)	Population B (*D. ananassae*)	Mating pairs				I_{PSI}	s.d.	P	F_{ST}
		N_{AA}	N_{BB}	N_{AB}	N_{BA}				
Malololelei	Apia	56	75	0	0	0.929	0.031	< 0.0001	0.623
Malololelei	Bogor	45	13	4	0	0.864	0.068	< 0.0001	0.049
Malololelei	Moorea	20	31	21	0	0.461*	0.997	< 0.0001	0.313
Malololelei	Nadi	32	15	16	17	0.146	0.113	0.19	0.385
Malololelei	Thursday Island	50	32	26	3	0.585	0.069	< 0.0001	0.323
Malololelei	Trinity Beach	38	62	19	4	0.666	0.064	< 0.0001	0.434
Nadi	Apia	35	24	0	0	0.937	0.045	< 0.0001	0.806
Nadi	Bogor	24	36	0	1	0.923	0.054	< 0.0001	0.524
Nadi	Moorea	67	51	2	0	0.967	0.023	< 0.0001	0.486
Nadi	Nadi	61	44	6	0	0.883*	0.041	< 0.0001	0.191
Nadi	Thursday Island	43	28	8	1	0.808	0.06	< 0.0001	0.232
Nadi	Trinity Beach	41	15	0	0	0.797*	0.072	< 0.0001	0.285
Nadi	Malololelei (*D. pallidosa*)	22	23	22	23	0.001	0.106	0.998	0.363

Drosophila pallidosa populations from Malololelei, Samoa and Nadi, Fiji. All other populations are *Drosopila ananassae*. I_{PSI} is the discrimination index (Rolan-Alvarez & Caballero 2000) and P is based on 10 000 permutations. *Assymetry statistically significant ($P < 0.05$). I_{PSI} calculated for contingency tables with zero cells by adding 1 to each cell.

only the ancestral and Asia-peripheral populations was also positive and significant (Spearman's $r = 0.22$, $P = 0.04$). Thus, 22% (0.47²) and 5% (0.22²) of the variance in mate discrimination appears to be a function of genetic distance across the species range and within the ancestral and Asia-peripheral range, respectively.

Mate discrimination between species

Drosophila pallidosa and *D. ananassae* showed the highest levels of discrimination, but isolation was complete in only three of the 12 pairwise comparisons (Table 4). Mating isolation was high within islands where the species occur
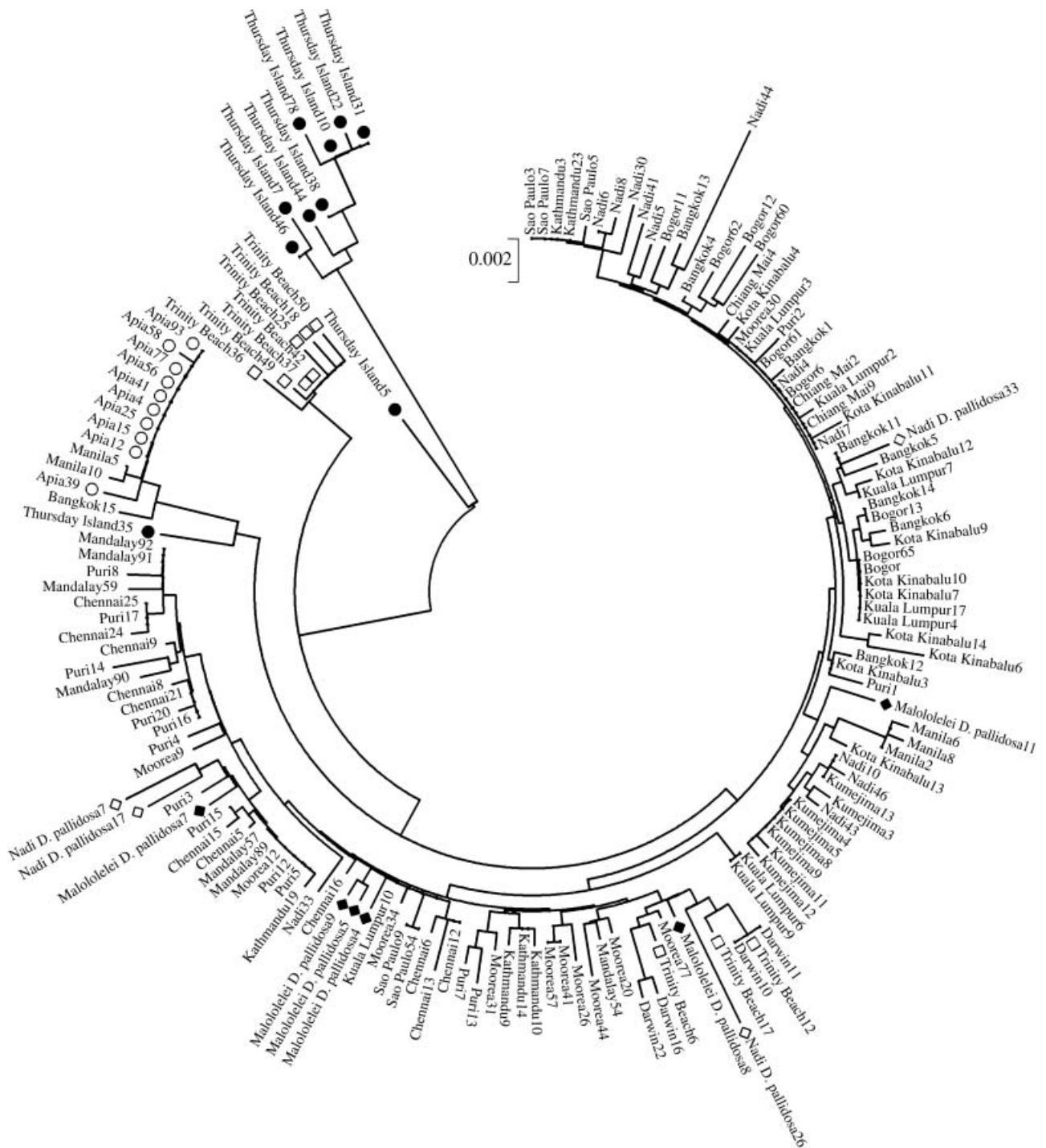
Fig. 3 Evolutionary relationships of 163 individuals based on mtDNA haplotypes. Individuals from Thursday Island are shown with closed circles (•), from Trinity Beach with open squares (□), and from Apia with open circles (○). *Drosophila pallidosa* individuals are shown with open diamonds (◇, Fiji) and closed diamonds (◆, Samoa). The optimal tree with the sum of branch length = 0.2084 is shown. The tree is rooted using Thursday Island which shows strongest similarity to the most closely related species, *Drosophila atripex* and *Drosophila phaeopleura* (see Fig. 6). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.
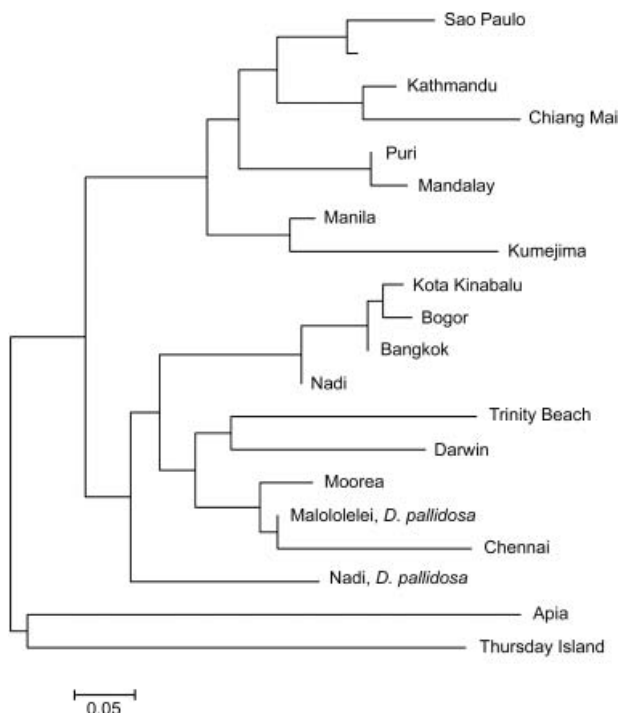
Fig. 4 Evolutionary relationships of 18 populations of *Drosophila ananassae* and two populations of *Drosophila pallidosa* based on pairwise F_{ST} (in the Materials and Methods). The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 2.810 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

in sympatry; complete in Samoa (Malololelei *D. pallidosa* vs. Apia *D. ananassae*) and nearly complete in Fiji (where Nadi *D. ananassae* females discriminated completely against *D. pallidosa* and *D. pallidosa* females mated with *D. ananassae* at a low rate). In contrast, *D. pallidosa* from Fiji and Samoa did not discriminate.

Discussion

We have conducted one of the largest geographical surveys of mate discrimination in any species, performing 219 assortative mating experiments for 67 pairwise population comparisons and recording 5105 copulations between 18 *Drosophila ananassae* and two *Drosophila pallidosa* populations from throughout their worldwide geographical range. Our results show that (i) in contrast to a similar study in *Drosophila melanogaster* (Henderson & Lambert 1982), substantial variation in mate discrimination exists, the strongest of which appears in island populations in the South Pacific; (ii) mate discrimination is significantly correlated with genetic differentiation between populations, but other evolutionary forces must also be involved, and at least two of the populations we assayed (Apia and Thursday Island) appear to be incipient species.
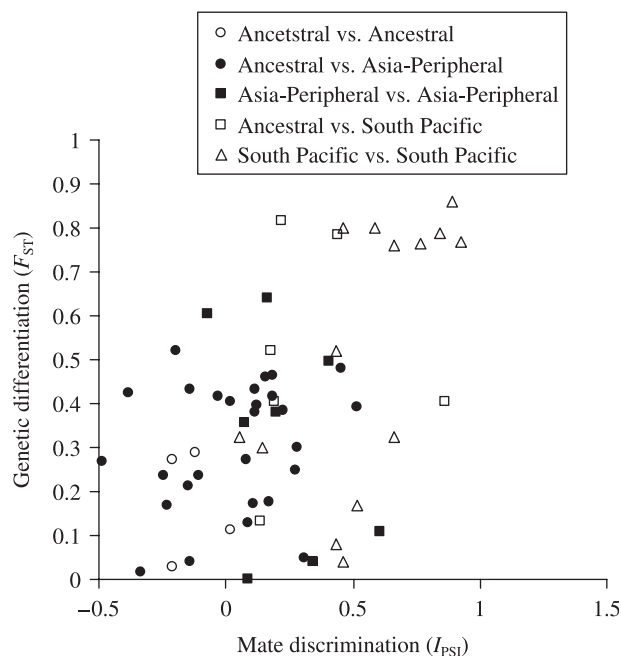
Fig. 5 Scatterplot of genetic differentiation (F_{ST}) based on mtDNA polymorphism and mate discrimination (I_{PSI}) among 18 populations of *Drosophila ananassae* (r = 0.54, $P < 0.0001$).

Geographical patterns of mate discrimination

Our results show that mate discrimination varies considerably across the geographical range of *D. ananassae* and is strongest between island populations in the South Pacific and in northeast Australia. Furthermore, mate discrimination occurs between some of the populations in the continuous geographical range in Asia (Puri vs. Kathmandu, and Chennai vs. Bangkok). These results contrast with the lack of mate discrimination between *D. melanogaster* populations in this same region (Henderson *et al.* 1982), and are similar in magnitude to discrimination between cosmopolitan and Zimbabwe *D. melanogaster* populations (Wu *et al.* 1995; Hollocher *et al.* 1997). Thus, the patterns of marked geographical population structure that are a distinguishing feature of *D. ananassae* populations (Das *et al.* 2004; Tobari 1993; Vogl *et al.* 2003; Schug *et al.* 2007) appear to be accompanied by structure in the patterns of mate discrimination as well. Despite the fact that *D. ananassae* is one of the most common species throughout the tropical and subtropical regions of the world (Tobari 1993), the patterns of mate discrimination are more characteristic of drosophilids that exist in restricted habitats, such as *Drosophila immigrans* in the USA and Australia (Ehrman & Parsons 1980), *Drosophila willistoni* in Central and South America (Gleason *et al.* 1998), and *Drosophila paulistorum* in Central America (Dobzhansky *et al.* 1964; Anderson & Ehrman 1969; Ehrman & Powell 1982) for a variety of other drosophilid species).
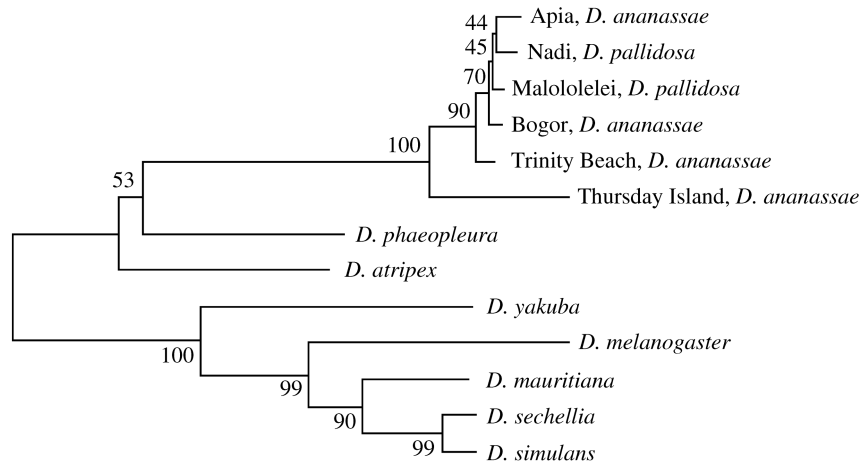
Fig. 6 Evolutionary relationships of six *Drosophila ananassae* and *Drosophila pallidosa* populations analyzed in this study for which phylogenetic signal was resolvable (see Fig. 3). Included are the most closely related species available (*Drosophila atripex* and *Drosophila phaeopleura*) and members of the *Drosophila melanogaster* subgroup for comparison of evolutionary distance. The evolutionary history was inferred using the neighbour-joining method (1). The optimal tree with the sum of branch length = 0.35022732 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1025 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. There were a total of 808 positions of the cytochrome *b* gene in the final data set.

Although the populations of *D. ananassae* in isolated habitats on islands show the highest levels of mate discrimination, we also find significant levels of mate discrimination within the continuous geographical range in Asia, in addition to some apparently isolated populations with relatively low levels of mate discrimination. These inconsistencies imply that there may be multiple reasons underlying mate discrimination. In one respect, our results are consistent with the study of the European meadow grasshopper Tregenza *et al*. (2000), which concluded that some aspect of the colonization process appears to be associated with the origin of prezygotic sexual isolation. However, in another respect, they are inconsistent because we find that mate discrimination has evolved in the continuous geographical region from the Sundaland islands, northwest where gene flow has had an important impact on genetic polymorphism. Indeed, observations of mate discrimination between populations with patterns of isolation by distance are uncommon (Tilley *et al*. 1990; Tregenza 2002). One possibility is that divergent mating behaviour may have evolved during a period of allopatry and has been maintained subsequently even in the presence of low levels of gene flow. There is growing evidence that mate discrimination may evolve rapidly as a by-product of diverse ecological conditions (e.g. Langerhans *et al*. 2007), suggesting that prolonged periods of allopatry may not be required for divergent mating behaviour to evolve.

The most striking patterns of mate discrimination we observe are between *D. ananassae* populations from Apia, Trinity Beach, and Thursday Island. These populations may have become established during the last 5000 years following human migration (Schug *et al*. 2007). Colonization is a process that is likely accompanied by population bottlenecks. However, evidence for recent population bottlenecks is not apparent in the patterns of mtDNA polymorphism or microsatellites (Schug *et al*. 2007) for these populations. Tajima's *D* values at mtDNA were generally negative for the mtDNA sequences, consistent with population expansion (besides purifying selection mentioned above). The Apia and Thursday Island populations show high levels of mate discrimination against each other and all other populations, and phylogenetic analysis suggests that they are independent lineages. Since the negative Tajima's *D* values indicate that it is unlikely that recent bottlenecks are the cause of these high levels of mate discrimination, these behaviours may have evolved previous to the colonization of the islands in a different geographical region, or these populations are much more ancient, existing on the islands before human migration like species of other drosophilid genera that are not peridomestic (e.g. *Mycodrosophila* spp., McEvey & Polak 2005; *Samoaia* spp., Malloch 1934). Interestingly, two haplotypes from the Manila population, which is in the ancestral species range, cluster with Apia haplotypes, suggesting that the Apia population may have diverged within the ancestral range before colonization. Alternatively, recent gene flow between Apia and Manila populations may be reflected in some individuals. Identifying the ancestral source of the populations by further sampling and molecular analysis of populations within the ancestral Indonesian geographical region may help resolve this question.

Trinity Beach also shows high levels of mate discrimination, but lower than Apia and Thursday Island. While this population is currently highly isolated from surrounding geographical regions due to a strict agricultural quarantine in place since the 1970s, we observe mtDNA haplotypes that indicate the population is a mixture of at least two independent ancestral lineages, which is supported by the microsatellite analysis of Schug *et al.* (2007). Many colonization/migration scenarios are thus possible, but the relatively high pairwise F_{ST} values we observe indicate that population isolation may play a role in generating or maintaining behaviours underlying mate discrimination in this population. This interpretation is supported by the lower levels of genetic differentiation we observe in Nadi, Fiji, and Moorea, French Polynesia, which both show only modest and insignificant mate discrimination against each other and the ancestral population. The mtDNA haplotypes of these two populations appear scattered throughout the tree and pairwise F_{ST} values are relatively low, indicating that they are either of very recent origin or experience gene flow with populations in the ancestral geographical range. Ongoing gene flow seems possible because of the international tourist and agricultural trade in Fiji and French Polynesia.

Genetic differentiation and mate discrimination

Our results suggest that genetic differentiation may play a role in the divergence of mate discrimination behaviours. In the entire data set, genetic differentiation explains 22% of the variance in mate discrimination, but is highly influenced by the strong mate discrimination between Apia, Thursday Island, and Trinity Beach, which may be nascent species (see below) and ancestral to all other populations (Fig. 3). Within the ancestral Indonesian and Asian geographical range, genetic differentiation explains only 5% of the variation in mate discrimination. This is perhaps not surprising because the range in genetic differentiation is relatively small in these regions, presumably representing current and historic gene flow among the populations which show a pattern of isolation by distance. However, this is the only study of which we are aware that has observed any relationship between genetic distance and mate discrimination (Claridge *et al.* 1985, 1988; Tilley *et al.* 1990; Gleason *et al.* 1998; Tregenza *et al.* 2000; Panhuis *et al.* 2001; Tregenza 2002). This may reflect the breadth of the geographical sampling we performed relative to other studies and suggests that broad geographical surveys may reveal insight into patterns of genetic differentiation that may underlie behavioural divergence.

We cannot, however, conclude that genetic differentiation is a prerequisite to the evolution of mate discrimination. In fact, our results show that some populations with high levels of mate discrimination do not always display high levels of genetic differentiation. For example, both the Trinity

Beach vs. Nadi ($I_{PSI} = 0.517$, $F_{ST} = 0.168$) and Puri vs. Kathmandu ($I_{PSI} = 0.604$, $F_{ST} = 0.109$) assays show statistically significant mate discrimination and levels of genetic differentiation that are low relative to most of the other comparisons between populations, including those that show no evidence of mate discrimination. Thus, on a restricted geographical scale, our results are consistent with studies of various other organisms that found that genetic differentiation and mate discrimination were not correlated (Claridge *et al.* 1985, 1988; Tilley *et al.* 1990; Gleason *et al.* 1998; Tregenza *et al.* 2000; Panhuis *et al.* 2001; Tregenza 2002). However, on a broader geographical scale, and in populations where strong mate discrimination is observed, there appears to be some relationship between the level of genetic differentiation and the evolution of mate discrimination.

Mate discrimination and adaptive evolution

There is a growing body of evidence that mate discrimination may evolve as a by-product of adaptation to environmental conditions while populations are in allopatry (e.g. Funk 1998; Vines & Schluter 2006; Langerhans *et al.* 2007). Tests for such an effect require measurement of environmental variables that influence fitness which may be multifarious and are often difficult to identify in natural populations. In the case of the Asian *D. ananassae* populations for which we observe significant mate discrimination (Chennai, Kathmandu, Puri and Bangkok), putative environmental variables have not yet been identified, but may be related to temperature and elevation in particular geographical regions. We know, for example, that *D. ananassae* is a stenotherm and is reactive to differences in temperature (e.g. Das *et al.* 1995; Joshi 1999; Sisodia & Singh 2002). In the South Pacific populations, the only potential environmental variable we can examine at this time is the presence of a sister species. The effect of a sympatric sister species on the evolution of mate discrimination was proposed by Zouros & d'Entremont (1980) based on their observation that *Drosophila mojavensis* in sympatry with *Drosophila arizonensis* showed higher levels of mate discrimination with other *D. mojavensis* populations than populations that were not in sympatry. This same phenomenon was also recently reported for populations of *Drosophila subquinaria* in sympatry with *Drosophila recens* (Jaenike *et al.* 2006).

We assayed two *D. ananassae* populations which show complete (Apia) or nearly complete (Nadi) discrimination with a sister species, *D. pallidosa*, in sympatry. In Apia, sympatry with a sister species is consistent with the hypothesis because it discriminates strongly against all other *D. ananassae* populations. However, in Nadi, sympatry with a sister species is not consistent with the hypothesis because it does not discriminate against another South Pacific-peripheral population, Moorea, or the ancestral population from Indonesia (Bogor). In fact, it is difficult to

distinguish the potential influence of a sister species from the effects of demography and biogeographical history that may underlie mate discrimination in Apia. As we discussed previously, mate discrimination in Apia may have evolved before or after colonization and it is not possible to determine the extent to which the demographic events that accompanied and followed colonization vs. the presence of a sister species affected mate discrimination. Thus, while our results are consistent with the Zouros & d'Entremont (1980) hypothesis in Apia, we cannot rule out other scenarios that may cause a similar pattern of mate discrimination. Furthermore, the lack of mate discrimination of Nadi with other populations may reflect gene flow which could prevent the effects of divergence in mate discrimination behaviours.

Another clue to the effects of adaptation on the evolution of mate discrimination may lie in patterns of asymmetric choice of females in ancestral and derived populations. Kaneshiro (1980, 1983) proposed that asymmetry should evolve in founder populations, presumably as previously co-adapted gene complexes break up during the founder event, followed by adaptation to new niches. In such a case, he argued that females in derived populations should become less choosy than those in the ancestral population as they seek new mates in previously uninhabited niches. Such a pattern of asymmetry was observed in Hawaiian drosophilids (Kaneshiro 1980, 1983) and in the ancestral African and derived (Cosmopolitan) strains of *D.* melanogaster (Hollocher *et al.* 1997). Although the mechanisms underlying the asymmetry have been debated (Iwasa & Pomiankowski 1995; Hollocher *et al.* 1997) it either appears to be irrelevant to the evolution of mate discrimination in *D. ananassae* populations or ancestral patterns of asymmetry that may have previously existed are not apparent in present-day populations. We found only six cases of asymmetry in mate discrimination and in two cases in the South Pacific where founder events are most likely to have occurred (Apia vs. Bogor, and Trinity Beach vs. Bogor). The asymmetry is in the opposite direction than Kaneshiro predicted and consistent with Watanabe & Kawanishi (1979) who observed similar patterns across a wider range of *Drosophila* in the *melanogaster* and *virilis* subgroups and proposed that a failure of females of a newly derived species to mate with ancestral males may drive asymmetry.

Influence of biogeographical history

Our sampling regime was designed to include populations from the ancestral, Asia-peripheral, and South Pacific ranges with the intent of examining the data for patterns of mate discrimination that may reflect both the biogeographical migration of the populations out of Indonesia and into surrounding habitats during the past 20 000 years (Das *et al.* 2004; Schug *et al.* 2007). We were particularly interested in these comparisons because of the evidence that natural selection may have a strong influence on broad expanses of the genome in populations from the regions in Northern vs. Southern Asia (Stephan *et al.* 1998; Chen *et al.* 2000; Kim & Stephan 2000; Baines *et al.* 2004) and because of the obvious genetic drift that may accompany population isolation on the South Pacific islands and potentially some of the peripheral populations in Southeast Asia that surround the ancestral geographical range in Indonesia. The trend towards higher levels of mate discrimination in peripheral populations (Fig. 2) is intriguing, particularly since it does not appear to correlate with levels of genetic differentiation or patterns of natural selection that were previously detected between the north and south populations. If this pattern is borne out in a more rigorous sampling and statistical analysis, it would suggest that some aspect of the biogeographical migration patterns has an important effect on the evolution of mate discrimination, even within a continuous geographical range.

Potential incipient speciation

Our results suggest that we may have identified three incipient species that show strong behavioural and molecular divergence from other *D. ananassae* populations and from each other. We are not the first to suggest this. Three other studies reached similar conclusions: Futch (1966) presented cytological evidence of a cryptic species in Papua New Guinea, Tobari *et al.* (1993) found a second cytological variant in Papua New Guinea, and Van Klinken *et al.* (2002) noted morphological variance in *D. ananassae* from Cairns and from Darwin. In our study, Thursday Island and Apia populations show strong mate discrimination with each other and all other populations including the ancestral Bogor population, and are also highly divergent at both mtDNA (this study) and microsatellites (Schug *et al.* 2007). Trinity Beach, a population that also shows high levels of genetic differentiation, shows strong mate discrimination from most other populations, and females from this population discriminate against the ancestral Bogor population (reflected by the statistically significant asymmetry). Both mtDNA differentiation (this study) and microsatellites (Schug *et al.* 2007) show higher levels of genetic differentiation between these populations. Furthermore, all three of the populations are more clearly distinguished in a distance and haplotype tree than *D. pallidosa* from both Fiji and Samoa.

Cleary the level of divergence between the *D. ananassae* samples is much younger than between any of the *D. melanogaster* species group. The phylogeny supports the hypothesis that Thursday Island and Trinity Beach populations are ancestral and diverged before the colonization of Asia and the South Pacific, perhaps in the Indonesian geographical area (Sundaland). The deeper ancestry of these populations may underlie the strong divergence in mating behaviours.

However among the many of the remaining populations, the extent of behavioural divergence is substantial and has likely occurred within the past 20 000 years.

The behavioural and phylogeographical results raise the possibility that Thursday Island, Trinity Beach, and Apia are true species, more divergent even that *D. pallidosa*. Body colour varies considerably in the South Pacific region (Tobari 1993). Cosmopolitan *D. ananassae* in the ancestral and peripheral ranges have a typically light body colour similar to *D. melanogaster*. Thursday Island and Apia flies have a very dark body colour, and may be the undescribed species reported by Tobari (1993) called *D. pallidosa*-like or *Drosophila papuensis*-like. The Nadi population of *D. ananassae* also displays a slightly darker body colour than the cosmopolitan populations, and thus may additionally be a member of Tobari's undescribed species. This scenario is supported by our data, suggesting that in fact these populations may be currently in the very early stages of speciation. Trinity Beach populations are similar in colour to the cosmopolitan populations, but may also be an incipient species.

Prezygotic mating isolation between *D. ananassae* and *D. pallidosa* has been well documented previously (reviewed in Tobari 1993) and appears to involve both differences in the male courtship song (Yamada *et al*. 2002), female discrimination (Doi *et al*. 2001), and potentially body size (Sisoda & Singh 2004). The female discrimination behaviour has been mapped to a region of the sex chromosome that carries an inversion (Doi *et al*. 2001; Sawamura *et al*. 2006, 2007), which is fixed in the Samoa population (Futch 1973). Thus, the recent speciation, yet close overall genetic relatedness of the two species may involve the fixation of a polymorphic inversion in the *D. pallidosa* lineage. The identification of the underlying genetic factors leading to rapid behavioural divergence between *D. ananassae* and *D. pallidosa*, and studies of chromosomal inversion frequencies throughout the ancestral, peripheral, and South Pacific range should provide insight into the evolutionary mechanisms involved.

We have performed crosses among these South Pacific populations, *D. pallidosa*, and the ancestral Bogor population in the laboratory and have noticed no evidence of postzygotic sexual isolation (hybrid viability or sterility). Crosses between *D. ananassae* and *D. pallidosa* stocks from Fiji show strong behavioural isolation but no obvious postzygotic sexual isolation (M. Schug, C. Pantazis, unpublished results; M. Matsuda personal communication). Furthermore, relative to the *D. melanogaster* subgroup, the divergence among populations is clearly very young, which is consistent with previous microsatellite (Schug *et al*. 2007) and intron (Das *et al*. 2004) studies. The results suggest that Thursday Island, Trinity Beach, and Apia are nascent species and that the exuberant expression of tarsal sex combs that distinguishes *D. pallidosa* from *D. ananassae* may not be a reliable indicator of a 'good' species. Furthermore, they suggest that behavioural divergence may evolve very rapidly.

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