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Differences in Morphological Traits between Two Sibling Species, *Drosophila ananassae* and *D. pallidosa*

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Chavali Vishalakshi and Bashisth Narayan Singh (2008) Differences in morphological traits between two sibling species, *Drosophila ananassae* and *D. pallidosa*. *Zoological Studies* **47**(3): 352-359. Capturing the mechanisms of speciation that appear in the early stages of reproductive isolation has been of recent interest to evolutionary biologists. In the present study, we investigated interspecific variations in several morphological traits, the degree of crossability, productivity, and the sex ratio in 2 sibling species, *Drosophila ananassae* and *D. pallidosa*, and their hybrids. The present species pair is unique due to the presence of strong sexual isolation and the absence of postmating barriers such as hybrid inviability or sterility. The 2 sibling species significantly differed in the following morphological traits: thorax length, sternopleural bristle number, wing length, wing-to-thorax ratio, sex comb tooth number, and ovariole number in males and females. Interspecific hybrids also significantly differed from their parental species in all morphological traits. Further, the degree of crossability and productivity was greater with conspecific matings than with heterospecific matings. Moreover, we found no sex ratio distortion in interspecific hybrids suggesting that there are fewer genetic incompatibilities between these 2 sibling species. These results are interpreted in terms of the evolutionary divergence between *D. ananassae* and its sibling species, *D. pallidosa*. http://zoolstud.sinica.edu.tw/Journals/47.3/352.pdf

Key words: Sibling species, *Drosophila ananassae*, *Drosophila pallidosa*, Morphological traits, Evolutionary divergence.

A recent resurgence of interest in speciation has revealed several important mysteries, particularly about reproductive isolation within *Drosophila* (Singh 1994, Sawamura and Tomaru 2002, Coyne and Orr 2004, Mishra and Singh 2005). However, several milestone achievements are still awaited, one of which is the elucidation of mechanisms involved in the early stages of speciation. Species maintain their identity through various reproductive isolating mechanisms (Dobzhansky 1937, Mayr 1942) and consequently undergo independent evolutionary fates (Orr and Presgraves 2000). Many speciation genetic studies have employed *Drosophila*, most of which focused on closely related species pairs with varying levels of divergence (Coyne and Orr 1989, 1997). But species pairs that are in the early or incipient stage of speciation have great potential for quantitative evolutionary analyses with particular reference to morphological and genetic divergence (Moraes et al. 2004, Kopp and Frank 2005) and also allow us to capture the process of speciation early enough to determine the initial causes of reproductive isolation (Reed and Markow 2004).

In the present study, we used a species pair, *D. ananassae* and *D. pallidosa*, which are unique due to the presence of strong sexual isolation and the absence of postmating barriers such as hybrid inviability or sterility in interspecific hybrids and their descendents (Futch 1973, Doi et al. 2001, Vishalakshi and Singh 2008). Both of these species belong to the *D. ananassae* complex of the *ananassae* species subgroup of the *melanogaster* species group (Bock and Wheeler 1972). *Drosophila ananassae* is a cosmopolitan species, whereas *D. pallidosa* is endemic to New

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Caledonia, Samoa, Tonga, and Fiji where these 2 species are sympatric (Futch 1966, Tobari 1993). Both species are genetically distinct in nature, and strong sexual isolation has been considered to be crucial in maintaining the integrity of the gene pool of the 2 species (Yamada et al. 2002a). Strong sexual isolation between *D*. *ananassae* and *D*. *pallidosa* was confirmed in the laboratory (Futch 1973, Doi et al. 2001, Vishalakshi and Singh 2006). However, sexual isolation was not affected by different experimental conditions (no, male, female, and multiple choice), but mating propensity was influenced by the sex ratio in these 2 sibling species (Vishalakshi and Singh 2006). These species are difficult to distinguish, as the only diagnostic traits in sympatric populations are the body color and sex comb tooth number (Bock and Wheeler 1972). Female sex pheromones (Nemoto et al. 1994, Doi et al. 1997) and male courtship songs (Yamada et al. 2002a b) also differ between these 2 species, which allows them to maintain genetically isolated in nature. Recently, Sawamura et al. (2007) reported that speciation genes (i.e., genes of premating and postmating isolation) are linked to inversions, which are species-specific in *D*. *ananassae* and *D*. *pallidosa*.

In this paper, we attempted to elucidate the demarcation among *D*. *ananassae*, *D*. *pallidosa* and their interspecific hybrids by examining various data on different morphological traits, the degree of crossability, productivity, and sex ratio in the parental species and their interspecific hybrids. The different morphological traits used were thorax length, wing length, wing-to-thorax ratio, sternopleural bristle number, sex comb tooth number, and ovariole number.

MATERIALS AND METHODS

Drosophila stocks

The mass culture stock (MYS) of *D*. *ananassae* was established in the laboratory from naturally impregnated females (n = 6) collected from Mysore, India in 2000. The stock of *D*. *pallidosa* (NAN 57) was kindly provided by Dr. M. Matsuda, Kyorin University, Tokyo, Japan, and is an isofemale line collected at Lautoka, Fiji. These stocks have been maintained in the laboratory on simple yeast culture medium at approximately 24°C.

Experimental design

Flies of *D*. *ananassae* and *D*. *pallidosa* were kept for two generations in an incubator, maintained at 25°C with continuous light. After 2 generations, 25 pairs of 7-d-old virgin females and males from both stocks were transferred to culture bottles. Flies were kept for 2 d to allow them to oviposit and were then discarded. Culture bottles were kept in the incubator at 25°C and were positioned at random and rotated daily in order to avoid any systematic macro-environmental effects. Virgin females and males from both stocks were separated under anesthesia within 2-4 h of eclosion and were kept in separate food vials of 3" (height) x 1" (diameter) for 7 d of aging. The 2 reciprocal crosses (throughout this paper, the maternal species is always indicated first) were made: i) *D*. *ananassae* ♀♀ x *D*. *pallidosa* ♂♂ (hereafter referred as AP) and ii) *D*. *pallidosa* ♀♀ x *D*. *ananassae* ♂♂ (hereafter referred as PA). For interspecific crosses, 25 virgin females of 1 species were crossed with 25 bachelor males of the other species. Since there is strong sexual isolation between these sibling species, the flies in both reciprocal crosses were kept for 2 d in food vials of 3" (height) x 1" (diameter) and then transferred to culture bottles and reared under the conditions described above.

Measurement of morphological traits

Different morphological traits (thorax length, wing length, wing-to-thorax ratio, sternopleural bristle number, sex comb tooth number, and ovariole number) were scored in 100 individuals (50 males and 50 females) of 5-d-old flies of *D*. *ananassae* and *D*. *pallidosa* (hereafter referred as pure species), and in F1 hybrids of both reciprocal crosses on both sides. Thorax length (TL) was measured from the anterior end of the thorax to the posterior end of the scutellum. For wing length (WL), the absolute length between the anterior crossvein to the distal tip of the 3rd longitudinal vein was measured under a microscope at 50x magnification using an ocular micrometer (1 unit = 16.67 μm). The wing-to-thorax (W/T) ratio was calculated from data of wing and thorax lengths. The sternopleural bristle number (SBN) in males and females was counted under stereo binocular. In females, the ovaries were dissected in insect saline (0.67% NaCl), stained with 2% acetocarmine, and mounted in 45% acetic acid,

and the ovariole number was counted under a microscope at 50x magnification. The sex comb in males of the *ananassae* subgroup is characterized by several transverse rows of stout blackish bristles on the ventral surface of the 1st, 2nd, and 3rd tarsal segments of the prothoracic legs (Bock and Wheeler 1972). Forelegs of males of both species and their hybrids were dissected and mounted in insect saline, and the total numbers of teeth (SCTN) on the 1st, 2nd, and 3rd tarsal segments were counted under a microscope.

Degree of crossability

The same stocks were used to observe the degree of crossability in pure species (means either A ♀ x A ♂ or P ♀ x P ♂ are involved) and interspecific crosses (means either A ♀ x P ♂ or P ♀ x A ♂). In each cross, 1 virgin female of 1 species was confined with the 3 bachelor males of the same (in pure crosses) or alien (in interspecific crosses) species in a food vial (size 3"x 1") and left for 10 d. After 10 d, the vials were observed, and those vials where the males and female were not alive were not counted. The vials with larval activity were counted as progeny obtained by that crossing. Altogether, 4 crosses were set up with 100 vials for each cross to accumulate sufficient data to analyze of degree of crossability in pure and interspecific crosses.

Number of progeny and the sex ratio

We measured the number of progeny produced and the sex ratio in parental species cultures and interspecific crosses to determine whether there were subtle distortions in the sex ratio indicating a deficit of males. Crosses were made in 20 vials at low density (5 pairs/vial), and offspring were scored until all had eclosed. Pearson correlation was performed to test the relation between the number of progeny and the sex ratio.

RESULTS

Details (mean ± S.E. and range) of 6 morphological traits of *D. ananassae* and *D. pallidosa* are given in table 1. Except for thorax length (TL), the mean values of sternopleural bristle number (SBN), wing length (WL), wing-to-thorax (W/T) ratio, sex comb tooth number (SCTN), and ovariole number (ON) were larger in *D. ananassae* than *D. pallidosa* of both sexes. There were significant differences in the various morphological traits between the 2 sibling species, except for thorax length in females and sternopleural bristle number in males (Table 1).

For each trait and species, phenotypic variability among individuals was indexed by the coefficient of variation (CV). Phenotypic variability was higher in males and females of *D. ananassae* than *D. pallidosa* (Fig. 1). The difference in the CV between the 2 sibling species was analyzed statistically by testing for the homogeneity of the CV. In males, there was a significant difference in WL (X^2 = 13.45, *d.f.*=1, $p < 0.001$), the W/T ratio (X^2 = 83.64, *d.f.*=1, $p < 0.001$), and SCTN (X^2 = 10.61, *d.f.* = 1, $p < 0.001$), but not TL (X^2 = 2.373, *d.f.* = 1,

Table 1. Details of different morphological traits in *Drosophila ananassae* and *D. pallidosa.* TL, Thorax length; SBN, Sternopleural bristle number; WL, Wing length W/T, Ratio of wing length and thorax length; SCTN, Sex comb tooth number; ON, Ovariole number

Sex	Trait	*D. ananassae* Mean ± S.E. (Range)	*D. pallidosaMean* ± S.E. (Range)	*t*-value
Males	TL	52.34 ± 0.36 (52 - 56)	55.07 ± 0.30 (51 - 60)	-7.28***
	SBN	13.92 ± 0.12 (12 - 16)	14.12 ± 0.11 (12 - 16)	-1.228
	WL	71.64 ± 0.46 (65 - 78)	67.22 ± 0.25 (72 - 81)	8.477***
	W/T	1.37 ± 0.11 (1.22 - 1.59)	1.20 ± 0.01 (1.08 -1.29)	12.768***
	SCTN	57.54 ± 1.08 (42 - 78)	52.42 ± 0.61 (43 - 64)	4.129***
Females	TL	59.86 ± 0.34 (53 - 63)	60.00 ± 0.29 (55 - 65)	-0.313
	SBN	15.22 ± 0.15 (13 - 17)	14.69 ± 0.12 (13 -17)	3.124**
	WL	82.5 ± 0.351 (76 - 86)	76.86 ± 0.29 (72 - 81)	12.404***
	W/T	1.38 ± 0.00 (1.29 - 1.51)	1.28 ± 0.01 (1.20 -1.41)	9.335***
	ON	23.2 ± 0.53 (16 - 31)	20.64 ± 0.29 (16 - 24)	4.230***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

$p > 0.05$) or SBN ($X^2 = 0.120$, *d.f.* = 1, $p > 0.05$). In contrast to males, in females the variability of the 2 species significantly differed for TL ($X^2 = 145.53$, *d.f.* = 1, $p < 0.001$) and ON ($X^2 = 354.94$, *d.f.* = 1, $p < 0.001$), but not for SBN ($X^2 = 1.328$, *d.f.* = 1, $p > 0.05$), WL ($X^2 = 3.113$, *d.f.* = 1, $p > 0.05$), or the W/T ratio ($X^2 = 0.538$, *d.f.* = 1, $p > 0.05$).

Further, we tested the correlation of 5 different morphological traits with body size (i.e., TL) in males and females of the 2 species. In *D. ananassae*, TL was positively correlated with WL (females, $r = 0.522$, $p = 0.001$; males, $r = 0.409$, $p = 0.003$), and ON ($r = 0.141$, $p = 0.328$) and negatively with the W/T ratio (females, $r = -0.704$, $p < 0.001$; males, $r = -0.604$, $p < 0.001$). Similarly in *D. pallidosa*, TL was positively correlated with WL (females, $r = 0.084$, $p = 0.561$; males, $r = 0.385$, $p = 0.006$), ON ($r = -0.189$, $p = 0.188$), and SCTN ($r = 0.009$, $p = 0.049$), and negatively correlated with the W/T ratio (females, $r = -0.770$, $p = 0.001$; males, $r = -0.755$, $p = 0.001$).

Table 2 presents the degree of crossability, productivity, and sex ratio in the parental species and their hybrids. The degree of crossability in pure-species crosses was greater than that of interspecific crosses. For example, when *D. ananassae* females were crossed with *D. ananassae* males, the crossability was 100%, but when they were crossed with *D. pallidosa* males, the crossability was 20%. Similarly, when *D. pallidosa* females were crossed with conspecific males, the crossability was 97% but decreased to 40% when crossed with heterospecific males. Also, the average crossability was greater in conspecific matings (98.5%) than in heterospecific matings (30%). In comparison to interspecific crosses, the productivity of both parental species was greater (Table 2). Interestingly, there was a sharp significant decrease in the number of offspring produced by females of a given

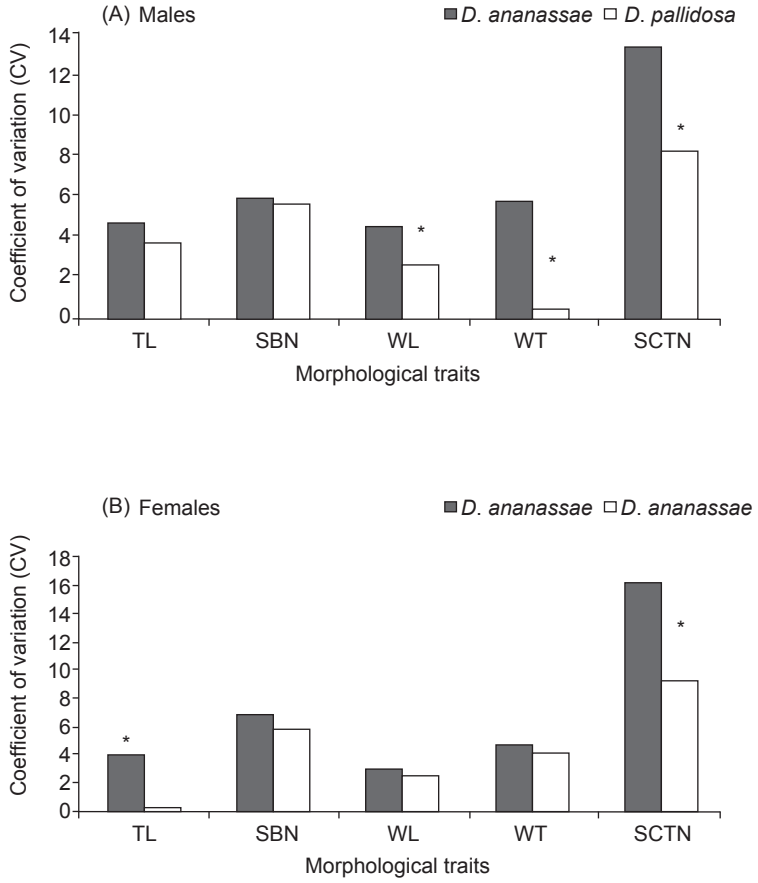

Fig. 1. Phenotypic coefficient of variation (CV) for morphological traits in (A) Males and (B) Females. TL, thorax length; SBN, sternopleural bristle number; WL, wing length; W/T, ratio of wing length and thorax length; SCTN, sex comb tooth number; ON, ovariole number. * $p < 0.001$

species when mated to conspecific compared to heterospecific males (X^2 = 76.32, p < 0.001). Of the 2 parental species, the productivity of *D*. *ananassae* was greater than that of *D*. *pallidosa*, which can be explained by the higher ovariole number (Table 1). This was also supported by data of the interspecific crosses, where the number of progeny produced was more when greater when *D*. *ananassae* females were involved in crosses compared to *D*. *pallidosa* females (Table 2). Both pure-species cultures and interspecific crosses produced males and females in a ratio which did not differ from 1:1, and in no set of crosses was there a significant correlation between offspring number and sex ratio among vials (r = -0.824, p = 0.176).

Various morphological traits of interspecific hybrids of both reciprocal crosses significantly varied from the parental species in males (except SBN) and females (Table 3). Moreover, we found increased mean values of morphological traits, when the hybrids were compared with mid-parent values (the average of means of the parent species involved in a cross) in both sexes (data not shown). To test this difference statistically, we made individual comparisons of hybrids and mid-parent values using the *t*-test (analyses not shown). In males, there were significant differences (p < 0.001) for WL, the W/T ratio, and SCTN but not for thorax length or sternopleural bristle number. Similarly in females there were significant differences in all traits. When the interspecific hybrids of both reciprocal crosses were compared by *t*-test, we found that there were differences (p < 0.001) in males for SBN, WL, the W/T ratio, and SCTN and only for WL and ON in females.

DISCUSSION

In the present study, we investigated interspecific variations between 2 sympatric sibling species, *D*. *ananassae* and *D*. *pallidosa*, with a recent origin of divergence (Bock and Wheeler 1972). It is evident from table 1 that the 2 species significantly differ in all morphological traits (thorax length, wing length, the W/T ratio, sternopleural bristle number, sex comb tooth number, and ovariole number) in males and females. However, except for thorax length, the mean values of the other traits were higher in *D*. *ananassae* (Table 1). The phenotypic variability, expressed in terms of the coefficient of variation, was higher in *D*. *ananassae* than in *D*. *pallidosa*, suggesting the cosmopolitan nature of its distribution.

The positive correlation between thorax and wing lengths in both species suggests that both traits are genetically correlated (David et al. 1994, Barker and Krebs 1995, Morin et al. 1997). Moreover, when species of very different sizes are compared, a strong correlation due to an allometric constraint is observed (i.e., moving a heavier body requires larger wings but also bigger flight muscles included in a larger thorax; Reiss 1989, Morin et al. 1997). Our comparative data, however demonstrate that these internal developmental constraints are not very strong when these 2 closely related were compared. Similar to our results, a negative correlation between thorax and wing lengths was found when 2 distantly related species, *D*. *melanogaster* and *D*. *ananassae*, were compared (Morin et al. 1997).

Further, the cost of transport or migratory activity for an adult is influenced by the ratio of wing length/thorax length and is likely to be subjected to genetic and evolutionary changes

Table 2. The degree of crossability, productivity, and sex ratio in the parental species and interspecific hybrids between *Drosophila ananassae* and *D*. *pallidosa*

	A♀ x A♂	P♀ x P♂	A♀ x P♂	P♀ x A♂
A) Crossability				
Number of females tested	100	100	100	100
Percentage crossability	100	97	20	40
B) Productivity				
Total number of progeny	2174	1018	1421	950
Male offsprings	1011	510	726	520
Ratio of males	0.465	0.500	0.511	0.547

that produce both intra- and intergenic variations (Barker and Krebs 1995, Morin et al. 1997). The higher value of the W/T ratio in *D. ananassae* (Table 1) suggests that its flight capacity is better than that of its sibling species, *D. pallidosa*. However, differences in the migratory activity of the 2 species under competitive constraints have previously been reported (Narise 1966). The 2 species significantly differ in sex comb tooth number with a higher number in *D. ananassae* supporting the previous findings of Bock and Wheeler (1972). Considering variations in the pattern of sex combs found in different species of the subgenus *Sophophora*, Stern (1954) speculated that the evolutionary process, which diversified the sex comb phenotypes in different species, began in response to a mutation in preexisting developmental prepatterns. Further, it has been documented that the number of teeth and their positioning are perhaps under sexual selection, which can cause rapid changes in sex comb morphology which are correlated with changes in mating behaviour (Carson and Lander 1984, Polak et al. 2004). Obeying this selection process, the sex combs strongly differed in the number of rows, and in the position and orientation among races and species. Similar to sex comb tooth number, there was a significant difference in the ovariole number in females with a higher number in *D. ananassae* (Table 1).

The phylogenetic proximity among species can also be reflected by the degree of crossability.

Table 3. Results of one-way ANOVA to test the difference between parental species and interspecific hybrids of *Drosophila ananassae* and *D. pallidosa*

Sex -Traits	Source of variation	SS	*d.f.*	MS	F
Male-TL					
	Between genotypes	291.70	3	97.23	19.29*
	Within genotype	987.72	196	5.04	
	Total	1279.42	199		
Female-TL	Between genotypes	122.26	3	40.75	8.286*
	Within genotype	963.96	196	4.92	
	Total	1086.22	199		
Male-SBN	Between genotypes	6.78	3	2.260	2.160^{ns}
	Within genotype	205.04	196	1.046	
	Total	211.82	199		
Female-SBN	Between genotypes	24.46	3	8.152	7.20*
	Within genotype	221.90	196	1.132	
	Total	246.36	199		
Male-WL	Between genotypes	536.29	3	178.77	31.97*
	Within genotype	1095.93	196	5.59	
	Total	1632.22	199		
Female-WL	Between genotypes	1454.58	3	484.86	51.44*
	Within genotype	1847.42	196	9.426	
	Total	3301.99	199		
Male-W/T	Between genotypes	0.710	3	0.237	59.25*
	Within genotype	0.714	196	0.004	
	Total	1.421	199		
Female-W/T	Between genotypes	0.245	3	0.0816	26.03*
	Within genotype	0.614	196	0.0031	
	Total	0.859	199		
Male-SCTN	Between genotypes	4341.36	3	1447.12	42.30*
	Within genotype	6705.36	196	34.21	
	Total	11046.72	199		
Female-ON	Between genotypes	347.32	3	115.77	12.87*
	Within genotype	1763.00	196	8.99	
	Total	2110.32	199		

* $p < 0.001$; ns,non-significant

In more closely related species, hybridization is frequent, while in distantly related species, it is difficult to obtain hybrids (Mishra and Singh 2006a). In the present case, the crossability was higher in conspecific matings (98.5%) than interspecific matings (30%) supporting the previous findings that there is preferential mating between males and females of the same species (Spieth 1966, Futch 1973, Doi et al. 2001, Yamada et al. 2002a, Vishalakshi and Singh 2006). The levels of crossability of interspecific crosses were 20% (*D. ananassae* females x *D. pallidosa* males) and 40% (*D. pallidosa* females x *D. ananassae* males). This suggests that *D. ananassae* females have a relatively higher discriminative ability than *D. pallidosa* females and therefore are less likely to mate with alien males (Spieth 1966, Futch 1973), which is in agreement with the results of our previous study of these 2 sibling species, where interspecific matings were 0.0% (*D. ananassae* female x *D. pallidosa* male) with both female and male choices, but they were 13.3% and 4% (*D. pallidosa* female x *D. ananassae* male) with respective female and male choices (Vishalakshi and Singh 2006). However, our results greatly differ from those of previous studies where the degree of crossability was tested in these 2 species (e.g., Doi et al. 2001, Yamada et al. 2002a), and the difference in results may have been due to factors involving different strains. It is known that populations of *D. ananassae* display a high population substructure across the entire distribution range throughout tropical, subtropical, and mildly temperate regions of the world (Das 2004, Schug et al. 2007, Singh and Singh 2007).

Numbers of offspring produced were greater in pure-species crosses than in interspecific crosses (Table 3). The productivity of *D. ananassae* females was higher than that of *D. pallidosa* females, which was correlated with the higher ovariole number in the former species (Table 1). The sex ratio of males and females was 1:1 in interspecific hybrids (Table 3). The higher the degree of genetic divergence between the hybridizing entities, the greater the chance there is for hybrids to be developmentally unstable (Garnier et al. 2006), which is reflected in a sex ratio distortion (Tao et al. 2001). Viewed from this perspective, our species pair must have recently diverged (Bock and Wheeler 1972), and on the basis of ribosomal intergenic spacer (IGS) length variation, these 2 sibling species vary in only 200 base pairs (Mateos and Markow 2005). Therefore, this might be the reason that there was no sex ratio distortion in interspecific hybrids of *D. ananassae* and *D. pallidosa*.

Mean values of morphological traits significantly differed among interspecific hybrids and parental species in males (except for SBN) and females. When compared with mid-parent values, the mean values of the morphological traits were higher in interspecific hybrids in both sexes (data not shown). In contrast to this, numbers of sex comb teeth in hybrids of 4 species of the *bipectinata* complex were intermediate of their parental values, which was due to a polygenic mode of inheritance for the sex comb (Mishra and Singh 2006b). However, hybrids show intermediate phenotypes for some mating traits suggesting a polygenic mode of inheritance of mating characters (see Futch 1973). The coevolution of a trait's expression and female preference will lead to premating isolation, which results in genetic isolation of species (Badyeav and Snell-Rood 2006). The behavioral divergence is then driven by selection to avoid unfit hybrid matings and is expected to be stronger in areas of sympatry (Gray 2004).

In conclusion, our results provide evidence for morphological divergence between *D. ananassae* and *D. pallidosa*, which along with strong premating isolation (as reported by Doi et al. 2001, Vishalakshi and Singh 2006), might be playing an important role in preventing gene flow between these 2 sibling species.

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