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Studies on Mating Preference and Productivity in *Drosophila ananassae* and *D. pallidosa* and Their Hybrids

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Abstract

During speciation different kinds of reproductive barriers originate to preclude gene flow between diverging populations. Reproductive isolation or barriers to gene flow can be categorized by the temporal nature of their effect: pre-zygotic barriers occur before fertilization and post-zygotic barriers occur after fertilization. In this study, we studied each components of reproductive isolation between *D. ananassae* and *D. pallidosa*, including both pre-zygotic and post-zygotic barriers. Because it might be possible that by dissecting these barriers one can get the answers of many unresolved questions related to the process of speciation of these two sibling species. We reported premating isolation because females of both *D. ananassae* and *D. pallidosa* were more discriminative for mating against the alien males rather than conspecific males, and this discrimination was much stronger in case of *D. ananassae* females for being the ancestral and cosmopolitan species. We also did not find any decrease in the production of progeny or viable offspring in comparison to conspecific males, indicating a lack of postmating prezygotic isolating barriers. Further, there is no complete lack of intrinsic post-zygotic isolation between these species or not complete presence of post-zygotic isolation, as both the hybrid sons were producing less number of progeny in comparison to all the crosses but it is near to significant but not significant and this is contrasting to the results of a previous study.

Keywords: *D. ananassae*, *D. pallidosa*, Mating Preference, Post-Zygotic Reproductive Isolation, Productivity

1. Introduction

After the debates of many decades in evolutionary biology, mechanistic understanding of speciation is yet a formidable challenge for the evolutionary biologists and speciation has been largely associated with several important mysteries, particularly to the reproductive isolation within *Drosophila*. Thus, the studies related to reproductive isolation are essential for modelling the process of speciation. During speciation different kinds of reproductive barriers originate to impede gene flow between diverging populations. Reproductive isolation or barriers to gene flow can be categorized by the temporal nature of their effect: pre-zygotic barriers occur before fertilization and post-zygotic barriers occur after fertilization (Coyne & Orr, 2004). Behavioural isolation is one of the most important pre-zygotic barriers to reproduction in sexually reproducing animal species, which reduce the attraction between heterospecific individuals by several ways and prevent matings between them. When pre-zygotic barriers will fail, postmating prezygotic barriers will act to prevent the generation of hybrid offspring by many ways (Palumbi, 1999; Price, 1997; Rice, 1996). If postmating prezygotic isolating barriers fail, then post-zygotic barriers come in picture. Post-zygotic barriers arise due to the genetic incompatibilities within the hybrid genome that result in a loss of viability or fertility of the hybrid offspring (Brideau et al., 2006; Cutter, 2012; Orr & Turelli, 2001). Thus, to understand speciation between two species it is must that one can quantify both pre-zygotic and post-zygotic barriers. It might be possible that one can get the answers of many unresolved questions related to the process of speciation by dissecting these barriers (Coyne & Orr, 1989, 1997).

By keeping this fact in view, we used a pair of sibling species of *Drosophila*: *D. ananassae* and *D. pallidosa*, in the present study. Because they are very unique species pair due to the presence of strong sexual isolation and the absence of postmating barriers such as hybrid inviability / sterility in the interspecific hybrids (Futch, 1966, 1973; Sawamura et al., 2008; Singh, 2016; Vishalakshi & Singh, 2006; Vishalakshi & Singh, 2009). Both these species are member of *D. ananassae* complex of the *ananassae* species subgroup of the *melanogaster* species group (Bock

& Wheeler, 1972). *D. ananassae* is cosmopolitan in nature whereas *D. pallidosa* is endemic to New Caledonia, Samoa, Tonga and Fiji Islands where these two species are sympatric (Futch, 1973; Tobari, 1993). Both these species are genetically distinct in nature and strong sexual isolation has been crucial in maintaining the integrity of gene pools of the two species (Yamada, Matsuda, & Oguma, 2002a). These species are difficult to distinguish, as only diagnostic traits in sympatric populations are the body colour and sex-comb tooth number (Bock & Wheeler, 1972). Female sex pheromones (Nemoto et al., 1994) and male courtship songs (Yamada et al., 2002b) also differ between these two species, which allows them to remain genetically isolated in nature.

In this study, we studied each components of reproductive isolation between *D. ananassae* and *D. pallidosa*, including both pre-zygotic and post-zygotic barriers to understand the mystery of these sibling species pair. Previous work suggested that hybrids produced by either reciprocal crosses are fully fertile and viable (Oguma, 1993; Sawamura et al., 2008). So, we believe that study of each component of reproductive isolation in these two sibling species would help in understanding the intricacies of mechanism of speciation of these two sibling species. Further, this work will provide the complete story behind the speciation of these sibling species in the nutshell. No-choice mating trials were first used to quantify the pre-zygotic barriers to hybridization. Further, postmating pre-zygotic barriers were determined by measuring number of matings in heterospecific crosses and progeny of females mated with heterospecific males in comparison to conspecific males. Finally, we tested post-zygotic isolation by quantifying mating preference and productivity of hybrids in comparison to pure species. It is known that maladaptive traits in hybrids prevent them from backcrossing to either parent population (Barton & Hewitt, 1985; Coyne & Orr, 2004). There is only one preliminary report where hybrids of these two sibling species were backcrossed to parental species to check whether there was a significant difference in the number of matings with each parental species (Futch, 1973). However, the conclusions drawn from this study were not very clear because no significant difference were found in the productivity of hybrids in comparison to pure species.

2. Materials and Methods

2.1 Drosophila Stocks

One mass culture stock of *D. ananassae*, established from flies collected from Pondicherry (PC) India was employed in the present study. One wild type strain of *D. pallidosa* was used. This strain is NOU 88 which was kindly provided by Prof. M. Matsuda of Kyorin University, Japan. These stocks are being maintained in the laboratory on the simple yeast agar culture medium at approximately 24ºC following 12 hours' light and dark cycle.

2.2 Experimental Design to Assay Mating Preference

All the experiments were categorized into two groups that was group I and group II. Group I comprises homogamic and heterogamic crosses of parental species. Group II comprises all the crosses of reciprocal hybrid daughters and sons with parental species and hybrid itself.

2.2.1 For Group I

20 pairs (females and males in equal number) of 8 days old females and males from each strain were transferred to the bottles. From these bottles virgin females and males were collected and aged in food vials for 8 days which were further used to set the culture bottles for the experiments. Flies were kept for 3 days to allow them to oviposit and were then discarded. Virgin females and males were collected and aged for 8 days in food vials. One day prior to experiments 15 females and 15 males of both the species were separated and kept in fresh food vials. After the completion of 8 days, 15 females and 15 males (same females and males for homogamic crosses whereas different females and males in reciprocal form for heterogamic crosses) were introduced into Elens-Wattiaux mating chamber. Matings were directly observed for sixty minutes, during the morning hours (7 A.M. to 11 A.M.). Once a pair commenced mating, it was aspirated out and kept in separate empty vials and data were recorded in the form of number of matings for that hour for each replicate. Five replicates were carried out for all the crosses.

2.2.2 For Group II

20 pairs (females and males in equal number) of 8 days old females and males from each strain were transferred to the bottles. From these bottles virgin females and males were collected and aged in food vials for 8 days which were further used to set the culture bottles for the experiments. Flies were kept for 3 days to allow them to oviposit and were then discarded. Virgin females and males were collected and aged for 8 days in food vials. After the completion of 8 days, reciprocal heterogamic crosses between *D. ananassae* PC and *D. pallidosa* NOU 88 (PC ♀ x NOU 88 ♂ and NOU 88 ♀ x PC ♂) were set in culture bottles by keeping 20 females with 20 males. From these bottles hybrid daughters and hybrid sons (daughters of PC ♀ x NOU 88 ♂ is called HD1, sons of PC ♀ x NOU 88 ♂ is called HS1, daughters of NOU 88 ♀ x PC ♂ is called HD2 and sons of NOU 88 ♀ x PC ♂ is called HS2) were

collected and aged in food vials for 8 days which were further used to check matings of both the reciprocal hybrid females and male with the parental males and females as well as hybrids themselves. One day prior to experiments 15 females and 15 males of each type (*D. ananassae* PC females, *D. ananassae* PC males, *D. pallidosa* NOU 88 females, *D. pallidosa* NOU 88 males, daughters of PC ♀ x NOU 88 ♂ (HD1), sons of PC ♀ x NOU 88 ♂ (HS1), daughters of NOU 88 ♀ x PC ♂ (HD2) and sons of NOU 88 ♀ x PC ♂ (HS2) were separated and kept in fresh food vials. After the completion of 8 days, 15 females and 15 males according to crosses (HD1 x PC ♂, HD1 x NOU 88 ♂, HD1 x HS1, HD1 x HS2, PC ♀ x HS1 and NOU 88 ♀ x HS1 from daughters and sons of PC ♀ x NOU 88 ♂ and HD2 x PC ♂, HD2 x NOU 88 ♂, HD2 x HS2, HD2 x HS1, PC ♀ x HS2 and NOU 88 ♀ x HS2 from the daughters and sons of NOU 88 ♀ x PC ♂) were introduced into Elens-Wattiaux mating chamber. Matings were directly observed for sixty minutes, during the morning hours (7 A.M. to 11 A.M.). Once a pair commences mating, it was aspirated out and kept in separate empty vials and data were recorded in the form of number of matings for that hour for each replicate. Five replicates were carried out for all the crosses.

2.3 Experimental Design to Assay Productivity

2.3.1 For Group I

20 pairs (females and males in equal number) of 8 days old females and males from each strain were transferred to the bottles. From these bottles virgin females and males were collected and aged in food vials for 8 days which were further used to set the culture bottles for the experiments. Flies were kept for 3 days to allow them to oviposit and were then discarded. Virgin females and males were collected and aged for 8 days in food vials. After the completion of 8 days, homogamic crosses of both the parental species (PC ♀ x PC ♂ and NOU 88 ♀ x NOU 88 ♂) and reciprocal heterogamic crosses between *D. ananassae* PC and *D. pallidosa* NOU 88 (PC ♀ x NOU 88 ♂ and NOU 88 ♀ x PC ♂) were set in 20 vials by keeping single female with single male (1 pair/vial). Total 80 vials were used to make first set of experiments (20 for PC ♀ x PC ♂, 20 for NOU 88 ♀ x NOU 88 ♂, 20 for PC ♀ x NOU 88 ♂ and 20 for NOU 88 ♀ x PC ♂). Flies were kept in vials for 3 days and were then transferred to the fresh food vials to set second set. Similarly, third and fourth sets of experiments were also set by transferring flies into fresh vials after each 3 days. Those vials where females and males were not alive or flight away during the transfer was excluded from the experiments. After about 10 days, the first sets of vials were inspected for larval activity. Vials in which larval activity was found were recorded as having a fertile female. The vials in which no larval activity was seen were kept for few more days and regularly checked for larval activity. The corresponding second, third and fourth sets of vials were also inspected for larval activity. Vials in which no larval activity was noted in all the four sets of vials were noted as having sterile females. Further, the vials which were recorded as fertile vials were kept to measure the number of females and males progeny separately in parental species as well as reciprocal interspecific crosses and the progeny were counted from each fertile vial of each cross until all had eclosed.

2.3.2 For Group II

20 pairs (females and males in equal number) of 8 days old females and males from each strain were transferred to the bottles. From these bottles virgin females and males were collected and aged in food vials for 8 days which were further used to set the culture bottles for the experiments. Flies were kept for 3 days to allow them to oviposit and were then discarded. Virgin females and males were collected and aged for 8 days in food vials. After the completion of 8 days, reciprocal heterogamic crosses between *D. ananassae* PC and *D. pallidosa* NOU 88 (PC ♀ x NOU 88 ♂ and NOU 88 ♀ x PC ♂) were set in culture bottles by keeping 20 females with 20 males. From these bottles hybrid daughters and hybrid sons (daughters of PC ♀ x NOU 88 ♂ is called HD1, sons of PC ♀ x NOU 88 ♂ is called HS1, daughters of NOU 88 ♀ x PC ♂ is called HD2 and sons of NOU 88 ♀ x PC ♂ is called HS2) were collected and aged in food vials for 8 days which were further used to set the experimental vials. From the hybrids of PC ♀ x NOU 88 ♂ cross, five sets of crosses were set with parental males, females and hybrid themselves: HD1 x PC ♂, HD1 x NOU 88 ♂, HD1 x HS1, PC ♀ x HS1, NOU 88 ♀ x HS1. Similarly, from the hybrids of NOU88 ♀ x PC ♂ cross, five sets of crosses were also set with parental males, females, and hybrid themselves: HD2 x PC ♂, HD2 x NOU 88 ♂, HD2 x HS2, PC ♀ x HS2, NOU 88 ♀ x HS2. Each cross was set in 20 vials by keeping single female with single male (1pair/vial). Flies were kept in vials for 3 days and were then transferred to the fresh food vials to set second set. Similarly, third and fourth sets of experiments were also set by transferring flies into fresh vials after each 3 days. Those vials where females and males were not alive or flight away during the transfer was excluded from the experiments. After about 10 days, the first sets of vials were inspected for larval activity. Vials in which larval activity was found were recorded as having a fertile female. The vials in which no larval activity was seen were kept for few more days and regularly checked for larval activity. The corresponding second, third and fourth sets of vials were also inspected for larval activity. Vials in which no larval activity was noted in all the four sets of vials were noted as having sterile female. Further, the vials which were recorded as fertile vials were

kept to measure the number of females and males progeny separately in different crosses of the hybrids of both the reciprocal cross and the progeny were counted from each fertile vial of each cross until all had eclosed.

2.4 Statistical Analysis

To test whether there are significant differences in mean number of matings, between the parental species (*D. ananassae* PC and *D. pallidosa* NOU 88) and reciprocal interspecific crosses, comparisons were made by applying one–way ANOVA followed by post hoc analysis with Bonferroni t-tests for pair wise comparisons. One-way ANOVA followed by post hoc analysis with Bonferroni t-tests for pair wise comparisons was also used to analyse the differences in the mean number of matings in different crosses of F1(PC ♀ x NOU 88 ♂) and F1(NOU 88 ♀ x PC ♂) relative to parental species. Student t-test was applied to compare mean number of matings of both the hybrids.

To test whether there are significant differences in mean number of progeny, in the parental species (*D. ananassae* and *D. pallidosa*) and reciprocal interspecific crosses, comparisons were made by applying one–way ANOVA followed by post hoc analysis with Bonferroni t-tests for pair wise comparisons. One-way ANOVA followed by post hoc analysis with Bonferroni t-tests for pair wise comparisons was also used to analyse the variations in the mean number of progeny in different crosses of F1(PC ♀ x NOU 88 ♂) and F1(NOU 88 ♀ x PC ♂) in relation to parental species. One–way ANOVA was also done followed by post hoc analysis with sequential Bonferroni t-tests for multiple comparisons to examine the differences in the combined mean number of progeny in different crosses of F1(PC ♀ x NOU 88 ♂) and F1(NOU 88 ♀ x PC ♂) relative to parental species. Student t-test was applied to compare mean number of progeny of both the reciprocal hybrids.

3. Results

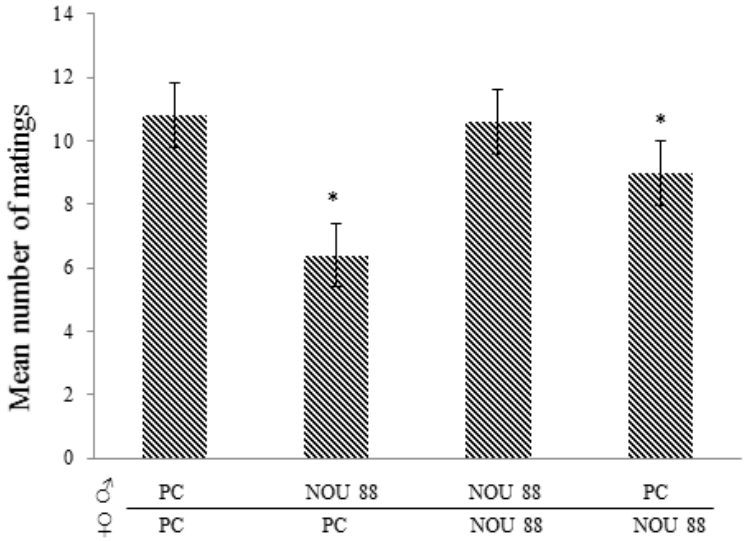

Figure 1. Mating rates within and between species in no choice trails

NOU 88 refers to *D. pallidosa* NOU 88 and PC refers to *D. ananassae* PC (*p< 0.05)

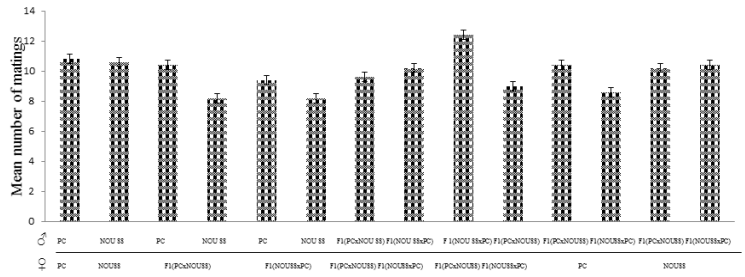

Figure 2. Mating rates of hybrids relative to pure species

F1(PC x NOU 88) are F1 hybrids from *D. ananassae* PC females, F1(NOU 88 x PC) are F1 hybrids from *D. pallidosa* NOU 88 females, PC refers to *D. ananassae* PC and NOU 88 refers to *D. pallidosa* NOU 88

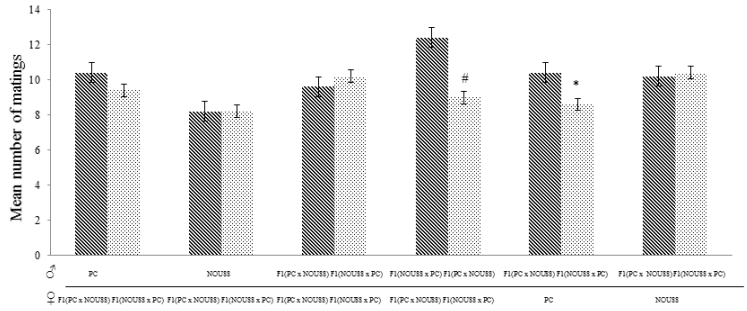

Figure 3. Comparison of mean number of matings between both the reciprocal hybrids

F1(PC x NOU88) are F1 hybrids from *D. ananassae* PC females, F1(NOU 88 x PC) are F1 hybrids from *D. pallidosa* NOU 88 females, PC refers to *D. ananassae* PC and NOU 88 refers to *D. pallidosa* NOU 88 (#p< 0.001, *p< 0.01)

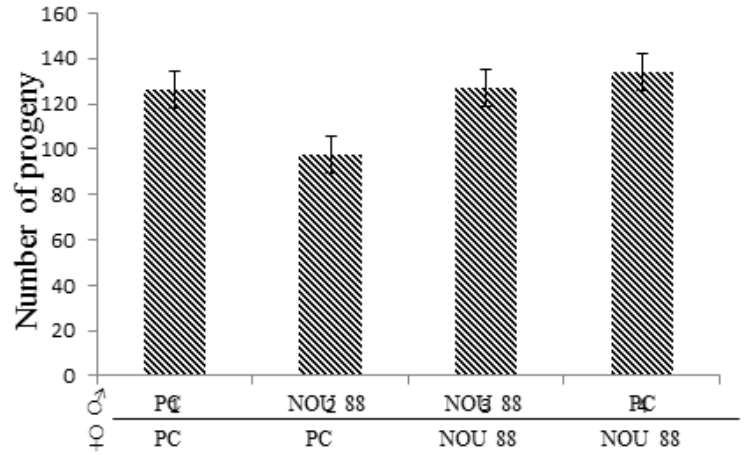

Figure 4. Progeny production in homogamic crosses relative to heterogamic crosses

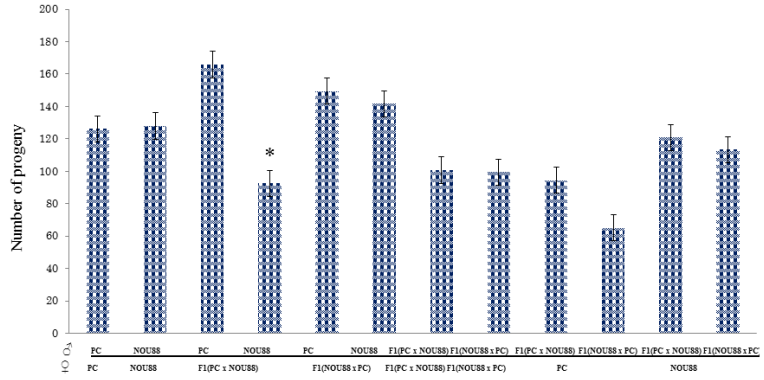

Figure 5. Number of progeny produced by hybrids relative to parental species

F1(PC x NOU 88) are F1 hybrids from *D. ananassae* PC females, F1(NOU 88 x PC) are F1 hybrids from *D. pallidosa* NOU88 females, PC refers to *D. ananassae* PC and NOU 88 refers to *D. pallidosa* NOU 88 (*p< 0.05)

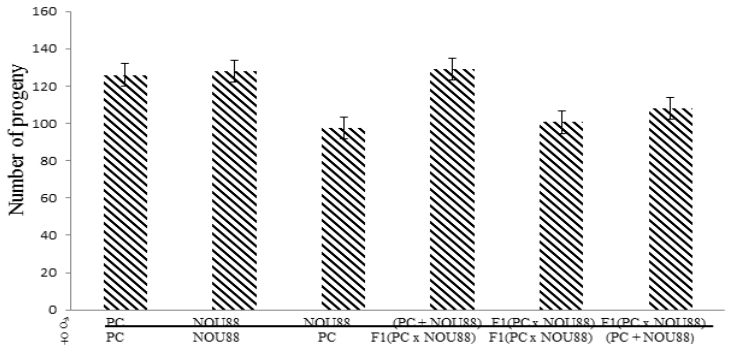

Figure 6. Combined progeny produced by F1(PC x NOU 88) relative to parental species

F1(PC x NOU 88) are F1 hybrids from *D. ananassae* PC females, PC refers to *D. ananassae* PC and NOU 88 refers to *D. pallidosa* NOU88

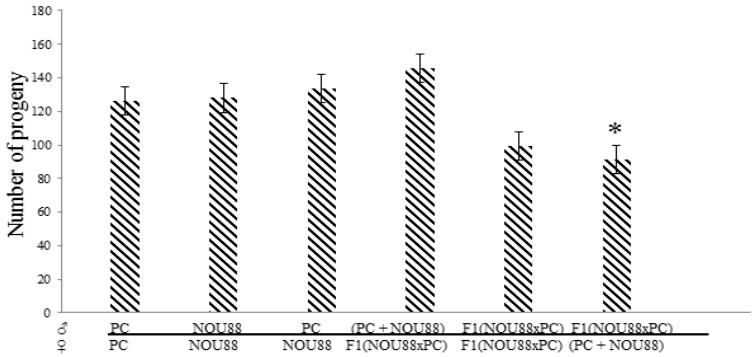

Figure 7. Combined progeny produced by F1(NOU 88 x PC) relative to parental species

F1(NOU 88 x PC) are F1 hybrids from *D. pallidosa* NOU 88 females, PC refers to *D. ananassae* PC and NOU 88 refers to *D. pallidosa* NOU 88 (*p< 0.05)

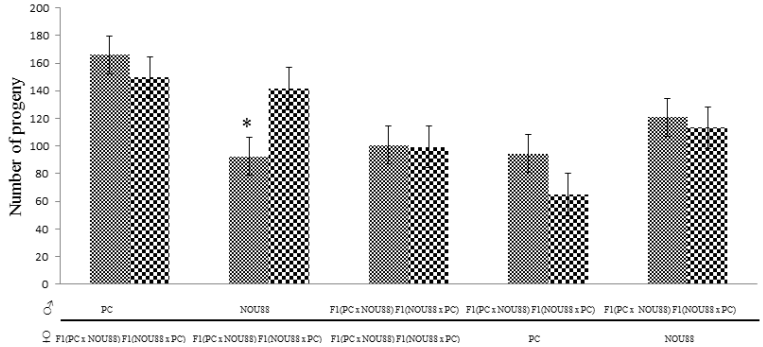

Figure 8. Comparison of progeny produced by both the reciprocal hybrids

F1(PC x NOU 88) are F1 hybrids from *D. ananassae* PC females, F1(NOU 88 x PC) are F1 hybrids from *D. pallidosa* NOU88 females, PC refers to *D. ananassae* PC and NOU 88 refers to *D. pallidosa* NOU 88

Table 1. Results of one-way ANOVA for comparing mean number of matings between homogamic and heterogamic crosses

Source of variation	df	SS	MS	F
Total	19	69.20	-	-
Between crosses	3	62.00	20.67	45.93*
Within crosses	6	7.20	0.45	

*p< 0.001.

Table 2. Results of one-way ANOVA for comparing mean number of matings in different crosses of F1 (PC x NOU 88) and F1 (NOU 88 x PC) relative to parental species

Crosses	Source of variation	df	SS	MS	F
PC ♀ x PC ♂	Total	44	150.44	-	-
NOU 88 ♀ x NOU 88 ♂	Between crosses	8	116.84	14.61	15.65*
PC ♀ x NOU 88 ♂	Within crosses	36	33.60	0.93	
HD1 x PC ♂					
HD1 x NOU 88 ♂					
HD1 x HS1					
HD1 x HS2					
PC ♀ x HS1					
NOU 88 ♀ x HS1					
PC ♀ x PC ♂	Total	44	70.98	-	-
NOU 88 ♀ x NOU 88 ♂	Between crosses	8	35.78	4.47	4.57*
NOU 88 ♀ x PC ♂	Within crosses	36	35.20	0.98	
HD2 x PC ♂					
HD2 x NOU 88 ♂					
HD2 x HS2					
HD2 x HS1					
PC ♀ x HS2					
NOU 88 ♀ x HS2					

*p< 0.001.

F1 (PC x NOU 88), F1 hybrids from *D. ananassae* PC females; F1 (NOU 88 x PC), F1 hybrids from *D. pallidosa* NOU 88 females; PC, *D. ananassae* PC; NOU 88, *D. pallidosa* NOU 88; HD1, hybrid daughters of *D. ananassae* PC ♀ x *D. pallidosa* NOU 88 ♂; HS1, hybrid sons of *D. ananassae* PC ♀ x *D. pallidosa* NOU 88 ♂; HD2, hybrid daughters of *D. pallidosa* NOU 88 ♀ x *D. ananassae* PC ♂; HS2, hybrid sons of *D. pallidosa* NOU 88 ♀ x *D. ananassae* PC ♂.

Table 3. Productivity of parental species, interspecific crosses between *D. ananassae* PC and *D. pallidosa* NOU 88 and hybrid females and males from both the reciprocal crosses with parental species as well as hybrids themselves

Type of crosses	n	Total number of progeny	Mean±S.E.	Number of female offsprings	Number of male offsprings
PC ♀ x PC ♂	18	2269	126.06	1163	1125
NOU 88 ♀ x NOU 88 ♂	14	1791	127.93	897	894
PC ♀ x NOU 88 ♂	14	1365	97.50	668	697
NOU 88 ♀ x PC ♂	20	2676	133.80	1339	1337
HD1 x PC ♂	17	2820	165.88	1468	1352
HD1 x NOU 88 ♂	17	1569	92.41	816	753
HD1 x HS1	17	1711	100.65	874	837
PC ♀ xHS1	16	1511	94.44	782	729
NOU 88 ♀ x HS1	17	2055	120.88	1072	983
HD2 x PC ♂	18	2690	149.44	1355	1335
HD2 x NOU 88 ♂	16	2356	141.69	1233	1123
HD2 x HS2	17	1690	99.41	837	853
PC ♀ x HS2	14	910	65.00	469	441
NOU 88 ♀ x HS2	17	2116	113.29	1062	1054

PC, *D. ananassae* PC; NOU 88, *D. pallidosa* NOU 88; HD1, hybrid daughters of *D. ananassae* PC ♀ x *D. pallidosa* NOU 88 ♂; HS1, hybrid sons of *D. ananassae* PC ♀ x *D. pallidosa* NOU 88 ♂; HD2, hybrid daughters of *D. pallidosa* NOU 88 ♀ x *D. ananassae* PC ♂; HS2 refers to hybrid sons of *D. pallidosa* NOU 88 ♀ x *D. ananassae* PC ♂.

Differences in the mean number of matings between homogamic and heterogenic crosses are statistically significant (p< 0.001, Table 1). *D. ananassae* PC females, mate with *D. pallidosa* NOU 88 males significantly less than conspecific males (t=10.371, p< 0.05) which is about half of the conspecific mating (Figure 1). In contrast, *D. pallidosa* NOU 88 females mate with *D. ananassae* PC males significantly less than conspecific matings (t=3.771,

$p< 0.05$, Figure 1) but their mating rate is higher in comparison to reciprocal cross (PC ♀ x NOU 88 ♂). Therefore, among heterogamic crosses, *D. pallidosa* NOU 88 females mated more often with *D. ananassae* PC males than *D. ananassae* PC females mated with *D. pallidosa* NOU 88 males (t= 6.128, $p< 0.05$). In contrast, both the homogamic crosses do not show significant differences in matings which indicate that rate of mating of both the species is almost same. So, it is clear that sexual isolation found between these two species is not biased because of differences in mating rates. Hybrid daughters from either reciprocal cross mate with *D. ananassae* PC males at similar rate (t=0.839, p=0.426, Figure 3) and this is similar to the mating rate observed between pure species *D. ananassae* PC females and males (t=0.655, $p> 0.05$ for HD1, t=2.239, $p> 0.05$ for HD2, Table 2, Figure 2) as well as *D. pallidosa* NOU 88 females and males (t=0.327, $p> 0.05$ for HD1, t=1.919, $p> 0.05$ for HD2, Table 2, Figure 2). The hybrid females from both the reciprocal crosses also mate with *D. pallidosa* NOU 88 males at similar rate (t=0.00, p= 1.0, Figure 3) and this is not similar to the mating rate observed between pure species *D. pallidosa* NOU 88 females and males (t=3.928, $p< 0.05$ for HD1, t=3.838, $p< 0.05$ for HD2, Table 2, Figure 2) as well as *D. ananassae* PC females and males (t=4.255, $p< 0.05$ for HD1, t=4.157, $p< 0.05$ for HD2, Table 2, Figure 2) . Hybrid daughters from either reciprocal crosses mate with respective hybrid sons at similar rate (t=-0.775, p= 0.461, Figure 3) and this is similar to the mating rates of both the parental species (Table 2, Figure 2). Hybrid daughters from either reciprocal crosses mate with reciprocal hybrid sons at significantly different rate (t=13.88, $p< 0.001$, Figure 3) and this is similar to the mating rates of both the parental species (Table 2, Figure 2). Hybrid sons from both the reciprocal crosses mate with *D. ananassae* PC females at significantly different rate (t=3.18, $p< 0.01$, Figure 3) and this is similar to the mating rates of both the parental species (Table 2, Figure 2) but hybrid sons from either reciprocal crosses mate with *D. pallidosa* NOU 88 females at similar rate (t=-0.447, p= 0.667, Figure 3) and this is similar to the mating rates of both the parental species (Table 2, Figure 2).

Table 4. Results of one-way ANOVA for comparing mean number of progeny in different crosses of F1 (PC x NOU 88) and F1 (NOU 88 x PC) relative to parental species

Type of crosses	Source of variation	df	SS	MS	F
PC ♀ x PC ♂	Total	129	408029.70	-	-
NOU 88 ♀ x NOU 88 ♂	Between crosses	7	72212.86	10316.12	3.75**
PC ♀ x NOU 88 ♂	Within crosses	122	335816.84	2752.60	
HD1 x PC ♂					
HD1 x NOU 88 ♂					
HD1 x HS1					
PC ♀ x HS1					
NOU 88 ♀ x HS1					
PC ♀ x PC ♂	Total	133	545730.81	-	-
NOU 88 ♀ x NOU 88 ♂	Between crosses	7	78656.21	11236.60	3.03*
NOU 88 ♀ x PC ♂	Within crosses	126	467074.60	3706.94	
HD2 x PC ♂					
HD2 x NOU 88 ♂					
HD2 x HS2					
PC ♀ x HS2					
NOU 88 ♀ x HS2					

**$p< 0.001$; *$p< 0.01$.

F1 (PC x NOU 88), F1 hybrids from *D. ananassae* PC females; F1 (NOU 88 x PC), F1 hybrids from *D. pallidosa* NOU 88 females; PC, *D. ananassae* PC; NOU 88, *D. pallidosa* NOU 88; HD1, hybrid daughters of *D. ananassae* PC ♀ x *D. pallidosa* NOU 88 ♂; HS1, hybrid sons of D. *ananassae* PC ♀ x *D. pallidosa* NOU 88 ♂; HD2, hybrid daughters of *D. pallidosa* NOU 88 ♀ x *D. ananassae* PC ♂; HS2, hybrid sons of *D. pallidosa* NOU 88 ♀ x *D. ananassae* PC ♂.

Females mated with conspecific males do not produce significantly more offspring than females mated with a heterospecific males but when *D. ananassae* PC ♀ were crossed with *D. pallidosa* NOU 88 ♂ less number of progeny were found in comparison to reciprocal cross (Figure 4). The occurrence of same number of progeny in conspecific and heterospecific mating indicate a lack of postmating prezygotic isolation in these sibling species pair. Significant differences were found in the mean number of progeny in different crosses of both the reciprocal hybrids relative to the parental species (Table 4). Hybrids from either reciprocal crosses yields almost similar

number of progeny with *D. ananassae* PC males (t=0.769, p= 0.448, Figure 8), and this is slightly higher to pure species (Figure 5). The hybrid females from both the reciprocal crosses also produce statistically different number of progeny with *D. pallidosa* NOU 88 males (t=-2.306, p< 0.05, Figure 8), and this is also less than the both the pure species but value is not significant (Figure 5). Hybrid daughters from either reciprocal crosses produced almost similar number of progeny with hybrid sons (t=0.059, p= 0.953, Figure 8) but this was slightly lower than the pure species (Figure 5). Hybrid sons from both the reciprocal crosses produced different number of progeny with *D. ananassae* PC females but this value was insignificant (t=1.543, p= 0.134, Figure 8) and this was lower than both the pure species but not significant (Figure 5). Hybrid sons from either reciprocal crosses produced almost similar number of offspring with *D. pallidosa* NOU 88 females (t=0.609, p= 0.547, Figure 8) and this is slightly lower than the both the pure species but not significant (Figure 5). Lowest number of progeny was found in the cross of hybrid sons of either reciprocal crosses with parental species in comparison to the cross of hybrid daughters with parental species and hybrid daughters with hybrid sons (Table 3). Hybrids which were produced by crossing *D. ananassae* PC females with *D. pallidosa* NOU 88 males, were found to produce more progeny in comparison to reciprocal hybrids in all the crosses except HD 1 x NOU 88 ♂ (Table 5, Figure 6, 7).

Table 5. Results of one-way ANOVA for comparing combined mean number of progeny in different crosses of F1 (PC x NOU 88) and F1 (NOU 88 x PC) relative to parental species

Type of crosses	Source of variation	df	SS	MS	F
PC ♀ x PC ♂	Total	129	408029.70	-	-
NOU 88 ♀ x NOU 88 ♂	Between the crosses	5	20566.30	4113.26	1.32^{NS}
PC ♀ x NOU 88 ♂	Within the crosses	124	387463.40	3124.71	
HD1 x (PC ♂ + NOU 88 ♂)					
HD1 x HS1					
(PC ♀ + NOU 88 ♀) x HS1					
PC ♀ x PC ♂	Total	133	545730.81	-	-
NOU 88 ♀ x NOU 88 ♂	Between the crosses	5	60240.322	12048.06	3.176*
NOU 88 ♀ x PC ♂	Within the crosses	128	485490.49	3792.89	
HD2 x (PC ♂ + NOU 88 ♂)					
HD2 x HS2					
(PC ♀ + NOU 88 ♀) x HD2					

NS, Not significant; *p< 0.01.

F1 (PC x NOU 88), F1 hybrids from *D. ananassae* PC females; F1 (NOU 88 x PC), F1 hybrids from *D. pallidosa* NOU 88 females; PC, *D. ananassae* PC; NOU 88, *D. pallidosa* NOU 88; HD1, hybrid daughters of *D. ananassae* PC ♀ x *D. pallidosa* NOU 88 ♂; HS1, hybrid sons of *D. ananassae* PC ♀ x *D. pallidosa* NOU 88 ♂; HD2, hybrid daughters of *D. pallidosa* NOU 88 ♀ x *D. ananassae* PC ♂; HS2, hybrid sons of *D. pallidosa* NOU 88 ♀ x *D. ananassae* PC ♂.

4. Discussion

D. ananassae and *D. pallidosa* were determined as different species based on their sexual isolation, chromosomal patterns, and differences in the pattern of sex-comb (Bock & Wheeler, 1972) but surprisingly they were not having any differences in male genitalia which is a fundamental basis of classification. Therefore, in the present study we tried to elucidate complete picture behind the speciation of these species by measuring each component of reproductive isolation by means of mating preference and productivity. In the present study, we report premating isolation and this is consistent with the previous finding (Futch, 1966, 1973; Vishalakshi & Singh, 2006). Occurrence of differences in courtship songs (Yamada, Tomaru, Matsuda, & Oguma, 2008) and cuticular hydrocarbon profiles (Doi, Matsuda, Tomaru, Matsubayashi, & Oguma, 2001) explain how premating isolation between *D. ananassae* and *D. pallidosa* exist even though postzygotic isolation is lacking. Females of both *D. ananassae* and *D. pallidosa* were more discriminative for mating against the alien males rather than conspecific males, and this discrimination was much stronger in case of *D. ananassae* females for being the ancestral and cosmopolitan species. However, it is known that females, who belong to the species of smaller population size may come across alien males at a high rate and thus pay greater cost of hybridization (Noor, 1995; Yukilevich, 2012). On the basis of this, *D. pallidosa*, an endemic species, should be more discriminative but we found that *D. ananassae*, which is cosmopolitan, was more choosy between these species. Thus, on the basis of our results, we predict that *D. ananassae* females may have developed rigid mate discrimination system to maintain the integrity

because of being cosmopolitan, *D. ananassae* have interactions with many species other than *D. pallidosa*. In heterospecific matings in the laboratory, we did not find any decrease in the production of progeny or viable offspring in comparison to conspecific males, indicating a lack of postmating prezygotic isolating barriers. This lack of postmating prezygotic isolation might be due to the lack of differences in the male genitalia, because possibly as a consequence of similar male genitalia, alien males might be equally capable to transfer sufficient amount of sperm during copulation.

Pattern of mating preference of hybrid daughters of both the reciprocal interspecific crosses involving *D. ananassae* and *D. pallidosa* was found to be similar which proves that, the sex of parental species has no influence on mate recognition in these species pair and genes responsible for mate recognition do not have any maternal effect. Nevertheless, our results contradict with an emerging body of evidence (Chawla & Werren, 2010; Clark, O'Hara, Humphreys, Rundle, & Dyer, 2016; Latour et al., 2014; Lemmon & Lemmon, 2010; Ritchie, 2000; Russell & Magurran, 2006; Santos, Pereira, Vicente, & Collares-Pereira, 2015; Schmidt & Pfennig, 2016; Schumer et al., 2017; Selz, Thommen, Maan, & Seehausen, 2014; Svedin, Wiley, Veen, Gustafsson, & Qvarnstrom, 2008) as, it is known that hybrids are unable to produce or respond to courtship signals because hybrids either possess intermediate preferences or select one parental species which is dominant over other (Charpentier et al., 2012; Culumber, Ochoa, & Rosenthal, 2014; Paczolt et al., 2015; Svensson et al., 2017; Veen, Faulks, Tyler, Lloyd, & Tregenza, 2012). Because in contrast to these facts, hybrid daughters of both the reciprocal crosses prefer both the parental species for matings but matings of both the hybrid daughters with *D. pallidosa* NOU 88 males is significantly less in comparison to both the parental species whereas HD1 (*D. ananassae* PC ♀ x *D. palliodsa* NOU 88 ♂) produced significantly less offspring in comparison to HD2 (*D. palliodsa* NOU 88 ♀ x *D. ananassae* PC ♂) with NOU 88 males. Hybrid daughters from either reciprocal crosses mate with respective hybrid sons at similar rate whereas hybrid daughters from either reciprocal crosses mate with reciprocal hybrid sons at significantly different rate. Mating of HD1 (*D. ananassae* PC ♀ x *D. palliodsa* NOU 88 ♂) with HS2 (*D. palliodsa* NOU 88 ♀ x *D. ananassae* PC ♂) is significantly high in comparison to HD2 (*D. palliodsa* NOU 88 ♀ x *D. ananassae* PC ♂) with HS1 (*D. ananassae* PC ♀ x *D. palliodsa* NOU 88 ♂), indicating that HD2 is not preferring HS1 as it contains X chromosome of *D. ananassae* PC and Y chromosome of *D. pallidosa* NOU 88 whereas HD1 prefers HS2. Thus, it might be possible that there is incompatibility between X and Y as X chromosome is from the ancestral species and Y chromosome from the derived one. Hybrid sons produce lowest number of progeny in comparison to all the crosses and this value is not significant but near to the significant, projecting light towards the asymmetric post-zygotic isolation by providing the hint of low fitness of the hybrid sons. Hybrid sons from both the reciprocal crosses mate with *D. ananassae* PC females at significantly different rate as HS2 prefer *D. ananassae* PC females less in comparison to HS1 whereas hybrid sons from both the reciprocal crosses produced different number of progeny with *D. ananassae* PC females and this value was insignificant but pattern was same to the mating preference. We did not find any differences in sex ratio in any cross of hybrids in comparison to parental species, indicating the absence of hybrid inviability. These results are consistent with the general pattern reported in the *Drosophila* that hybrid male sterility evolved earlier in the divergence of species (that is, more closely related species) than hybrid inviability (Price & Bouvier, 2002; Russell, 2003).

5. Conclusion

Thus, we can speculate that there is no complete lack of intrinsic post-zygotic isolation between these species or no complete presence of post-zygotic isolation, as both the hybrid sons were producing less number of progeny in comparison to all the crosses but it is near to significant but not significant and this is contrasting to the results of a previous study. However, species pair of the same divergence time is showing clear cut evidence of post-zygotic isolation (Masly & Presgraves, 2007), even though Jennings, Snook, & Hoikkala (2014) reported post-zygotic reproductive isolation between allopatric *Drosophila montana* populations by counting egg and progeny production and Kao, Zubair, Salomon, Nuzhdin & Campo (2015) reported the post-zygotic isolation between United States and Caribbean *Drosophila melanogaster*. Thus, it might be possible that rate of divergence between *D. ananassae* and *D. pallidosa* is very slow in comparison to other species pair or even races of some species so the hybrids are not so rigid to preclude gene flow because this species pair is not diverged so much despite of their 1.68 MYA divergence time (Russo, Beatriz, Frazão & Voloch, 2013) whereas this time is enough for the complete divergence of other species pair, making this species pair matchless. So if the rate of divergence is slow between them in comparison to races and races have not achieved the species status, then why have the *D. ananassae* and *D. pallidosa*, without very strong or complete post-zygotic isolation, have speciated or considered as a distinct species whereas races not evolved into distinct species? But it would be too early to give any hard and clear conclusion about speciation of these two sibling species. So further studies are needed to be fully confident in this assertion.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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