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RESEARCH ARTICLE

Differential mate choiceamong laboratory evolved cytoraces, members of *nasuta albomicans* complex of *Drosophila.*

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Abstract

Hybridization played an important role in diversification of many taxonomic group, as its known to accelerate speciation by introgression, slow or reverse differentiation by recombination. The cytoraces are the resultant of interracial hybridization between *Drosophila nasuta nasuta* and *Drosophila nasuta albomicans* of *nasuta* subgroup of *immigrans* species group of *Drosophila*. The extensive genetic studies on these cytoraces, which are inbred for more than 600 generations, have indicated the presence of divergence. Here, we investigate the mate choice among few cytoraces to establish the existence of racial divergencein preference for a mate and time taken to complete the copulation with the mate. The cytoraces showed differential preferences for homogamy, heterogamy and random mates among the cytoraces. Where the males of C-3 showed preference for homogamy, indicating the positive assortative mating, while the males of C-9 mates randomly. The males of C-3 and C-15 showed longer copulation duration and were able to overcome the female resistance. These behaviors of cytoraces renders them as a potential system to study the early onset of preference in raciation/speciation.

Introduction:-

Once hybridization was thought as the rare, grossest blunder in sexual preference of an organism, but as the time passed, the conception was changed. Now we know that in nature, species are often incompletely isolated for millions of years even after their formation and there about 25% of plant and 10% of animal species are known to involved in hybridization and introgression (Mallet 2005).There are few cases, where hybridization has led to the formation of new species, e.g. sunflowers (Riesberg et al. 1995)andHeliconius butterflies (Mava´reza et al. 2006).In most of the cases, hybrids in nature, would not survive as most of them are sterile; even if they are healthy they would be unable to find mates because of competition from parent species and their unique heritage. But if they can be experimented in a laboratory, an ideal environment where there is no threat of a competition from a parental species, they would be able to mate and flourish. But in amidstof hybrids, which hybrid would they prefer, homospecific or heterospecific?

The *Drosophila nasuta nasuta* (2n = 8) and *Drosophila nasuta albomicans* (2n = 6) are cross-fertile, allopatric, chromosomal races of *nasuta* subgroup of the *immigrans* species group of *Drosophila*. The interracial hybridization between *D. n. nasuta* and *D. n. albomicans* led to the formation hybrid, cytoraces(Ramachandra &Ranganath 1986; 1990; 1996). These cytoraces have inherited the chromosomes from both the parents and have eight karyotypes (Tanuja et al. 2003). They also show racial divergence among themselves in quantitative (Harini& Ramachandra 2000; 2003) and qualitative traits (Ranjini& Ramachandra 2009). Though, the cytoraces are being inbred for more than 600 generationsand are equivalent to geographically isolated populations, they are not reproductively isolated from the parental species or sibling races.In this study, we would determine the sexual compatibility amidstcytoraces, with their preference for a non-random mate and if that non-random mate is homospecific or heterospecific.

The mate choice manifests when there is a non-random mating, as they show preference for a specific mate while resisting the other mates. Usually, non-random mating implies that there is a benefit in mating a specific mate,thus numerous studies have explored the reason of such behavior (Reynolds and Gross 1990; Kirkpatrick and Ryan, 1991; Andersson1994; González *et al.* 1999; Kokko*et al.* 2003). The mate preference shows the chromosomal, gene and sexual compatibility among the cytoraces as they are originated from different parents.

Materials and methods:-

The cytoraces used in this study are as follows:

(a) Cytorace 2 (♂- 2n = 6, $2^n2^aX3^aY3^a4^a4^a$,♀- 2n = 6, $2^n2^aX3^aX3^a4^a4^a$) (Ramachandra &Ranganath 1986)
(b) Cytorace 3 (♂- 2n = 8, $2^n2^a3^n3^nX^nY^n4^a4^a$, ♀- 2n = 8, $2^n2^a3^n3^nX^nX^n4^a4^a$) (Ramachandra &Ranganath 1990)
Cytorace 9 (♂- 2n = 6, $2^n2^aX3^aY3^a4^a4^a$, ♀- 2n = 6, $2^n2^aX3^aX3^a4^a4^a$) (Ramachandra &Ranganath 1996)
(c)
(d) Cytorace 15 (♂- 2n = 8, $2^n2^a3^n3^nX^nY^n4^a4^a$, ♀- 2n = 8, $2^n2^a3^n3^nX^nX^n4^a4^a$) (Ramachandra &Ranganath 1996).

These cytoraces were serially cultured in a half-pint-milk bottles containing, wheat cream agar medium and are maintained at 22ºC±1. The virgin females and unmated males were isolated within four hours of their eclosioninto the media vials and were aged for 5 days prior to mating experiments. The flies were marked with a thin nail enamel to differentiate cytoraces, in male and female choice experiments.

Mating experiments:-

The flies were aspirated into the empty vials to avoid etherization before the experiment. The trails were observed to record the mate preference and copulation duration (initiation to termination) among the cytoraces. In the present study, we have used four different cytoraces for three mating preference experiments: no choice, male choice and female choice.

No choice: In this experiment, each female of four cytoraces were given a choice to mate with one of the males from four cytoraces and the males were homogamic/heterogamicorigin. There were 16experimental combinations and the matingtrails were observed for three hours (8:00 AM to 12:00 PM) to record the copulation duration. The differences observed in copulation duration between the females mated was analyzed using one-way ANOVA.

Male and Female choice: In these experiment, the males and females of cytoraceswere given a choice between females and males oftwo differentcytoraces respectively, which forms 24 experimental combinations in each of the choice experiments. The males and females of cytoraces were given a choice between homogamic and heterogamic mates and also between heterogamic mates. The number of successful mating, preference and the copulation duration were observed for three hours(8:00 AM to 12:00 PM). The degree of sexual isolation was measured for the homogamic/ heterogamic experimental combinations (Stalker 1942). The paired t-test was used to compute the difference of copulation duration between preferred and un-preferred mate. The generalized linear model was computed to discern the effects of males on the variation observed in copulation duration between the females of an experimental combinations.

$$\text{Isolation index} = \frac{\text{\% homogamic mating - \% heterogamic mating}}{\text{\% homogamic mating + \% heterogamic mating}}$$

The isolation index of 0 shows no isolation, +1 indicates complete isolation and -1 indicates heterogamic mating in cytoraces.

Results:-

In the present study, four cytoraces of NAC of *Drosophila* were subjected to 15,783 mate choice experiments, of which, 7032 were successful in mating (44.55%).The successful mating recorded in no choice,male choice and female choice were 29.77%, 46.51% and 64.3%respectively.

No choice experiments:

The no choice experimental combinations experienced lower percentage of mating success, when compared to male choice (56.5%) and female choice (116.4%). The 16 experimental combinations in no choice experiments revealed that, the males of C-3 had the least mating success with the females of cytoraces. Generally, the males of cytoraces showed least mating success across females, except in few cases (Table 1). When the males of C-2, C-9 and C-15 mated with females of C-15 showed higher mating success. The copulation duration of the cytoraces varied across no choice experiments. The males of C-2 and C-9 showed lower copulation duration than the males of C-3 and C-15 (Figure 1). One-way ANOVA showed the differencesin copulation duration among the males of cytoraces; C-2 ($F = 2.833$, $P = 0.038$), C-3 ($F = 9.931$, $P < 0.001$), C-9 ($F = 9.749$, $P < 0.001$) and C-15 ($F = 24.907$, $P < 0.001$).

Male choice experiments:-

The male choice experiments had higher mating success than no choice (36.2%), but lower than female choice (38.2%) experiments.The male choice experiments in cytoraces showed the preference for both homogamic and heterogamic mates (Table 2a and 2b). Each homogamic/heterogamic and heterogamic/heterogamicfemales were organized into 12 experimental combinations (Table 2a and 2b). The males of C-2 prefer C-3 females only in the presence of C-15 females, but they do not distinguish between C-3 and C-9 females in heterogamic trails (Table 2b), while showed no preference in homogamic trails (Table 2a). The males of C-3 could not distinguish between homo and hetero mates when C-9 is present (table 2) and prefer C-3 only in the presence of C-2 and C-15. The males of C-9 rejected the females of C-3 in presence of homogamic mate but preferred in presence of other heterogamic females. The males of C-15 preferred C-9 females over C-15 females in homogamic trails and preferred C-3 females only occurs in the presence ofheterogamic female (Table 2b).

The copulation duration of males varied across the females but the males of C-3 and C-15 mated longer than the C-2 and C-9 (Figure 2). The paired t-test was used to identify the differences in copulation duration of preferred and un-preferred mate. The significant copulation difference was observed between preferred and un-preferred females, when the males of C-3 mated to females of C-3 and C-15 ($t_{20} = 43.464$, $P=0.002$). The generalized linear model was computed to discern the effects of males on copulation duration in an experimental combination. The males of C-2 showed the effect on copulation duration in 10 of the 12 experimental combinations. The males of C-9 and C-15 showed the effect on seven and six experimental combinations respectively. While the males of C-3 showed to effect the copulation duration in two experimental combinations (Table 3).

Female choice experiments:-

The female choice experiments had higher mating success than no choice (53.8%) and male choice (27.7%) experiments. The female choice experiments in cytoracesshowed the preference for both homogamic and heterogamic mates (Table 4a and 4b). Each homogamic/heterogamic and heterogamic/heterogamicmales were organized into 12 experimental combinations (Table 4a and 4b). The females of cytoraces preferred to mate any males in presence of C-3, except C-9 females. The females of C-3 preferred heterogamic males in presence of homogamic, except C-9 and preferred C-2 males in presence of C-9. The females of C-2 and C-15 preferred homogamic mates in the presences of C-3 males, while the females of C-2 preferred to mate with males of C-15 than homogamic males. The C-9 females had no preference forthe homogamic mates but preferred other males in presence of C-3.The rest of the experimental combination of males failed to elicit female preference (Table 4a and 4b).

The copulation duration of females varied across the males and males of C-3 and C-15 mated longer than the C-2 and C-9 (Figure 3). The paired t-test was used to identify the differences in copulation duration of preferred and un-preferred mate. The significant copulation duration was observed between preferred and un-preferred males. The females of C-2 mated with males of C-2 and C-3 ($t_{20} = 28.011$, $P=0.002$), C-2 and C-15 ($t_{16} = 43.464$, $P=0.032$),and C-3 and C-9 ($t_{16} = 36.019$, $P=0.003$) showed significant difference between preferred and un-preferred male`s copulation duration. The females of C-3 ($t_{16} = 40.471$, $P=0.001$), C-9 ($t_{18} = 35.485$, $P=0.008$) and C-15 ($t_{13} = 24.207$, $P=0.029$) showed significant copulation duration between preferred and un-preferred mates in a choice of C-2 and C-3 males. The generalized linear model discerns the effects of females on copulation duration of males

(Table 5). The females of C-15 and C-3 showed to effect male`s copulation duration in 10 and 7 experimental combinations respectively. Whereas the females of C-2 and C-9 showed to effect male`s copulation duration in 4 experimental combinations.

Discussion:-

The cytoraces differ from their parents in morphometric and fitness features (Harini& Ramachandra 1999a; 1999b; 2000a; 2000b), and some of themhavelonger life spans than their parents (Ranjini& Ramachandra 2009). The Inter-Simple Sequence Repeat marker analysis between the cytoraces and their parents revealed, the increased genetic variability and greater fitness in C-1, C-2 and C-4, whereas C-3 has the least DNA polymorphism (Bijaya& Ramachandra 2010). The cytoraces have shown increased substitution rate in few coding genes (Ranjini& Ramachandra 2013; Radhika & Ramachandra 2014). The earlier studies on mate preferences between parental species and cytoraces have established the initiation of divergence in cytoraces (C-1 to C-4) from parents (Ramachandra &Ranganath 1994; Tanuja et al 2001). This study aims to ascertain the presence of racial divergence among the cytoraces.

The probability of mating between any pair depend on both the male attractiveness and female preference (Bateson 1983). The female mate choice can occur when the effects of traits expressed in one sex lead to nonrandom mating(Clutton-Brock 2007; Kokko 2003). The males and females of cytoraces were given a single potential mate, where the potential mate may or may not be a desirable mate to either sexes, resulting in lower mating success. In no choice, the males of C-3 showed least mating success with the females mated and shows the discrimination towards mating heterogamic females. Whereas the females of C-15 mated indiscriminately with the males of C-2 and C-9. The male choice has higher mating success, due to the presence of two potential females. The C-3 males showed preference for the homogamic females, except in presence of C-9 females and showed no preference for heterogamic females (between heterogamic choices). The males of C-2 mates indiscriminately except in one of the choice (Table 2b). The female choice has higher mating success than other choice experiments, owing to the presence of two potential males.The females of cytoraces showed preference to mate the other males in presence of C-3 males (Table 4a and 4b). This behavior of females implies that the males of C-3 are the least desirable mate of the cytoraces presented. The females of C-9 show no preference in presence of homogamic males, but preferred the heterogamic males (C-2 and C-15) in presence of C-3 males in heterogamic mate choice (Table 4b). The female preference changes depending on the number and quality of available males (Hoikkala&Aspi 1993).

In *Drosophila*, the courtship and copulation duration has significant costs to males (Byrne & Rice 2006), reason for discrimination in matingfemales and also because the current mating will affect the subsequent mating opportunities (Bondurianksy 2001). The genetic variance in both the sexes determine the copulation duration (Hirai et al 1999; Edward et al 2014). Thecopulation duration of a male also depends on female resistance and his ability to overcome the resistance (Mazzi et al 2009). The males mate for more than twice the duration necessary to complete the sperm transfer and the extra copulation time serves to delay the female re-mating, rather than to increase that rate at which of offspring are sired before re-mating (Gilchrist and Partridge 2000).The males of C-3 also showed higher copulation duration (Figure 1) when mated, this behavior of C-3 males may be to sire more offspring from the females. The males of C-3 has no effect on copulation duration differences found between the females mated in a choice.The males of C-2 and C-15 affected the copulation duration differences observed between the females of a choice in most of the experimental combinations. The males of C-2 and C-15 had the ability to overcome the female resistance in order to prolong the copulation duration, while C-3 and C-9 lack such ability.

Some of the males in female choice, prolonged the copulation duration byovercoming female resistance in few experimental combinations (Table 5). In few cases, the un-preferred males mated with the females longer than the preferred males (Table 4a, 4b and Figure 3). This demeanor of un-preferred males renders the delayed female re-mating, which increases the rate of offspring sired before re-mating (Gilchrist and Partridge 2000).The males of C-3 and C-15 mated to females longer than other cytoracesand one of the reasons for the longer copulation duration might be the males overcomingthe female resistance. The males of C-3 and C-15 mated to females longer than other cytoraces and one of the reasons for the longer copulation duration might be the males overcoming the female resistance.Mating under different sex ratio conditions, provide evidence that copulation duration is a form of male reproductive investment that responds to the perceived intensity of sperm competition as predicted by game theoretical models (Mazzi et al. 2009).

The assortative mating was observed only in males of C-3 and the assortment is the consequence of mutual mate choice or by the behavior of only males or females (McNamara & Collins 1990). The assortative mating plays a key role in speciation, contributing to premating isolation between phenotypically divergent populations (Felsenstein 1981; Kondrashov&Shpak 1998; Coyne &Orr 2004; Bolnick& Kirkpatrick 2012).The females of C-3 showed disassortative mating, as it increases heterozygosity and decreases inbreeding depression(Waser 1993; Pusey & Wolf 1996). The C-3 may be stepping on their first step towards the incipient isolation from other cytoraces.

This investigation showed that, the each cytoraces are racially diverging, where C-3 has diverged more than other cytoraces. The random or indiscrimination of mates in cytoraces may be due to the conserved or unchanged cues of reproduction. The cytoraces are also one of the best model to study mate preference, as we can investigate both assortative and random mating of organism. The unfinished products (hybrids) of species provides more information about the process of mate preference and speciation than the finished product of speciation (species).

Figure legends:-

Figure 1: The graph represents the mean copulation duration across no choice experiments. The four different patterned columns identify the males of cytoraces.
Figure 2: The graph represents the mean copulation duration across male choice experiment. The significant copulation duration between preferred and un-preferred mates is represented as pattern filled columns.
Figure 3: The graph represents the mean copulation duration across female choice experiment. The significant copulation duration between preferred and un-preferred mates is represented as pattern filled columns.

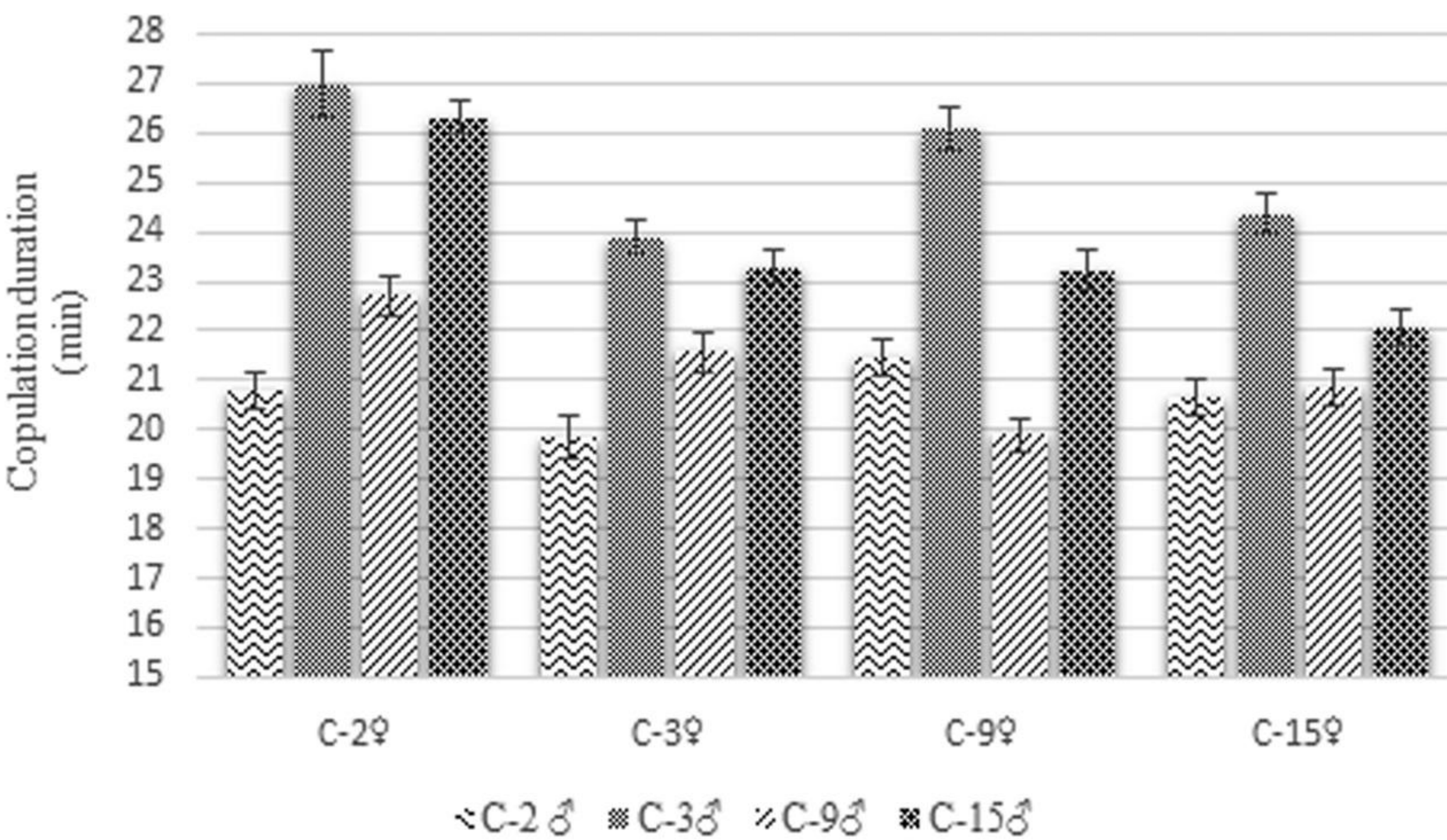

Figure 1

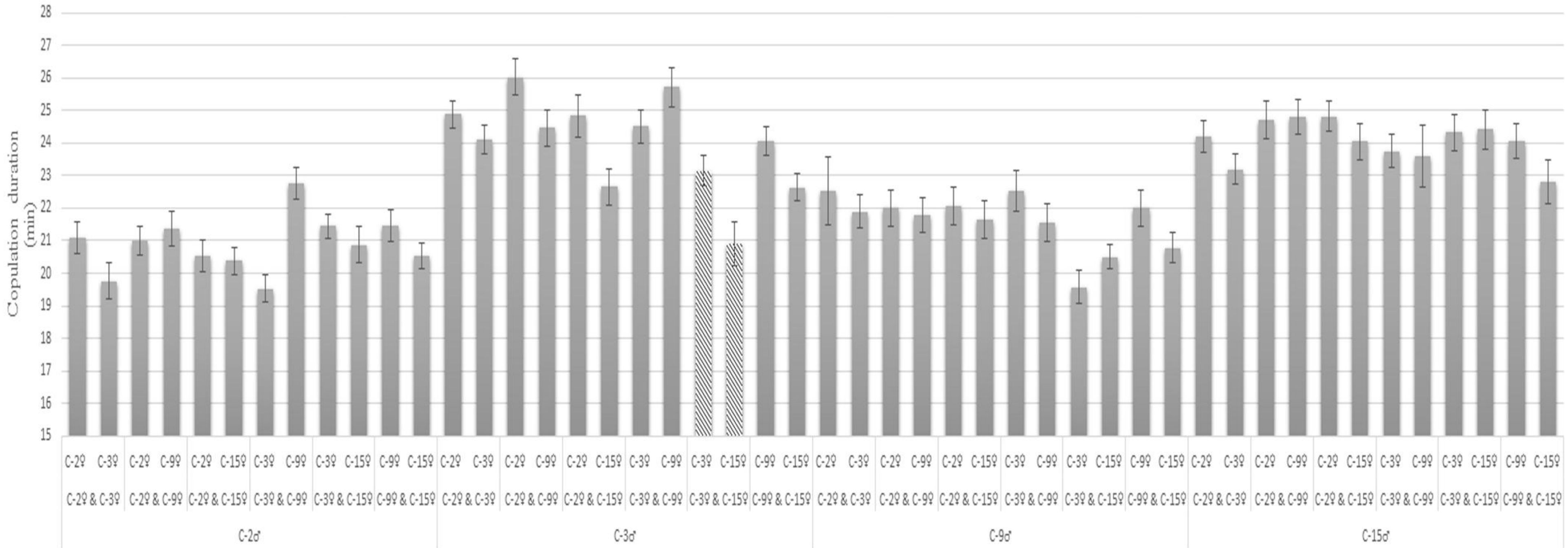

Figure 2

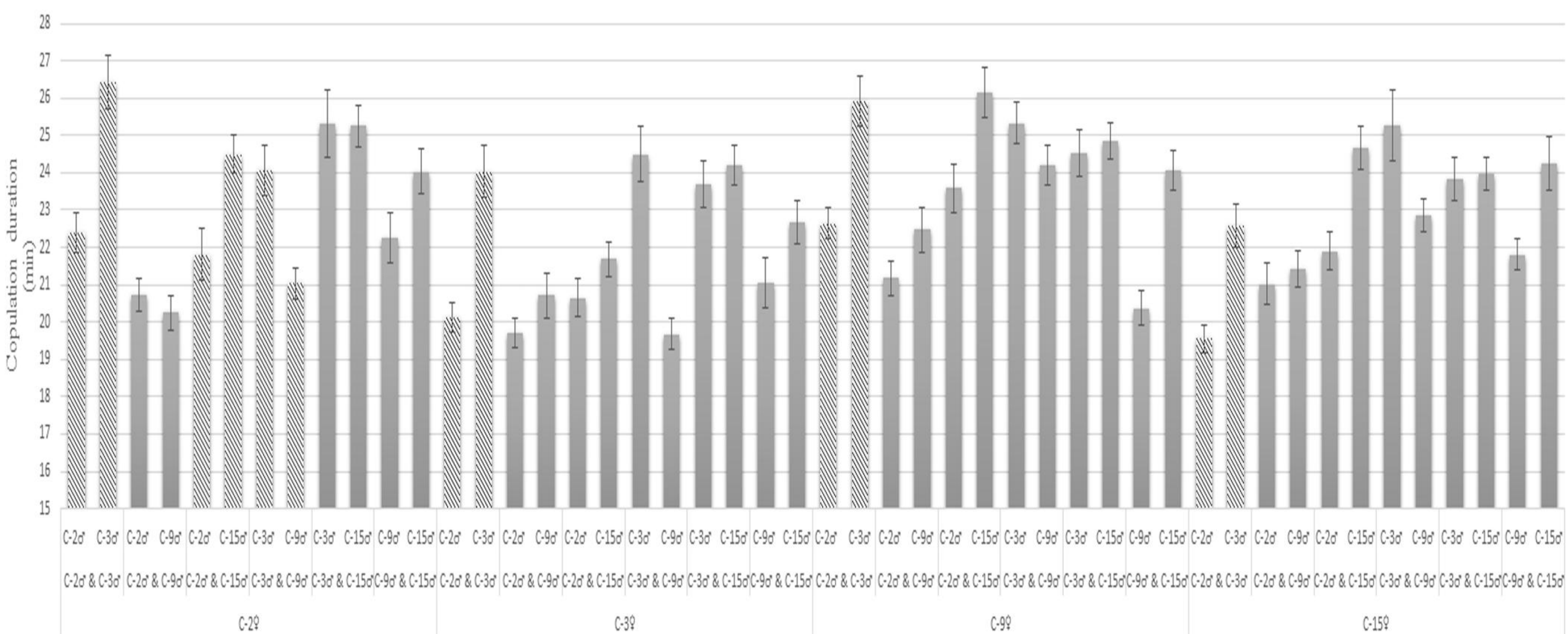
Copulation duration (min)
28
27
26
25
24
23
22
21
20
19
18
17
16
15
C-2♂ C-3♂ C-2♂ C-9♂ C-2♂ C-15♂ C-3♂ C-9♂ C-3♂ C-15♂ C-9♂ C-15♂
C-2♂ & C-3♂ C-2♂ & C-9♂ C-2♂ & C-15♂ C-3♂ & C-9♂ C-3♂ & C-15♂ C-9♂ & C-15♂
C-2♀
C-3♀
C-9♀
C-15♀

Figure 3

Tables:-

Table 1: The no choice experiments among four cytoraces of *nasuta-albomicans* complex of *Drosophila*

Crosses ♂	♀	Total no. of Crosses	No. of mating recorded N	%	χ^2	p-value
C-2	C-2	540	116	21.48	175.674	<0.001
	C-3	250	113	45.2	2.304	0.129
	C-9	551	120	21.77	175.537	<0.001
	C-15	225	114	50.66	0.040	0.841
C-3	C-2	612	111	18.13	245.987	<0.001
	C-3	297	105	35.35	25.485	<0.001
	C-9	633	103	16.27	288.039	<0.001
	C-15	574	119	20.73	196.683	<0.001
C-9	C-2	481	112	23.28	136.533	<0.001
	C-3	300	112	37.33	19.253	<0.001
	C-9	246	106	43.06	4.699	0.030
	C-15	230	114	49.56	0.017	0.895
C-15	C-2	249	108	43.37	4.373	0.037
	C-3	293	115	39.24	13.546	<0.001
	C-9	308	111	36.03	24.013	<0.001
	C-15	213	106	49.76	0.005	0.945

Table 2a: Male choice experiments revealed the preference in presence of a heterogamic mate

Crosses ♂	♀	Total no. of Crosses	No. of mating recorded N	%	Homogamic mating N	%	Heterogamic mating N	%	χ^2	P value	Isolation index
C-2	C-2, C-3	215	108	50.23	60	55.55	48	44.44	1.333	0.248	0.111
	C-2, C-9	232	104	44.82	48	46.15	56	53.84	0.615	0.433	-0.076
	C-2, C-15	195	111	56.92	58	52.25	53	47.74	1.385	0.239	0.045
C-3	C-3, C-2	301	118	39.20	76	64.40	42	35.59	9.796	0.002	0.288
	C-3, C-9	277	110	39.71	56	50.90	54	49.09	0.036	0.849	0.018
	C-3, C-15	318	108	33.96	73	67.59	35	32.40	13.37	<0.001	0.351
C-9	C-9, C-2	273	109	39.92	61	55.96	48	44.03	1.550	0.213	0.119
	C-9, C-3	211	111	52.60	68	61.26	43	38.73	5.631	0.018	-0.225
	C-9, C-15	197	106	53.80	50	47.16	56	52.83	0.340	0.560	-0.056
C-15	C-15, C-2	230	123	53.47	67	54.47	56	45.52	0.984	0.321	0.089
	C-15, C-3	237	117	49.36	55	47.00	62	52.99	0.419	0.518	-0.059
	C-15, C-9	229	106	46.28	39	36.79	67	63.20	7.396	0.007	-0.264

Table 2b: Male choice experiments revealed the preference for heterogamic mate

Crosses ♂	♀ I II	Total no. of Crosses	No. of mating recorded N	%	Heterogamic Mating I N	%	Heterogamic Mating II N	%	χ^2	P value
C-2	C-3, C-9	225	116	51.55	64	55.17	52	44.82	1.241	0.265
	C-3, C-15	169	117	69.23	82	70.08	35	29.91	18.88	<0.001
	C-9, C-15	179	115	64.24	49	42.6	66	57.39	2.513	0.113
C-3	C-2, C-9	402	113	28.1	59	52.21	54	47.78	0.221	0.638
	C-2, C-15	293	104	35.49	46	44.23	58	55.76	1.385	0.239
	C-9, C-15	327	108	33.02	49	45.37	59	54.62	0.926	0.336
C-9	C-2, C-3	162	110	67.9	34	30.9	76	69.09	16.036	<0.001
	C-2, C-15	230	110	47.82	57	51.81	53	48.18	0.145	0.703
	C-3, C-15	210	107	50.95	64	59.81	43	40.18	4.121	0.042
C-15	C-2, C-3	181	108	59.66	41	37.96	67	62.03	6.259	0.012
	C-2, C-9	242	107	44.21	54	50.46	53	49.53	0.009	0.925
	C-3, C-9	186	115	61.82	103	89.56	12	10.43	72.009	<0.001

Table 3: The effects of males on copulation duration between two females of an experimental combination

Experimental combinations						
	Choice[1]			Choice[2]		
♂	♀	Wald Chi-Square	P value	♀	Wald Chi-Square	P value
C-2	C-2	9.851	0.002	C-3	22.605	<0.001
	C-2	31.990	<0.001	C-9	21.736	<0.001
	C-2	22.338	<0.001	C-15	27.159	<0.001
	C-3	16.298	<0.001	C-9	0.641	0.423
	C-3	17.734	<0.001	C-15	19.264	<0.001
	C-9	2.733	0.098	C-15	8.583	0.003
C-3	C-2	7.503	0.006	C-3	2.138	0.144
	C-2	2.695	0.101	C-9	0.211	0.646
	C-2	0.867	0.352	C-15	3.013	0.083
	C-3	0.803	0.370	C-9	3.790	0.052
	C-3	2.870	0.090	C-15	15.226	<0.001
	C-9	2.511	0.113	C-15	0.043	0.835
C-9	C-2	0.361	0.548	C-3	3.622	0.057
	C-2	13.908	<0.001	C-9	16.607	<0.001
	C-2	6.086	0.014	C-15	6.889	0.009
	C-3	0.674	0.412	C-9	2.681	0.102
	C-3	38.161	<0.001	C-15	31.517	<0.001
	C-9	0.868	0.351	C-15	6.359	0.012
C-15	C-2	2.423	0.120	C-3	2625.909	<0.001
	C-2	0.017	0.897	C-9	2343.549	<0.001
	C-2	1.125	0.289	C-15	1825.515	<0.001
	C-3	0.022	0.882	C-9	662.349	<0.001
	C-3	0.012	0.914	C-15	1659.586	<0.001
	C-9	2.137	0.144	C-15	1175.027	<0.001

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Table 4a: Female choice experiments revealed the preference in presence of a heterogamic mate

Crosses		Total no. of Crosses	No. of mating recorded		Homogamic mating		Heterogamic mating		χ^2	P value	Isolation index
♀	♂		N	%	N	%	N	%			
C-2	C-2, C-3	229	113	49.34	78	69.02	35	30.97	16.363	<0.001	0.380
	C-2, C-9	249	126	50.60	67	53.17	59	46.82	0.508	0.476	0.063
	C-2, C-15	160	113	70.62	43	38.05	70	61.94	6.451	0.011	-0.238
C-3	C-3, C-2	196	132	67.34	36	27.27	96	72.72	27.273	<0.001	-0.454
	C-3, C-9	179	119	66.48	65	54.62	54	45.37	1.017	0.313	0.092
	C-3, C-15	165	109	66.06	43	39.44	66	60.55	4.853	0.028	-0.211
C-9	C-9, C-2	188	107	56.91	54	50.46	53	49.53	0.009	0.923	0.009
	C-9, C-3	169	113	66.86	55	48.67	58	51.32	0.080	0.778	-0.026
	C-9, C-15	159	112	70.44	56	50.0	56	50.0	0.000	1.000	0.000
C-15	C-15, C-2	168	127	75.59	65	51.18	62	48.81	0.071	0.790	0.023
	C-15, C-3	200	123	61.50	87	70.73	36	29.26	21.146	<0.001	0.414
	C-15, C-9	146	111	76.02	49	44.14	62	55.85	1.523	0.217	-0.117

Table 4b: Female choice experiments revealed the preference for heterogamic mate

Crosses ♀	♂ I II	Total no. of Crosses	No. of mating recorded N	%	Heterogamic Mating I N	%	Heterogamic Mating II N	%	χ^2	P value
C-2	C-3, C-9	214	111	51.86	29	26.12	82	73.87	25.306	<0.001
	C-3, C-15	201	101	50.24	28	27.72	73	72.27	27.252	<0.001
	C-9, C-15	157	114	72.61	55	48.24	59	51.75	0.140	0.708
C-3	C-2, C-9	183	130	71.03	77	59.23	53	40.76	4.431	0.035
	C-2, C-15	157	120	76.43	52	43.33	68	56.66	2.133	0.144
	C-9, C-15	171	129	75.43	57	44.18	72	55.81	1.744	0.187
C-9	C-2, C-3	197	106	53.8	76	71.69	30	28.3	16.036	<0.001
	C-2, C-15	191	118	61.78	64	54.23	54	45.76	0.847	0.357
	C-3, C-15	188	111	59.04	42	37.83	69	62.16	6.568	0.010
C-15	C-2, C-3	192	132	68.75	99	75	33	25	33.000	<0.001
	C-2, C-9	159	127	79.87	61	48.03	66	51.96	0.197	0.657
	C-3, C-9	188	116	61.7	32	27.58	84	72.41	23.310	<0.001

Table 5: The effects of females on copulation duration between two males of a choice combination in cytoraces

Experimental combinations						
	Choice[1]			Choice[2]		
♀	♂	Wald Chi-Square	P value	♂	Wald Chi-Square	P value
C-2	C-2	0.064	0.800	C-3	17.766	<0.001
	C-2	1.083	0.298	C-9	2.915	0.088
	C-2	10.088	0.001	C-15	0.046	0.830
	C-3	2.359	0.125	C-9	9.416	0.002
	C-3	1.819	0.177	C-15	3.148	0.076
	C-9	4.243	0.039	C-15	0.060	0.807
C-3	C-2	12.888	<0.001	C-3	2.593	0.107
	C-2	7.333	0.007	C-9	0.865	0.352
	C-2	26.491	<0.001	C-15	15.817	<0.001
	C-3	3.734	0.053	C-9	29.338	<0.001
	C-3	0.148	0.701	C-15	0.101	0.751
	C-9	10.912	0.001	C-15	2.967	0.085
C-9	C-2	0.005	0.944	C-3	14.587	<0.001
	C-2	0.115	0.734	C-9	1.964	0.161
	C-2	1.488	0.222	C-15	2.816	0.093
	C-3	12.601	<0.001	C-9	3.840	0.050
	C-3	0.552	0.458	C-15	1.711	0.191
	C-9	20.676	<0.001	C-15	0.053	0.817
C-15	C-2	20.244	<0.001	C-3	1586.655	<0.001
	C-2	0.291	0.590	C-9	1941.008	<0.001
	C-2	12.791	<0.001	C-15	1722.106	<0.001
	C-3	5.441	0.020	C-9	2820.409	<0.001
	C-3	0.032	0.859	C-15	2881.709	<0.001
	C-9	8.967	0.003	C-15	1165.882	<0.001

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