The role of sexual conflict, sexual selection and ancestry in the evolution of chemical sexual signals

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Abstract

The ability of sexual conflict to facilitate speciation has been widely anticipated in speciation research. However, mathematical models based on mating rate predict only two scenarios that result in speciation out of six possible evolutionary outcomes of sexual conflict (Gavrilets and Hayashi, 2005). There have been only two instances where experimental evolutionary studies have convincingly demonstrated the possibility of speciation linked to sexual conflict (Martin and Hosken, 2003; Syed et al., 2017). The study by (Syed et al., 2017) involving replicates of *Drosophila melanogaster* populations under differential operational sex ratio have shown that both premating and postmating-prezygotic isolation evolved in the male biased (high sexual conflict) regime but not in the female biased regime (low sexual conflict). This indicates possibility of coevolution of sexual signals such as cuticular lipids in form of precopulatory isolating mechanism since they evolve rapidly and are involved in *Drosophila* mate recognition. We studied cuticular lipid divergence in the same *Drosophila* populations undergoing reproductive isolation and found that their cuticular lipid profiles are sexually dimorphic indicating past and/or ongoing sexually antagonistic coevolution. Although male biased replicates evolved isolation in reproductive traits due to high sexual conflict, the patterns of cuticular lipid divergence in high and low sexual conflict regimes suggest that sexual selection is the dominant selection pressure rather than sexual conflict affecting the cuticular lipid trait. Although there is evidence of divergence of cuticular lipid profiles among both sexes of male biased replicates, the divergence pattern is defined by ancestry of the different replicates and cannot be unambiguously credited to sexual conflict.

Keywords*:* Sexual conflict, Sexual selection, Ancestry, Speciation, Divergence, Drosophila, Cuticular hydrocarbon, Random forest analysis, Gas chromatography

Introduction

The ability of sexual conflict to facilitate speciation has been widely anticipated in speciation research (Gavrilets, 2014). Mathematical models of sexual conflict based on mating rate envisage six possible evolutionary outcomes out of which two lead to speciation: allopatric speciation as a byproduct of sexually antagonistic coevolution (SAC) and sympatric speciation as a byproduct of divergence in the reproductive traits of females followed by the male sex (Gavrilets and Hayashi, 2005). A comparative study on groups of insect species has shown that prevalence of high sexual conflict makes a taxonomic group more speciose than those with low or no sexual conflict (Arnqvist et al., 2000). However, the support for speciation through sexual conflict is not universal and comparative studies on other groups of species have not found a similar trend (Gage et al., 2002; Morrow et al., 2003). Experimental studies that directly investigate speciation linked to sexual conflict too are inconsistent in their support for the hypothesis presumably due to the occurrence of four other possible dynamic outcomes predicted in the mathematical models that do not result in speciation (Gavrilets, 2014).

By evolving independent replicates of a dung fly population for 35 generations under monogamous (low) and polygamous (high) sexual conflict regime, (Martin and Hosken, 2003) showed that mating success between individuals of the same population was significantly higher than that of pairs consisting of males and females from different populations under polygamous regime. In the monogamous regime, there was no difference in mating success between individuals from hetero-population and same population. Recently, a study on *Drosophila melanogaster* populations using male biased (high sexual conflict) and female biased (low sexual conflict) operational sex ratio regimes showed that evolution of both premating and post mating prezygotic isolation was possible within 105 generations in the high sexual conflict regime but not in the low sexual conflict regime (Syed

et al., 2017). Contrary to these two studies, few other studies were unable to find any support for sexual conflict driven speciation leaving us to contemplate the potential of sexual conflict as an "engine of speciation" (Gage et al., 2002; Morrow et al., 2003; Plesnar-Bielak et al., 2013).

In the *Drosophila* populations under high sexual conflict regime studied by (Syed et al., 2017), assortative mating among 'within replicate' mating pairs indicates independent evolution of improved mating compatibility among individuals of the same population and reproductive incompatibilities between males and females from different replicate populations. These incompatibilities can be brought about by divergence in any of the large number of traits relevant to mating success. Reproductive isolation between the replicates also provides evidence of genetic divergence underlying mating ability among these *Drosophila* populations that is most likely a result of sexual selection either in form of male-male competition or female choice. Therefore, it remains to be seen if the *Drosophila* populations studied by (Syed et al., 2017) have also undergone divergence in secondary sexual characters that contribute to reproductive isolation among the populations under high sexual conflict regime. The current study investigates divergence in one such secondary sexual character, namely, cuticular hydrocarbon profiles using the same *Drosophila* populations that evolved reproductive isolation due to sexual conflict. Cuticular lipids form a thin waxy layer over the cuticle of an insect and can be detected over a short distance by another insect through olfactory/taste receptors present on the antennae, proboscis, maxillary palp and legs (Ferveur, 2005). Cuticular lipids in insects primarily serves the purpose of an anti-desiccating agent (Nelson, 1978) but they also have a significant secondary role as chemical cues in their reproductive biology (Ferveur and Cobb, 2009). In many studies on the genus *Drosophila*, cuticular lipid profiles have been found to act as species delimiting factor (Coyne et al., 1994; Howard and Blomquist, 2005; Jallon and David, 1987).

It is important to note that a population that is subjected to high sexual conflict, also experience high selection pressure due to sexual selection simultaneously. Previous study by (Syed et al., 2017) indicates that divergence among allopatric *Drosophila* populations in mate preference, copulation duration and sperm defense ability (P1) evolve due to sexually antagonistic coevolution that occurs within each populations. This divergence then creates reproductive isolation among these populations due to high sexual selection that appears between two populations. If cuticular lipid divergence occurs in a similar fashion, then at least males of high sexual conflict regimes will form separate groups while males of relaxed sexual conflict regimes will form a singular group. However, different reproductive traits (here cuticular lipid profiles) have the possibility of being affected differently (by sexual conflict and sexual selection combine) than the reproductive traits studied by (Syed et al., 2017) such that they show different divergence patterns altogether. For example, if high sexual conflict has weak influence on cuticular lipid composition, then sexual selection pressure will force the males of different high sexual conflict regimes to show similar cuticular lipid profiles such that they cluster together. The divergence pattern may also be influenced by independent evolution in allopatry due to random rapid mutations in cuticular lipid associated genes. In reality, it is highly possible all three factors contribute to the divergence pattern and different levels of contribution from different factors may lead to pattern not explainable by single such process. Therefore, the different possible patterns of divergence of cuticular lipids that we expect are as follows:

1. Cuticular lipid divergence as a byproduct of sexually antagonistic coevolution: In this case, males under high sexual conflict regimes will form separate groups (showing high divergence) while males under low sexual conflict regimes will form a singular group (showing no divergence). The females of the high and low sexual conflict regime may also follow the same pattern.

2. Divergence due to sexual selection: In this case, males under high sexual selection pressure will move towards an optimum point and maintain profiles that is preferred by most females whereas males under low sexual selection pressure can potentially diversify. The females under high or low sexual selection regime may diversify and may not form clusters since the selection pressure is primarily on males.
3. Divergence due to rapid mutation in allopatry: In this case, populations under both high and low sexual conflict regimes may form separate groups but the divergence pattern may be random depending on the instance of mutations.

Previous studies have also recorded dimorphism in cuticular lipid profiles between males and females that can be attributed to past sexually antagonistic coevolution and possible signatures of current sexual conflict (Bedhomme et al., 2011; Chenoweth et al., 2008; Ferveur, 2005; Savarit and Ferveur, 2002). The cuticular lipids in *Drosophila melanogaster* are known to play an important role in sexual signals that may lead to speciation through sexual selection (Chenoweth and Blows, 2003; Coyne, 1996). Hence, in the current study, an obvious possibility is that the divergence of cuticular lipid profile may potentially drive sexual selection in high sexual conflict regime and reinforce already isolated but closely related populations. We expect the replicate male biased populations that show evidence of reproductive isolation to form separate groups while the female biased replicate populations that do not show any signs of isolation in the previous study should cluster together. The difference in cuticular lipid profile of populations under high sexual conflict regime will indicate the basis for sexual selection where females of a population choose a particular bouquet of lipids presented only by males of that population. We also expect *Drosophila melanogaster* males and females to form separate groups based on cuticular lipid profiles irrespective of the population to which they belong. This is because cuticular lipid

dimorphism is thought to be associated with various mechanisms to resolve sexual conflict in two sexes by evolving independent genetic control of cuticular lipid biosynthesis (Bedhomme et al., 2011). The objectives of the present study that investigates divergence of cuticular lipids in *Drosophila melanogaster* populations maintained under high and low sexual conflict regime are as follows:

1. To characterise cuticular lipid profiles of the *Drosophila* populations maintained at differential operational sex ratio.
2. To investigate differences and variation in male and female cuticular lipids in the same *Drosophila* population.
3. To investigate divergence in cuticular lipid profiles in both the sexes of high and low sexual conflict *Drosophila* regimes.

Methods

Drosophila population maintenance

In this study, we used nine populations of *Drosophila melanogaster* belonging to 168-170 generation raised under three different selection regimes. These three selection regimes were identical in all respect, except for the adult sex-ratio. The male biased regime (henceforth M) had a 1:3 :: female:male adult sex ratio, while the female biased regime (henceforth F) had a 3:1 :: female:male adult sex ratio. The control regime (henceforth C) had a 1:1 adult sex ratio. Each regime had three replicate populations ($M_{1,2,3}$ in the M regime, $F_{1,2,3}$ in the F regime and $C_{1,2,3}$ in the C regime). All nine populations ultimately trace their ancestry to a large, laboratory adapted population, LH_{st} which has an autosomal, recessive scarlet eye-colour marker. We first derived three populations from the LH_{st} population and labeled them $C_{1,2,3}$. The $C_{1,2,3}$ populations were maintained at a 1:1 adult sex ratio for five generations. Subsequently, from each C population, we derived one population for the M regime, one for the F regime and one for the C regime. M, C and F populations with the same subscript were derived from the same C population and therefore are closely related in terms of ancestry. For example, M_1, F_1 and C_1 were derived from C_1. Moreover, during subsequent population maintenance, M, C and F populations with the same subscript are handled on the same day and therefore can be treated as statistical blocks. The details of maintenance of M, C and F populations has been described elsewhere (Nandy et al., 2013a). Briefly, these populations are maintained on a 14-day discrete generation cycle, at 25˚C and 60% relative humidity (RH), 12:12 hours light/dark cycle and standard cornmeal– molasses–yeast food in standard vials (90-mm length × 30-mm diameter). In each regime, every generation we collect virgin males and females and combine them in appropriate sex ratios. Males and females interact for around 48 hours, after which they are transferred to fresh vials for oviposition. Eggs laid are

trimmed to a density of around 140-160 eggs per vial containing 6-8 ml food. These eggs are used to start the next generation.

Generating flies for cuticular lipid extraction

Before cuticular lipid extraction from virgin M, C, and F flies, we allowed one generation of "standardization" or common-garden rearing at a 1:1 sex ratio following (Nandy et al., 2013a) to account for any potential non-genetic parental effect (Rose, 1984). In order to generate flies for cuticular lipid extraction, we collected eggs from "standardised" flies. On the 10th day after egg collection from the standardized M, C, and F populations, we collected flies as virgins within 6 hours of eclosion under light CO_2 anesthesia. The flies were isolated by keeping each of them singly in a vial for 2 days. On 12th day (ensuring flies of same age to minimize age-specific variation of cuticular lipids) the cuticular lipids extraction assay was conducted.

Cuticular lipid profiling

Cuticular lipid extraction

We transferred each virgin individual to a 1.5 ml gas chromatography (GC) vial without anesthetising it. We then poured a 200 µl Hexane solvent containing 10ng/µl Pentadecane ($C_{15}H_{32}$, internal standard) into the GC vials. The vials were left undisturbed for 4 minutes and then vortexed for 1 minute. We removed the dead individual from each GC vial and subsequently left the hexane to dry out. The extracted samples in the GC vials were then stored at 4˚C until gas chromatography was performed. Cuticular lipids were extracted from the male and female *Drosophila* flies belonging to all blocks of MCF population [n for M = 119 (60 males:59 females), n for F = 115 (58 males:57 females) and n for C = 118 (59 males:59 females)].

Gas chromatography

Before gas chromatography, the dried extract adhering to the glass GC vials was brought to solution by pipetting 20 µl Hexane and vortexed for 30 sec. We injected 2µl of this solution manually into splitless Shimadzu GC-2010 Plus instrument fitted with a Restek Rxi-5Sil MS (30m x 0.25µm df x 0.25mm ID) capillary column. The 24.88 minutes' temperature program was set to increase from initial 57˚C held for 1.1 minutes to 190˚C held for 1.2 minutes at 100˚C per minute. The temperature increased from 190˚C to 270˚C at 5˚C per minute and finally raised to 300˚C at 120˚C per minute with hold time of 5 minutes at 300˚C (Bedhomme et al., 2011). The carrier gas was Nitrogen with column flow rate fixed at 2ml per minute.

Cuticular lipid identification

Before gas chromatography of *Drosophila* samples, a chromatographic run was performed with a mix of known straight chained alkane standards (C_{10} to C_{40}) increasing in even numbers of Carbon. This chromatogram was used to measure Kovats Retention Index (KI) for all relevant unknown sample peaks by referring to retention time for internal standard (Pentadecane: C_{15}) and hydrocarbon compounds in the standard mix. The KI was used to identify cuticular lipid peaks based on already published KIs of cuticular lipids.

Random Forest analysis

Multivariate analysis like principal component analysis and discriminant function analysis are generally used in studies that investigates group patterns within samples based on cuticular profiles (Kather and Martin, 2012). However, these statistical techniques are adversely impacted by small sample-size(n):variables(p), non-independence of cuticular lipids in a sample (since many cuticular lipids share common biochemical pathways) and disproportionate effects of cuticular lipids found in trace amounts (Martin and Drijfhout, 2009). To overcome this drawbacks of traditional techniques, we used Random Forest

analysis (R package “Party”) that performs well under “small n large p” conditions, unaffected by highly correlated variables and is unbiased towards the nature of variables used (Strobl et al., 2009a; Strobl et al., 2009b). Random Forest Analysis is a machine learning algorithm that forms numerous classification schemes without prior expectation of the data structure by repeated subsampling of the data and random selection of a few predictor variables (Strobl et al., 2009b). Using these classification schemes (also called base learners), individuals are reassigned their identity based on their probability to appear as a particular group in the base learners. When these new identities of the samples are compared to their already known group identity, we calculate the percentage correct prediction of individual identities from Random Forest analysis. Based on the probabilities of individual identities, proximity analysis (identical to similarity-dissimilarity matrix/ correlation matrix in traditional multivariate analysis) gives us a measure of samples’ group affinity. Multidimensional scaling (identical to Principal Component Analysis) of the proximity matrix is then illustrated in a scatterplot to show how experimental individuals group with respect to each other.

We performed Random Forest analysis using standardized area under the peak value for all relevant peaks. All peaks that consistently appeared in the samples when compared with a blank chromatographic run (using Hexane that was used in the extraction process) were considered relevant to this study. The area under the peak value was calculated by drawing a baseline to each relevant peak using the Shimadzu GCSolutions software. We manually checked the integrated peaks in all the sample chromatograms for any irregularities. Seed (a random number) was set to 1220. We checked the stability of the classification scheme by increasing the number of subsampling events gradually from 5000 to 20000 and decided to set the number of trees (ntree) parameter in the analysis at 15000. The number of variables (mtry = 6) to be used in the analysis was fixed to rounded square root of the number of

predictor variables (in the present study, the number of peaks analysed in chromatograms) in the data (Strobl et al., 2009b). Multi-dimensional scaling (MDS) of proximity matrix was used to produce two dimensional scatterplots showing clustering of individuals.

Results

Results from gas chromatography

We identified a total of 44 peaks that consistently appeared in MCF chromatograms by comparing their calculated Kovats Retention Index (KI) (see Table S1) to already published KIs (calculated using DB-5 column as in the present study) in literature on insect cuticular lipids (Dembeck et al., 2015; Everaerts et al., 2010; Fockink et al., 2013; Gries et al., 2003; Gries et al., 2004; Lockey, 1976; Lockey, 1985b; Marques et al., 2000; Pavković-Lučić et al., 2016; Steiger et al., 2007; Susset et al., 2013; Zhang et al., 2014). Out of the 44 peaks, 37 were cuticular hydrocarbons (CHCs), 3 belonged to other chemical groups found on insect cuticle and 4 were not identified. Most of the CHCs had hydrocarbons chain lengths between C_{20} and C_{30}. There are also isomeric forms of few alkenes (such as Heneicosene, Pentacosene, Heptacosadiene, etc.) and their corresponding alkanes and methyl alkanes that appear as 4-5 tightly placed bouquet of peaks on the chromatogram as well.

The area under peak data of the 44 peaks in each of the 352 samples was standardized by dividing the peak value with that of internal standard (Pentadecane) from respective chromatograms. Each standardized value was then multiplied by 10^5 to enable us to use more significant figures in further statistical tests. Male and female chromatographic profiles significantly differed in relative abundance of 35 peaks out of the 44 identified peaks based on Welch two sample T test ($p<0.05$). Peak 19 (Pentacosene) that is always present in males was totally absent in females. Peak 33 (9,13-Heptacosadiene) which was generally absent from male chromatograms were also absent in few females. Apart from Peak 19 and Peak 33, all other peaks appeared consistently in all female samples while except Peak 33 and Peak 39 (Trimethyloctacosane), all peaks appeared consistently in all male chromatograms (see Table S1).

Results from Random Forest analysis

We used 37 peaks out of 44 cuticular lipids for the Random Forest analysis. Peak 15, 25-29 and 42 were ambiguous since they consistently appeared as more than one peak. As indicated in the Table S1, MDS of proximity matrix showed sexes formed distinct groups in *Drosophila* M, C and F populations with 100 % correct sex identification in random forest analysis. In C population, Random Forest analysis produced correct prediction of individual block affinity equal to 93.2%. It also shows C males are more dispersed than females (Fig. 1). Since C populations are derived from LH_{st} and, M and F populations are derived from the C populations, this pattern (Fig. 1) can be considered as the ancestral pattern and is subsequently used as reference pattern to that we see in M and F. In the same analysis, the M females are more dispersed than the M males with 91.6 % correct identification of block-affinity of the M individuals (Fig. 2). This means due to sexual selection in M regime, M males have evolved CHC profiles which are more similar to each other than M females whose CHC profiles have diverged becoming more dissimilar and variable. In F population, we see an opposite trend where the males are dispersed more than the females with 90.4 % correct identification of block-affinity of the F individuals (Fig. 3). This pattern (Fig. 3) also indicate that relaxation of sexual selection has led to diversification of male CHC profiles such that they are more dissimilar to each other than what we find in M regime. Cluster of C females are, however, looser than those in F population which means F females have evolved similar CHC profiles among themselves due to some indirect mechanism that is not obvious.

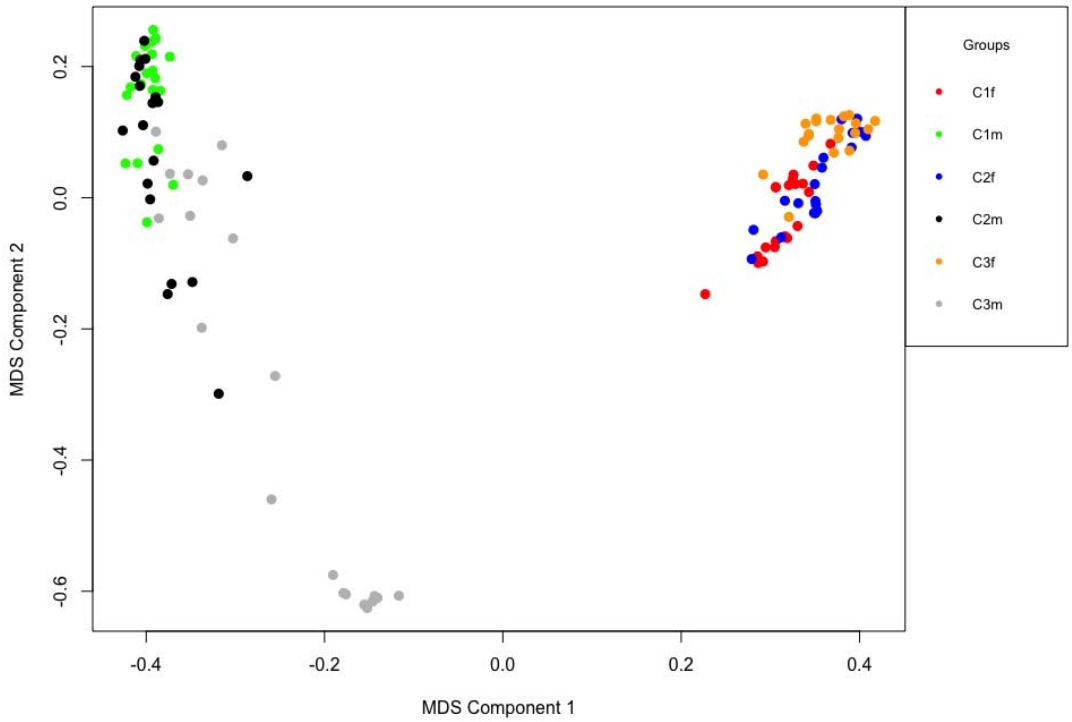

Fig. 1 Multi-dimensional scaling (MDS) of the proximity matrix of C individuals ($C_{1/2/3}$ are C blocks, m= males and f= females)

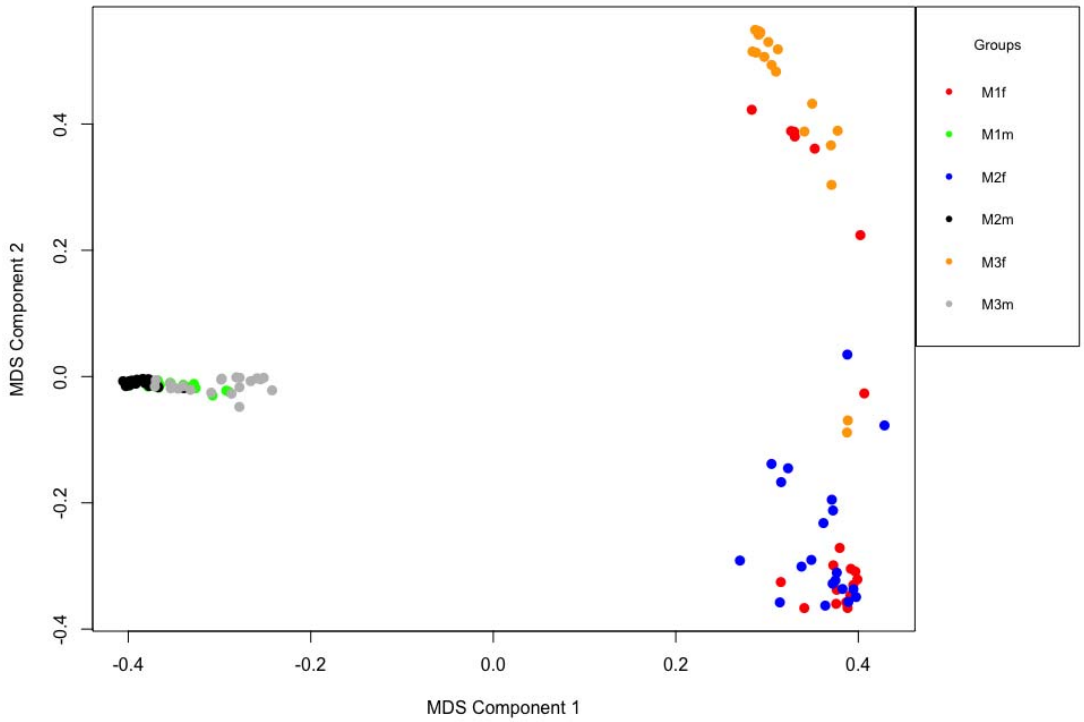

Fig. 2 Multi-dimensional scaling (MDS) of the proximity matrix of M individuals ($M_{1/2/3}$ are M blocks, m= males and f= females)

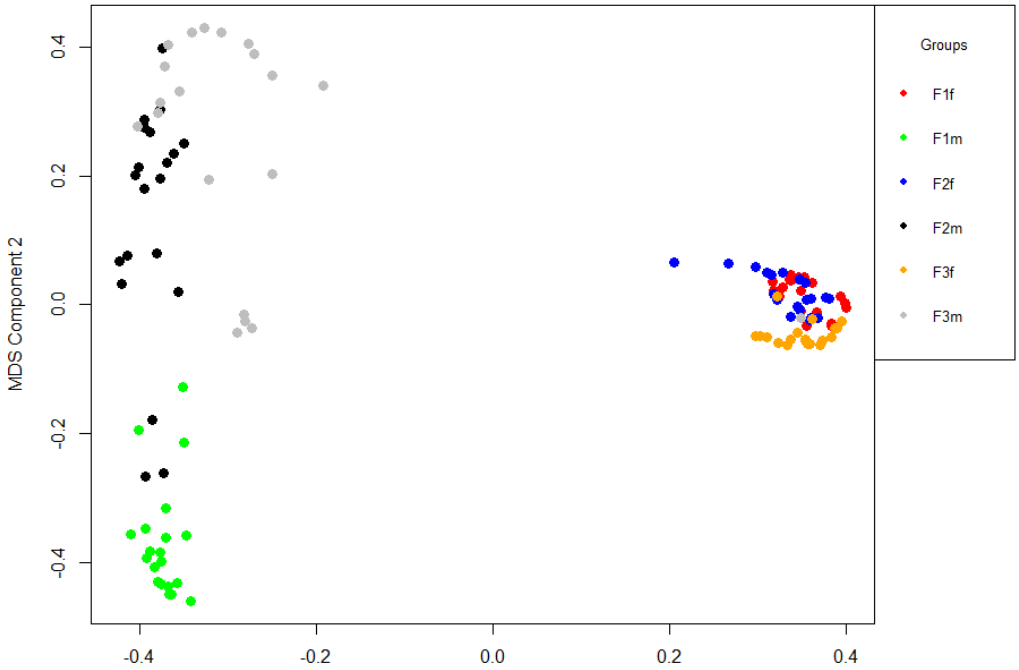

Fig. 3 Multi-dimensional scaling (MDS) of the proximity matrix of F individuals ($F_{1/2/3}$ are F blocks, m= males and f= females)

When we used males from M with their block identities and F population as a whole, Random Forest analysis predicted the group affinity of M and F males with 66.9% correct identification. Upon plotting MDS component 1 against MDS component 2 to examine divergence of males of M blocks with respect to F males, we found that M blocks formed separate groups with few overlaps among themselves. However, F males did not group separately from the M blocks and overlapped substantially with all M blocks (Fig. 4). Upon closer examination using block identity of F males (not shown), we found that most F males that overlapped with different M blocks also belonged to the corresponding blocks in F i.e. there seems to be a strong block wise grouping pattern in males which may be a result of some ancestral condition. In this analyse, using block identity of M and F males, the correct prediction of M and F males by Random Forest analysis improved to 90.7 %.

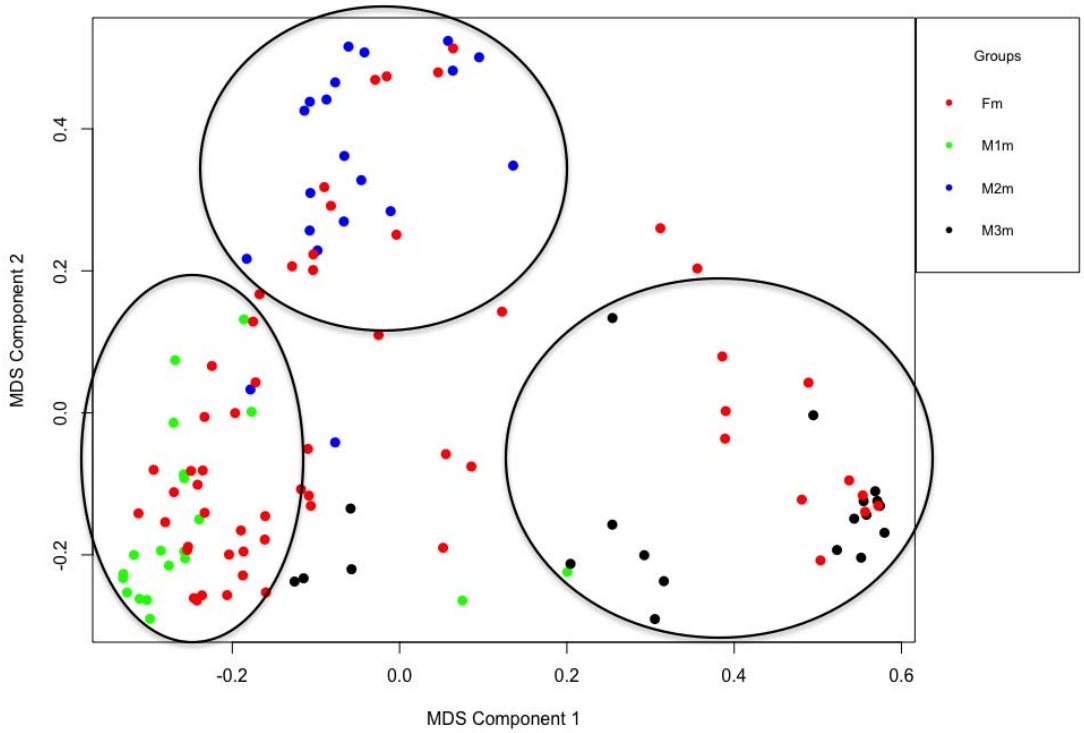

Fig. 4 MDS of proximity matrix derived from Random Forest analysis of males from 3 separate M blocks and F population as single group

In Random Forest analysis involving females of M blocks and F population as a single group, we could identify M and F females with 72.4% correct prediction of M blocks and F regime. MDS component 1 versus MDS component 2 plot revealed that M_3 females formed a separate group with few overlaps while $M_{1,2}$ appeared to form a single group. We again find considerable overlap of F females on all M blocks (Fig. 5) but they appear to form a separate group away from all the M blocks. When F blocks were used instead of F females as a single population, Random Forest analysis identified the individuals into blocks with 84.5 % correct prediction.

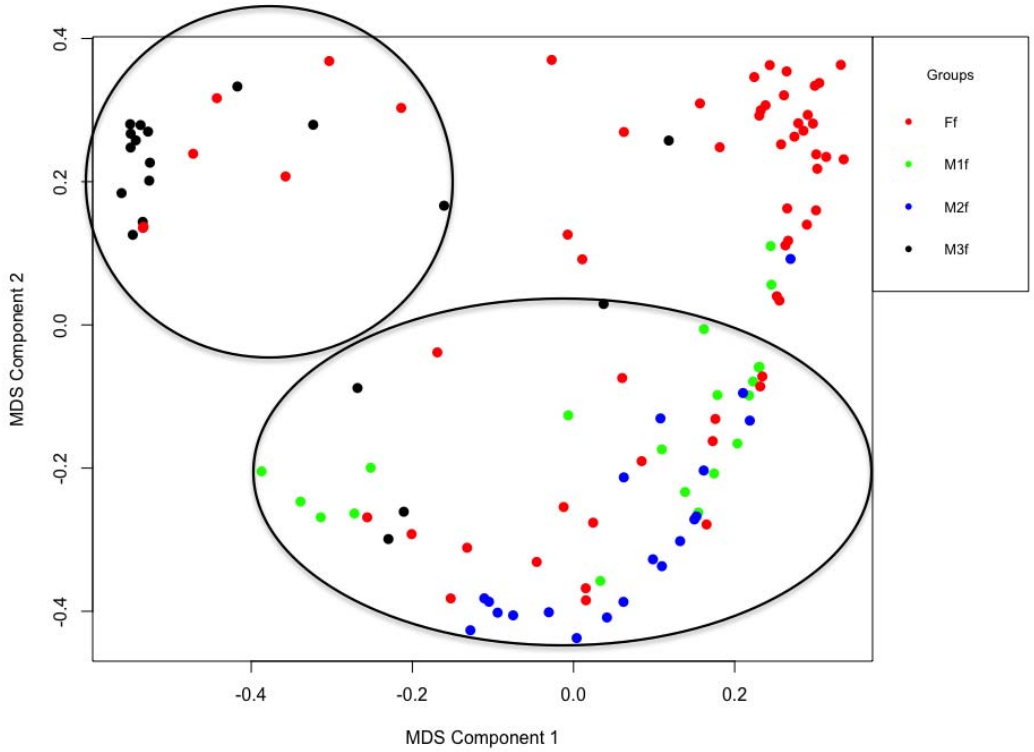

Fig. 5 MDS of proximity matrix derived from Random Forest analysis of females from 3 separate M blocks and F population as single group

Discussion

Characterisation of cuticular lipids

Cuticular lipids that are found on insect cuticle consists of variety of chemical compounds, namely, alcohols, aldehydes, esters, glycerides, fatty acids, sterols and hydrocarbons (Lockey, 1985a). Hydrocarbons that form the bulk of the cuticular lipids (Bedhomme et al., 2011) and prevent desiccation (Nelson, 1978) are also known to have secondary role as chemical cues. We found 44 cuticular lipids in *Drosophila melanogaster* population under the current study, out of which 37 peaks belonged to hydrocarbon class. The CHCs (alkanes, alkenes and methyl alkanes) of *Drosophila* usually have chain length between C_{20} to C_{40}. They exhibit great variety in number and position of double bonds (Ferveur, 2005). Similar trend was noticed among CHCs in the current study (see Table S1). The cuticular lipid profiles are highly dimorphic in *Drosophila* with known genetic basis for behavioural isolation (Fang et al., 2002) and independent links to sex determination genes in both sexes (Bedhomme et al., 2011; Ferveur, 2005; Foley et al., 2006). As shown in Table S1, difference between sexes over 36 cuticular lipids (32 hydrocarbons + 3 unidentified lipids + 1 alcohol) were statistically significant. It may be noted that peak 19 (Pentacosene) is completely absent in females and peak 33 (9,13-Heptacosadiene) is mostly absent in males compared to females. Contact through different body parts provides information about sex, mating status, age, etc. and therefore plays an immense role in reproductive biology of an insect. Such categorical difference in cuticular lipid profiles of *Drosophila* males and females in the current study indicates possible incidence of sexual conflict in their evolutionary past as shown by (Bedhomme et al., 2011) and resultant prevalence of mate choice in their reproductive biology. Isolation due to divergent reproductive behavior evident among the M populations is possibly due to sexual selection in form of mate choice (Syed et al., 2017). Since cuticular lipid profile in *Drosophila* sp. is dimorphic, one possible mechanism for this

reproductive isolation among M replicates could be mate choice over the divergent cuticular lipid profiles.

Variation of cuticular lipids in sexes of *Drosophila*

Experimental studies and comparative analysis of taxonomic groups suggest that speciation is possible under sexual conflict. The mathematical models support this possibility but also suggest that dynamics of evolution under sexual conflict is quite varied and stochastic to a point that evolution of reproductive isolation in allopatry is only one of the several other outcomes (Gavrilets, 2014). Apart from speciation, a population under sexual conflict may follow a different evolutionary stable strategy to resolve the conflict between sexes. For example, there might be an extensive diversification of female traits with respect to male traits (Buridan's ass regime) such that males need to adapt to the variability in such traits and are caught in limbo unable to increase its own fitness in the population as a whole. This is relevant to the results from the current study where multivariate analysis show M females are more dispersed and M males form a tight group with respect to their CHC profile. If we compare this result with the CHC profile of males and females of C regime, we see that the CHC profiles of M females have diverged while the CHC profile of M males have converged considerably. This can potentially indicate that under increased sexual selection, males are pushed towards a common optimum point in terms of CHC profile. On the contrary, F males and F females show a diametrically opposite pattern to that of the M regime. Under low sexual selection it appears that selection pressure is possibly weaker and hence the males can diversify and/or maintain their cuticular lipid variation as seen in the control group. The F females appear to be more tightly packed in terms of their CHC profile (contrary to M females) than C population which indicates that F females are probably under selection pressure to maintain similarity of their cuticular lipid profiles. The reason for this is not obvious and may be a result of correlated evolution in some other trait affected by sexual

selection. F females may also be affected by sexual selection due to altered sex ratio where they have to maintain identical profile to be acceptable to males that are fewer in number. To conclude, the M and F individuals appear to be significantly affected by sexual selection rather than sexual conflict that appears to have less or no effect on secondary sexual characters such as cuticular lipids. We may also conclude that since cuticular lipids form a basis for mate recognition, M males under high sexual selection tend to keep their cuticular lipid profiles similar to be acceptable by the limited number of females in the male biased regime. The same may also be true for the females of female biased regimes or their CHC divergence pattern may be by-product of sexual selection pressure on any other hitherto unknown trait.

Pattern of cuticular lipid divergence in M regime compared to F regime

The third objective of the study was to find out whether males and females of M regime that are under high sexual conflict formed separate groups compared to F regime that are under weak sexual conflict. Cuticular lipid profiles are species specific and while different species profiles may differ in absence or presence of certain cuticular lipids, closely related species often differ in relative abundance of the constituent lipids (Kather and Martin, 2012). In the current study, we find that all investigated cuticular lipid peaks occur in M, F and C population indicating that divergence, if any, would be due to relative abundance of the studied peaks. If reproductive isolation at the level of conspecific identity based on cuticular lipids is strong under high sexual conflict, we expect the groups undergoing reproductive isolation to form separate clusters. Since reproductive isolation is evolving in M regime in terms of mating behavior while F regime is not, it may be expected that cuticular lipids have diverged in M population but not in F population. The Random Forest analysis of the three replicate M populations shows that they form 3 distinct groups in case of males (Fig. 4). However, F males do not form a single group and seems to be dispersed all over (Fig. 4).

This pattern corroborates with previous pattern (Fig. 3) where F males seems more diversified than the F females. This divergence pattern becomes interesting when we take into consideration that M males also show overall similarity in cuticular lipid profiles compared to females (Fig. 2). This indicates that even though M males display similar profile in general they also keep their ancestral identity intact. Besides, few F males that overlap with males from different M blocks mostly belonged to the same blocks i.e. F1 males overlapped with M1 males etc. It is to be noted here that C population was derived from LH_{st} population while M and F blocks have been derived from the C population maintained in triplicates. Although M, C and F population were established using a large population size (n ~ 2250, N_e= 450; (Nandy et al., 2013b)), there seems to be a founder effect especially affecting the cuticular lipid profiles of the MCF population. Overall, block effect (since $M_{1,2,3}$ and $F_{1,2,3}$ are derived from $C_{1,2,3}$ respectively where subscript denotes common ancestry) seems to be contributing to the divergence pattern with some effect of sexual selection on these lipid profiles. M females do not show clear distinction between block 2 and block 3 presumably due to selection pressure acting against ancestral grouping pattern (due to founder effect) on them to maintain distinct cuticular lipid identity (Fig. 5). However, F females do loosely appear to form a separate group away from M females which may be due to sexual conflict. However, there is substantial overlap of F females on M females following the effect of ancestry that we also see in M and F males. (Ferveur and Cobb, 2009) suggest that the kind of variability in the CHC profiles of different populations under different selection pressure (as is observed in this study) is possibly due to different initial and undergoing evolutionary mechanism. The founder effect on CHC profiles of MCF population during initial establishment was not predicted simply because the populations were established using a large number of individuals. However, the maintenance of block identity appears to be highly influenced by the ancestral pattern. Fact that this definite identity of M

and F blocks are breaking down is possibly because of high sexual selection pressure on these populations. On the other hand, overall effect of sexual conflict on the cuticular lipid profiles seems minimal. This study is an example of different reproductive traits being under different levels of selection pressure such that the pattern predicted due to isolation based on sexual conflict involving mate preference, copulation duration and sperm defense ability is not reflected on the mate recognition cue such as cuticular lipids. The fact that individuals from M blocks choose individuals from the same block during mating can thus be attributed to female choice of conspecific CHC profiles which in turn is the result of ancestry rather than divergence due to sexual conflict. On the other hand, F males and F females seems to be breaking out of their initial cause of divergence (i.e. ancestry) and forming predominantly separate single groups due to low sexual conflict and heightened sexual selection. It is possible that given more time, as the reproductive isolation will become more consolidated to initiate speciation, these patterns will get sorted out more discretely with each trait involved following their specific evolutionary trajectory.

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Conflict of Interest Declaration: The authors declare no competing interest.

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