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ScienceDirect
Insect Biochemistry and Molecular Biology 38 (2008) 244–255
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Evolution of a desaturase involved in female pheromonal cuticular hydrocarbon biosynthesis and courtship behavior in *Drosophila* ☆

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Received 27 September 2007; received in revised form 8 November 2007; accepted 15 November 2007

Abstract

Drosophila species exhibit polymorphism in female pheromonal cuticular hydrocarbons, with 7-monoenes produced in *Drosophila simulans* and 7,11-dienes in most populations of *Drosophila melanogaster* (5,9-dienes in several African populations). A female-biased desaturase, *desatF*, expressed only in *D. melanogaster* is involved in the synthesis of 7,11-dienes. We investigated the role of *desatF* in 5,9-diene flies. We constructed a 5,9-diene strain knock-down for *desatF* and showed that *desatF* is involved in 5,9-diene formation. We also studied *D. melanogaster*/*D. simulans* hybrids. These hybrid females produced dienes and received normal courtship from *D. melanogaster* males, but copulation success was reduced. With *D. simulans* males, courtship was decreased and no copulation occurred. Hybrids with a chromosomal deletion of the *D. melanogaster desatF* gene had no dienes and received normal courtship from *D. simulans* males; depending on the *D. simulans* parental strain, 7–19% of them succeeded in mating. *D. simulans desatF* promoter region shows 21–23% gaps and 86–89% identity with *D. melanogaster* promoter region, the coding region 93–94% identity, depending on the strain. These differences could explain the functional polymorphism of *desatF* observed between both species, contributing to different cuticular hydrocarbon profiles, that constitute an effective barrier between species.

Keywords: Pheromones; Desaturase; *Drosophila*; Promoter; Evolution

1. Introduction

Understanding sexual isolation that contributes to speciation is one of the most important—yet unsolved-issues in speciation biology. Mate recognition and courtship are the first steps that may lead to sexual isolation. In Drosophila, active courtship behavior is performed by the male and is composed of stereotypic sequences that include orientation of the male toward the female, tapping of the female abdomen by his forelegs, performing a courtship song by wing vibration, licking of the female's genitalia by his proboscis and eventually, copulation (Hall, 1994; Greenspan, 1995). Drosophila courtship is largely dependent on female pheromones, although visual and auditory signals are also involved (Antony and Jallon, 1982). Pheromones are long-chain hydrocarbons secreted by the epidermis and act by contact or at short distances (Antony et al., 1985). In *Drosophila simulans* and *Drosophila melanogaster* the predominant female hydrocarbons have been shown to induce courtship by conspecific males and also to be important for species recognition (Jallon, 1984; Coyne and Oyama, 1995).

D. simulans and *D. melanogaster* are closely related species, show a worldwide geographic distribution and occupy almost the same ecological niche (David and Tsacas, 1981). They differ markedly in their profiles of long-chain cuticular hydrocarbons (HC; see Fig. 1): *D. simulans* is sexually monomorphic, with large quantities of 7-tricosene (7-T, 23:1) and 7-pentacosene (7-P, 25:1) in

☆ Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AJ271414, AM773238, AM773239, AM773240, AM773241, AM773627.

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doi:10.1016/j.ibmb.2007.11.005

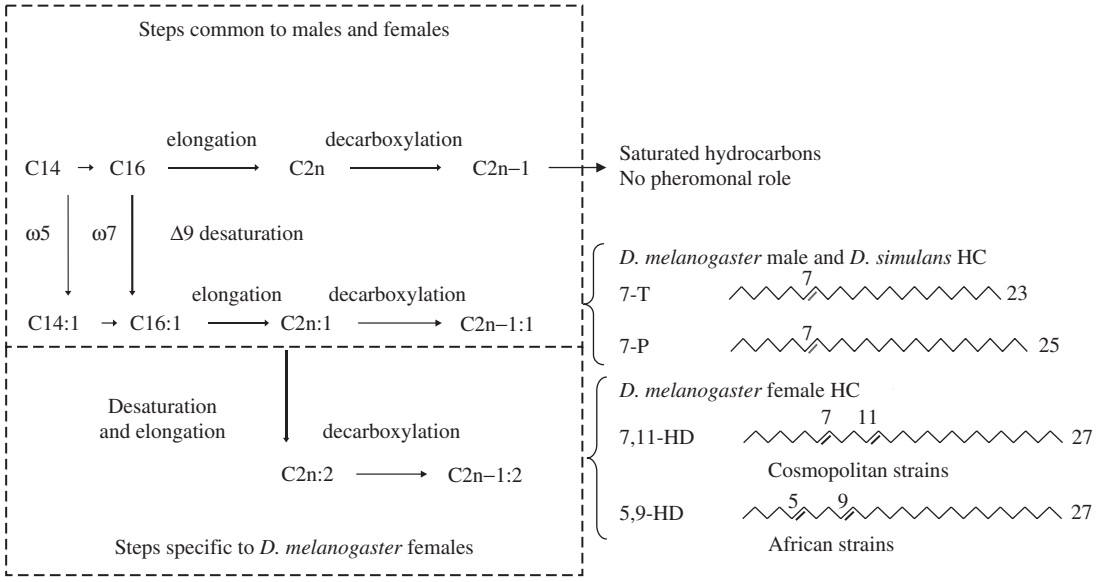

Fig. 1. Biosynthesis and main cuticular hydrocarbons (HC) in *D. simulans* and *D. melanogaster*. The desaturation of palmitic acid is performed by Desat1 and leads to ω7 fatty acids and HC insaturated in position 7. Desat2, expressed in 5,9-morph females, acts on myristic acid and leads to ω5 fatty acids. The steps specific to *D. melanogaster* females involve a female-specific desaturase (DesatF) and elongase (EloF).

both sexes. *D. melanogaster*, on the other hand, is sexually dimorphic, with males having large amounts of 7-T and 7-P and females being characterized by the predominance of dienes, usually 7,11-heptacosadiene (7,11-HD, 27:2) and 7,11-nonacosadiene (7,11-ND, 29:2) (Jallon and David, 1987). 7-T has been shown to induce wing vibration in *D. simulans* and to inhibit male courtship in *D. melanogaster*, while 7,11-HD, the major female hydrocarbon in *D. melanogaster*, shows the highest pheromonal activity toward conspecific males (Jallon, 1984). Other hydrocarbons, such as 7,11-nonacosadiene (7,11-ND, 29:2), 7-P and 7-heptacosene (7-H, 27:1) were also found to have less but significant behavioral activity toward *D. melanogaster* males (Antony et al., 1985; Ferveur and Sureau, 1996). The 7,11-dienes, specific to *D. melanogaster* females, strongly repress mating by *D. simulans* males (Coyne et al., 1994; Coyne and Oyama, 1995; Savarit et al., 1999). There is also a geographical polymorphism with female *D. melanogaster* pheromones: females from central-southern Africa and those from the rest of the world carry different types of pheromones, which differ only in the position of the unsaturations: 5,9-heptacosadiene (5,9-HD, 27:2) in African females and 7,11-HD in cosmopolitan ones (Jallon and Péchiné, 1989; Ferveur et al., 1996). The role of 5,9-HD has not been thoroughly investigated, although 5,9-HD seems less potent than 7,11-HD in inducing Canton-S (7,11-HD morph) male wing vibration (Arienti and Jallon, unpublished data).

The production of 7-monoenes and of the first double bond of 7,11-dienes is controlled by a desaturase gene, *desat1*, which introduces a double bond in position ω7 in the precursor fatty acids (Wicker-Thomas et al., 1997; Dallerac et al., 2000). The (5,9)/(7,11) difference has been mapped to a single locus close to the *desat1* gene (Coyne et al., 1999) and is due to the presence of a second desaturase gene, *desat2*, responsible for the synthesis of ω5 precursor fatty acids and expressed only in 5,9-HD females (Dallerac et al., 2000). The gene is present in the genome of both pheromonal morphs, but the loss of the promoter in the *desat2* gene has resulted in the 7,11-morph among cosmopolitan flies (Takahashi et al., 2001). The formation of the second double bond in position 11, specific to *D. melanogaster* females, is due to a second desaturase gene, *desatF*, also expressed in females of the 5,9-morph (Chertemps et al., 2006). *RNAi* knock-down of *desatF* in *D. melanogaster* females of the cosmopolitan type led to a dramatic decrease in 7,11-dienes and increase in 7-monoenes, showing that *desatF* is involved in the second desaturation step. The production of 7,11-dienes however was not completely absent, reaching about 5% of the total hydrocarbons (instead of about 40% in control females) and might be due to a low expression of *desatF* in *RNAi* knock-down flies, although *desatF* expression was not detectable by PCR (Chertemps et al., 2006). This suggests two possibilities: either the expression of *desatF* persisted to a small level in *desatF RNAi* females, or *desatF* is not the sole gene involved in diene hydrocarbon synthesis. To address these alternatives we took advantage of the possibility of making hybrids between *D. melanogaster* and *D. simulans*. We investigated pheromone production in hybrid females resulting from the cross between *D. simulans* males and *D. melanogaster* females, either wild-type or carrying a chromosomal deletion including *desatF* gene (the deletion is lethal when homozygous). We also tested these hybrid females toward parent males to evaluate the impact of diene production on courtship.

Regarding the geographic polymorphism in *D. melanogaster*, we considered if *desatF* was also involved in

5,9-diene production by the African morph. To answer this question, we constructed a line derived from African flies (5,9-morph), carrying a *P-GAL4* element targeting expression in fat body and oenocytes and we crossed this line with a *UAS-desatF RNAi* line to analyze the impact of *desatF* knock-down on 5,9-diene production.

As the *desatF* gene is present in *D. simulans* genome but not expressed in this species, we sequenced the promoter regions of *desatF* in two *D. simulans* strains and two *D. melanogaster* strains to see whether differences in the promoter region could explain the lack of *desatF* expression in *D. simulans*. Gene analysis shows that DesatF protein sequences show about 8% divergence between both species and *desatF* promoter sequences have evolved more rapidly among strains and species.

The results show that *desatF* is the only desaturase gene involved in the second desaturation step leading to diene pheromones and is capable of synthesizing both 7,11- and 5,9-dienes. As in our experiments, *D. simulans* males could mate with hybrid females only if these were devoid of 7,11-dienes, *desatF* might have played an important role, the loss of its function contributing to different hydrocarbon profiles and to sexual isolation.

2. Materials and methods

2.1. Drosophila strains and species

Flies were reared on standard yeast/cornmeal/agar media at 25 °C with a 12:12 h light/dark cycle. Experiments were performed on 4-day-old flies. The abbreviations of wild-type and mutant strains used in this study are indicated in Table 1.

The two *D. melanogaster* strains, Canton-S, characterized by a high 7,11-HD phenotype and Tai, characterized by a high 5,9-HD phenotype (Jallon and Péchiné, 1989) have been described previously (Dallerac et al., 2000). The two *D. simulans* strains, Seychelles, characterized by a high 7-T phenotype, and Yaoundé, characterized by a high 7-P phenotype have been described elsewhere (Rouault et al., 2004).

The *RNAi* line, *UAS-RNAi desatF/Cy*, was constructed and described previously (Chertemps et al., 2006).

The mutant *D. melanogaster* strains were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN) and the Kyoto Drosophila Stock Center (Kyoto Institute of Technology, Kyoto, Japan):

- Df(3L)desatF: *Df(3L)lxd6/TM3* [Df (067E05-07;068C0-2-04)].
- *OK72-GAL4*: w^{*}; P{w[mW.hs] = GawB}OK72. This strain was shown to target expression in fat body and oenocytes (Chertemps et al., 2006).

2.2. Drosophila genetics

2.2.1. Construction of a 5,9-HD morph, desatF RNAi line

A *w*; *OK72-GAL4*; Tai line was constructed by successive crosses. The 5,9-HD morph is due to *desat2* gene, located on the third chromosome. Females of this line are homozygous for *desat2* and therefore of 5,9-HD morph. They were crossed with w; *UAS-RNAi desatF*/Cy males to give progeny of two different genotypes: *w*; *OK72-GAL4/Cy*; Tai/CS, which has both 5,9-HD and 7,11-HD, because *desat2* is expressed and is partially dominant in 7,11-HD/5,9-HD hybrids (Coyne et al., 1999). The other genotype: *w*; *OK72-GAL4/UAS-RNAi desatF*; Tai/CS expresses *desatF RNAi* under the *OK72-GAL4* promoter.

2.2.2. Interspecific crosses

Hybrid females were obtained by crossing *D. melanogaster* females with *D. simulans* males. In these conditions only sterile female hybrids were produced (Sturtevant, 1920). Dmel Df(3L)desatF females and Dsim Sey or Dsim YB males were used as parental strains, producing offspring with two different phenotypes: *TM3* (noted Dmel desatF+/Dsim Sey or Dmel desatF+/Dsim YB) and + (noted Dmel desatF−/Dsim Sey or Dmel desatF−/Dsim YB). The former females had one dose of functional *desatF* (brought by *TM3*), whereas the latter females had no functional *desatF* (due to the Df(3L)desatF deletion).

Table 1
Species and strains used in the study

Abbreviation	Species/strain	Geographic origin	Genotype
Dmel CS	*D. melanogaster*/Canton-S	Ohio (USA)	Dmel/wild-type
Dmel Tai	*D. melanogaster*/Tai	Ivory Coast	Dmel/wild-type
Dmel BDGP	*D. melanogaster*/14021.0231.36		y[1] oc[R3.2]; Gr22b[1] Gr22d[1] cn[1] CG33964[R4.2] bw[1] sp[1]; LysC[1] lab[R4.2] MstProx[1] GstD5[1] Rh6[1]
Dsim Sey	*D. simulans*/Seychelles	Seychelles	Dsim/wild-type
Dsim YB	*D. simulans*/Yaoundé	Yaounde (Cameroun)	Dsim/wild-type
Dsim WUG	*D. simulans*/14021.0251.195	North America	Dsim/w^{501}
Dsec BI	*D. sechellia*/14021.0248.25	Seychelles	Dsec/wild-type
Dmel Df(3L)desatF	*D. melanogaster*/*Df(3L)lxd6*		*Df(3L)lxd6/TM3*
Dmel TM3	*D. melanogaster*/*TM3*		Dmel/*TM3*

2.3. Hydrocarbon analyses

Cuticular hydrocarbons were extracted from flies and analyzed by gas chromatography (GC) as described previously (Dallerac et al., 2000). All data are presented as mean percentages of hydrocarbons ($n = 10$ for all tests). Only the hydrocarbons which show variations are represented.

2.4. Observation of courtship and copulation

In these experiments, one 4-day-old virgin female (control, mutant or hybrid) was tested with one 4-day-old wild-type (Dmel CS, Dsim Sey or Dsim YB) male, as described (Jallon and Hotta, 1979). Three courtship parameters were measured: courtship latency: time from the introduction of the male into the female-containing observation chamber until wing vibration occurs (in minutes); number of attempted copulations measured during a 20 min observation period measured from the introduction of the male into the chamber; copulation latency: time in minutes from the introduction of the male into the chamber until copulation ($n \geqslant 30$ for all tests).

2.5. Reverse transcription-PCR (RT-PCR)

cDNA synthesis and PCR conditions were the same as previously described (Dallerac et al., 2000). The *desatF* open reading frame was amplified by PCR from cDNA using the same primers as described and *rp49* was used as a control (Chertemps et al., 2006).

2.6. Nucleotide and protein sequence analysis

DNA sequences from three *D. melanogaster* strains, three *D. simulans* strains, and one *Drosophila sechellia* strain were used: Dmel CS, Dmel Tai, Dmel BDGP; Dsim YB, Dsim Sey and Dsim WUG, Dsec BI (see Table 1). Dmel BDGP, DsimWUG and Dsec BI were sequenced by the Berkeley Drosophila Genome Project (BDGP), the Washington University Genome (WUG) and the Broad Institute (BI), respectively. Sequences were extracted from the complete genome sequences of these species (Crosby et al., 2007; genomes available at http://flybase.bio.indiana.edu). The other sequences were obtained after PCR amplification, using *desatF* open reading frame primers and a primer situated just before the sequences shown in Figure S2 (position −899 to −879, relatively to the Dmel BDGP sequence: GACGACACGACGACAAATCG). Nucleotide and amino acid sequences were aligned with the Clustal-W program (Thompson et al., 1994). Pairwise scores were calculated as the number of identities in the best alignment divided by the number of residues compared (gap positions were excluded). Putative transcription factor sites were analyzed, using TFsitescan program (Ghosh, 2000). A phylogenetic tree was then constructed by the neighbor-joining method based on a Kimura 2-parameter matrix in the MEGA program, version 3.1 (Kumar et al., 2004). The confidence values of branches of the phylogenetic tree were determined using bootstrap analysis based on 1000 re-samplings.

3. Results

3.1. Expression of desatF RNAi in Tai flies

Two genotypes were compared: *w*; *OK72-GAL4/Cy*; Tai/CS (named Dmel Tai-Control) and *w*; *OK72-GAL4/UAS-RNAi desatF*; Tai/CS (named Dmel Tai-RNAi). The latter genotype expresses *RNAi desatF* under the *OK72* promoter.

In Dmel Tai-RNAi females, the expression of *desatF* was no longer detectable by RT-PCR, contrary to Dmel Tai-Control females (Fig. 2A).

After GC analysis, all the compounds were well separated from each others with the exception of 5,9-dienes, which migrated with the methylated HC and could not be separated from them (Fig. 2B). The percentages of 5,9-dienes were thus evaluated by GC–MS. In our experiments, the proportions of methylated HC were very constant and the differences in (5,9-dienes + methylated-HC) between can be attributed to 5,9-diene differences. The Dmel Tai-Control females had about 21% 7,11-dienes (mainly in C27 and C29) and 25% 5,9-dienes (mainly in C25 and C27), as determined by GC–MS. After expression of *desatF RNAi*, the percentage of 7,11-HC decreased to a value of 4% and the 5,9-HC were no more detectable by GC–MS. The proportion of methylated hydrocarbons was not affected. There was also a sixfold increase in 7-monoenes and sevenfold increase in 5-monoenes (especially in C23, C25 and C27). The overall percentages of saturated linear compounds did not change, with more C29 at the expense of C27.

3.2. Hydrocarbons in D. melanogaster and D. simulans females

Dmel TM3 females, obtained by crossing Dmel Df(3L)desatF line with Dmel CS showed a hydrocarbon profile characteristic of wild-type *D. melanogaster* females, with about 2%, 21% and 13% 7,11-dienes in C25, C27 and C29, respectively and only 10% 7-monoenes equally distributed in C23, C25 and C27 (Fig. 3A).

D. melanogaster females with one dose of cytological deletion Df(3L)desatF had half dienes and twice monoenes, with no significant modification of branched or linear saturated HC, compared to control (Dmel TM3) females. The effect on unsaturated HC was the same whatever the chain length (Fig. 3A).

Both *D. simulans* strains, Dsim Sey and Dsim YB had similar hydrocarbon pattern, with no dienes and 70% monoenes (exclusively in C23 and C25). They differed mainly from each other by the presence of less 7-T (−23%)

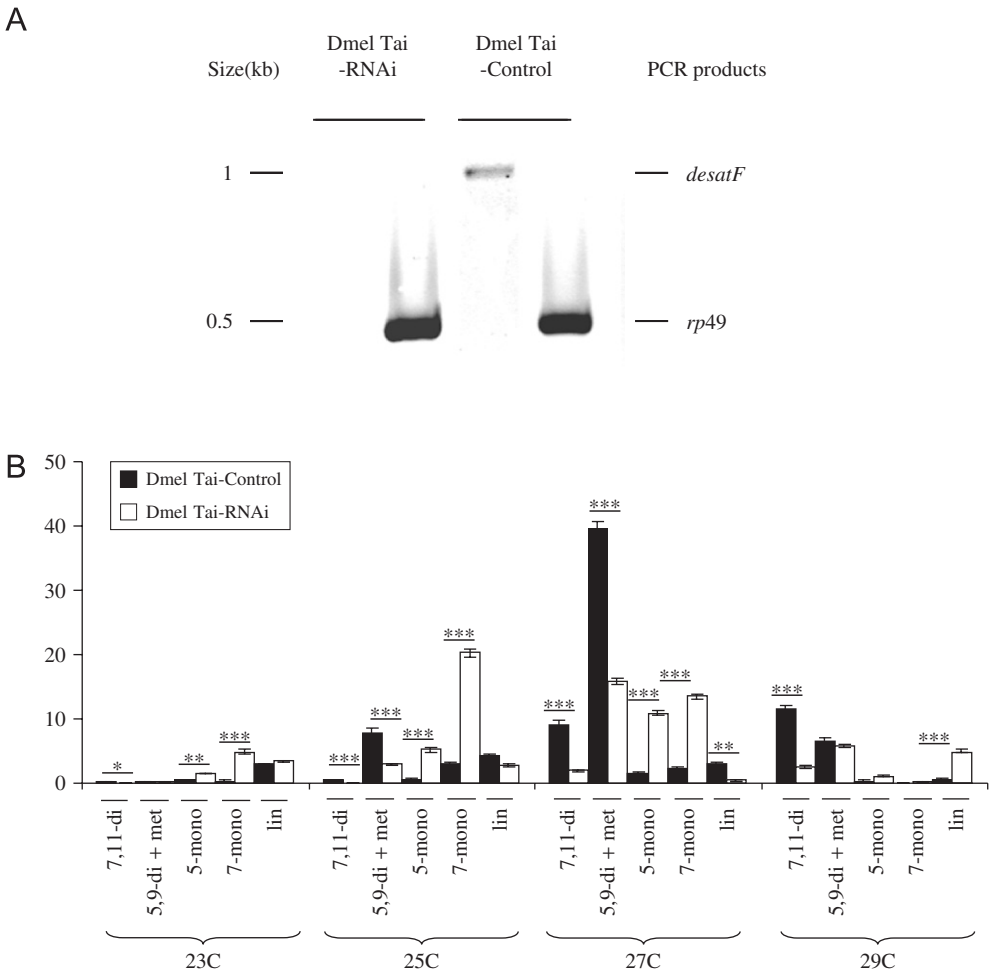

Fig. 2. Effect of *desatF* knock-down on *desatF* expression and hydrocarbons in 4-day-old 5,9-HC females. (A) Products of RT-PCR from Dmel Tai-RNAi and Dmel Tai-Control females, using *desatF* specific primers. Amplification of *rp49* was used as a positive control. (B) Mean hydrocarbons (±S.E.) of Dmel Tai-RNAi and Dmel Tai-Control females. Abbreviations were as follows: di, dienes; mono, monoenes; lin, saturated linear; met, methylated. Means with (*), (**) and (***) were significantly different with the Mann–Whitney *U*-test ($P = 0.05$, 0.01 and 0.001, respectively).

and more 7-P (+163%) in Dsim YB, compared to Dsim Sey strain.

3.3. Hydrocarbons in females from interspecific crosses

HC profiles of interspecific female hybrids were intermediate between the two parental strains, with large quantities of C23 and C25 HC.

Dmel desatF+/Dsim Sey hybrids had 45% monoenes, mainly in C23 and Dmel desatF+/Dsim YB hybrids 41% monoenes, mainly in C25. Both hybrids had also 12–15% 7,11-dienes, but these dienes were shorter than those in Dmel TM3 females: there were about three times more C23 and C25 dienes, three times less C27 dienes and 25 times less C29 dienes (Fig. 3C).

Hybrids carrying the Df(3L)desatF deletion were characterized by the absence of dienes, at the expense of monoenes in C23, C25 and C27. In particular, 7-P level in Dmel desatF−/Dsim Sey was three times higher than in Dmel TM3 and two times higher than in Dsim Sey; 7-P level in Dmel desatF−/Dsim YB was two times higher than in Dmel TM3 and 1.5 times higher than in Dsim YB (Fig. 3D).

3.4. Behavioral experiments using D. melanogaster males as tester males (Fig. 4)

Dmel TM3 females were subject to intense courtship from Dmel CS males, with 100% females inducing male wing vibration (in less than 2 min) and 92% copulation (in about 5 min mean time). In comparison, 95% and 69% Dmel Df(3L)desatF females induced male wing vibration and copulation, respectively. Both courtship and copulation latencies were doubled, compared to Dmel TM3 females. The number of copulation attempts from males was doubled too (Fig. 4).

In all, 85–88% of *D. simulans* females induced courtship from *D. melanogaster* males, but courtship latency was twice that toward Dmel TM3 females. There were more copulation attempts for Dsim YB, compared to Dmel TM3 control. But no copulation occurred in 20 min for either *D. simulans* strain.

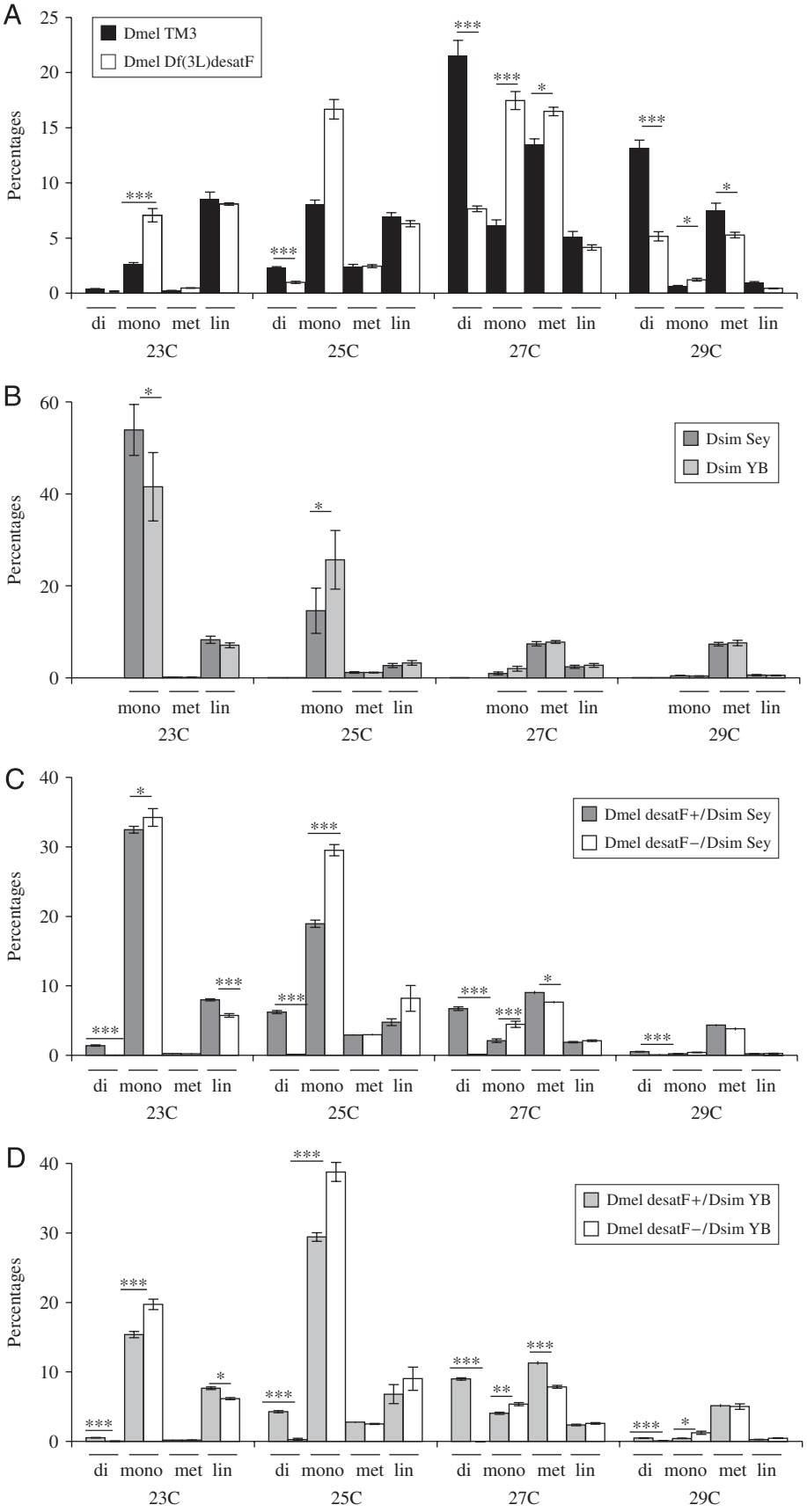

Fig. 3. Mean hydrocarbons (±S.E.) of 4-day-old *D. melanogaster* (Dmel Df(3L)desatF and Dmel TM3) (A), *D. simulans* (Dsim Sey and DsimYB) females (B) and of *D. melanogaster*/*D. simulans* hybrids (C, D). Abbreviations are the same as in Fig. 2.

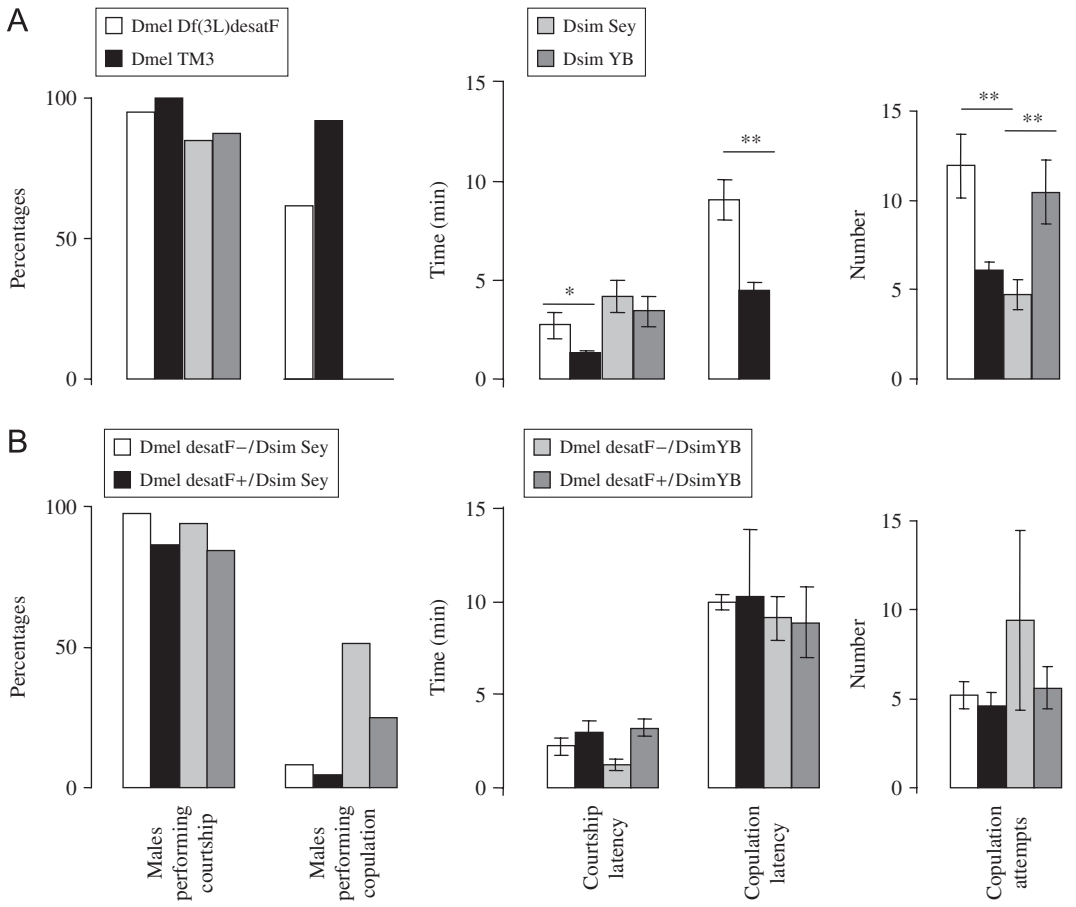

Fig. 4. Mean courtship parameters (±S.E.) of 4-day-old tester Dmel CS male flies with females of different genotypes: (A) Dmel Df(3L)desatF ($n = 39$), Dmel TM3 ($n = 38$), Dsim Sey ($n = 40$) and Dsim YB ($n = 32$); (B) *D. melanogaster*/*D. simulans* hybrids Dmel desatF−/Dsim Sey ($n = 37$), Dmel desatF+/Dsim Sey ($n = 43$), Dmel desatF+/Dsim YB ($n = 32$) and Dmel desatF−/Dsim YB ($n = 33$). Means with (*) and (**) were significantly different with the Mann–Whitney *U*-test ($P = 0.05$ and 0.01, respectively).

With Dmel desatF+ hybrid females, the percentage of females leading to courtship and courtship latency were not significantly different from Dmel TM3 parental females. Copulation latency was doubled and copulation success much decreased. Males copulated more with Dsim YB hybrid females (25%) than with Dsim Sey ones (4.7%).

Dmel desatF− hybrid females induced more courtship from Dmel CS males than Dmel desatF+ hybrid ones. Courtship and copulation latencies were not significantly different from those observed with Dmel TM3 but Dmel desatF−/Dsim had twice the copulation success as Dmel desatF+/Dsim females.

3.5. Behavioral experiments using *D. simulans* males as tester males (Fig. 5)

Courtship behavior of *D. simulans* males toward females from the same strain was roughly similar for Dsim Sey and Dsim YB: courtship occurred for 92–95% females and copulation for only 26% and 32% Dsim Sey and Dsim YB females, respectively. Copulation latency was about the same for both strains (9–10 min) (Fig. 5).

Toward a Dmel TM3 hybrid female, courtship parameters were modified: courtship percentage was decreased a little for Dmel desatF+/Dsim Sey, there were very few copulation attempts and no copulation. With Dmel desatF+/Dsim YB females, courtship was dramatically decreased, with only 21% females inducing male wing vibration. Courtship latency was doubled, compared with Dsim YB females; there was no copulation attempt and no copulation in 20 min.

With Dmel desatF−/Dsim females courtship parameters were not significantly different from those with Dsim females; however, there were fewer copulation attempts (−45%) for Dmel desatF−/Dsim YB and less copulation success for both types of hybrids (−75% and −42% for Dmel desatF−/Dsim Sey and Dmel desatF−/Dsim YB females, respectively).

3.6. Sequences of desatF gene in *D. melanogaster* and *D. simulans* strains

3.6.1. Analysis of the coding region

The ORF was 1065 bp long, encoding 355 aa (Figure S1). At the nucleotide level, there were 80 positions within the

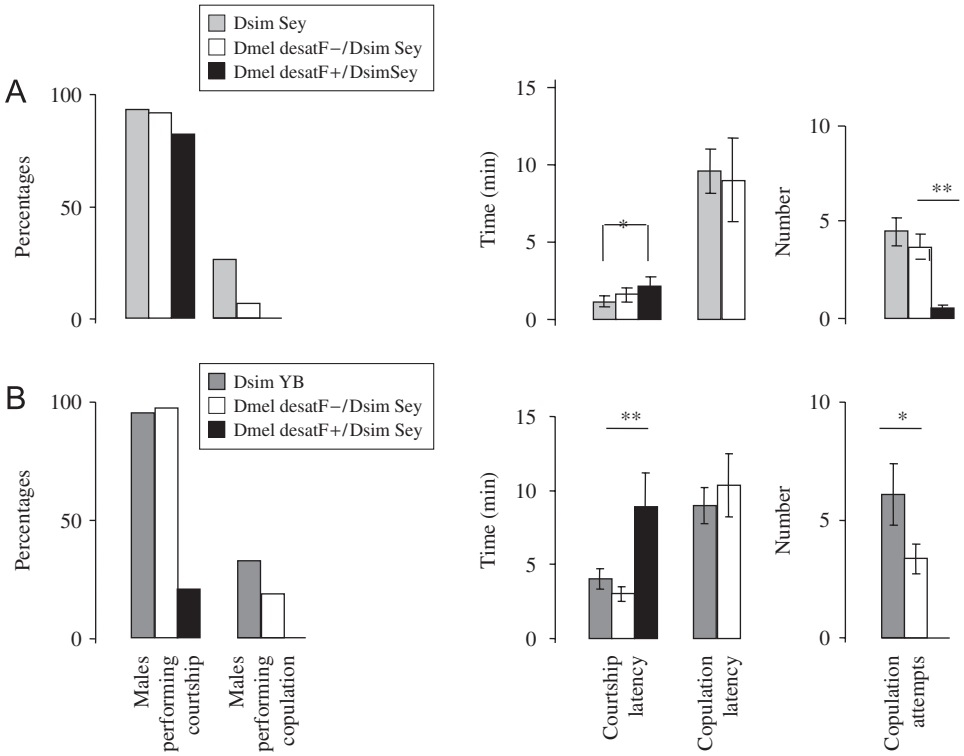

Fig. 5. Mean courtship parameters (±S.E.) of 4-day-old tester Dsim Sey (A) and Dsim YB (B) male flies with females of different genotypes: Dsim Sey ($n = 57$), Dmel desatF−/Dsim Sey ($n = 46$), Dmel desatF+/Dsim Sey ($n = 44$), YB ($n = 40$), Dmel desatF+/Dsim YB ($n = 34$) and Dmel desatF−/Dsim YB ($n = 32$). Means with (*) and (**) were significantly different with the Mann–Whitney *U*-test ($P = 0.05$ and 0.01, respectively).

coding region that were either polymorphic within or different between the two species (92% identity). Aligning the deduced amino acid sequence of *desatF*, there were three histidine boxes conserved at the same place. *D. melanogaster* sequences were highly conserved, with one and two modifications in the Dmel Tai and Dmel BDGP sequences, compared to Dmel CS (identities >99%). *D. simulans* protein sequences were less conserved, with one modification in Dsim YB, 5 in Dsim Sey and 5 in Dsim WUG, compared to the *D. simulans* consensus sequence (common to two or three *D. simulans* sequences). Nucleic acid sequences differed from Dmel CS sequences by 23, 24 and 25 modifications in Dsim YB, Dsim Sey and Dsim WUG, respectively (93–94% identity) (Figure S2). All these genes are intronless.

The alignment of the DesatF coding sequences of the three *D. melanogaster* and the three *D. simulans* strains shows 20 fixed replacement substitutions. Adding the *D. sechellia* sequence shows that only five of them are *D. simulans* specific. Since *desatF* does not seem to be transcribed in *D. simulans*, a low codon usage bias could be expected in the *desatF* gene (Shields et al., 1988). In fact, the codon bias estimated by the effective number of codons (ENC; Wright, 1990) is almost identical in *D. melanogaster* (53.0) and *D. simulans* (52.2). The ENC for *D. sechellia desatF* is a bit lower (i.e. stronger bias): 49.9. The GC 3% percentage is identical in the three species (62.2%). Thus, the silencing of *desatF* in *D. simulans* is not reflected in the base composition, which could suggest a recent silencing. In order to compare the rate of evolution of *desatF* in *D. melanogaster* and *D. simulans*, we have performed a relative rate test (Sarich and Wilson, 1973; Robinson et al., 1998). We included *D. sechellia* and we chose *Drosophila erecta* as the outgroup. The test showed no significant difference in the rate of evolution in any of the three species. In addition, to test departure from neutral evolution of *desatF*, a McDonald–Kreitman test (McDonald and Kreitman, 1991) was performed, in spite of the small number of sequences. The result was not statistically significant (not shown).

3.6.2. Analysis of the upstream non-coding region

Regarding the region upstream of the ORF, the *D. simulans* sequences were about 10% longer than the *D. melanogaster* ones, due to numerous small insertions of 1–26 nt in *D. simulans*. The three *D. melanogaster* sequences were 96–99% identical, the three *D. simulans* were 94–95% identical and the *D. simulans* sequences share 86–89% identity with *D. melanogaster* sequences (Figure S2 and Fig. 6).

The putative transcription start was found at −21 nt from the coding ATG. A modified TATA box (ATAAATA) was located at position −51 for the six sequences and no putative CAAT box was found in the seven sequences. Several transcription factor binding sites that have been described for *Drosophila* were found: in particular, one ADH-site d5 was present in all the sequences and seven Adf-1 (Adh transcription factor)

A

	Dmel Tai	Dmel CS	Dmel BDGP	Dsim WUG	Dsim YB	Dsim Sey	Dsec BI
Dmel Tai	100	**99**	**99**	**92**	**92**	**92**	**93**
Dmel CS	*98 (2.2)*	100	**99**	**92**	**92**	**92**	**93**
Dmel BDGP	*97 (4.9)*	*96 (5.9)*	100	**92**	**92**	**92**	**93**
Dsim WUG	*89 (20.8)*	*88 (22.3)*	*89 (20.9)*	100	**98**	**97**	**96**
Dsim YB	*87 (21.9)*	*87 (23.3)*	*88 (21.1)*	*94 (8.6)*	100	**98**	**96**
Dsim Sey	*86 (23.1)*	*86 (24.7)*	*87 (23.2)*	*95 (6.6)*	*94 (8.6)*	100	**96**
Dsec BI	*68 (20.2)*	*75 (20.8)*	*68 (21.1)*	*88 (16.8)*	*85 (20.6)*	*85 (18.3)*	100

B

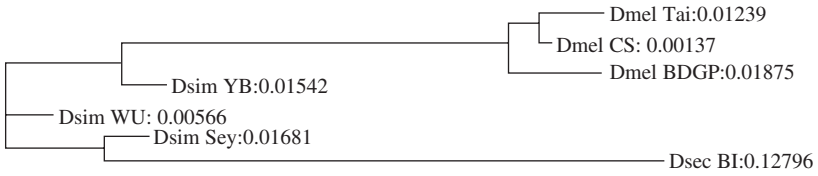

Fig. 6. Phylogeny of *Drosophila desatF*. (A) Percentages of identities and gaps (in brackets) between the promoter sequences (bottom of the diagonal, in italics) and the deduced protein sequences (top of the diagonal, in bold). (B) Phylogenetic tree in Phylip format (multiline) obtained with the seven sequences, using the Clustal-W program.

response elements occurred in Dmel CS sequence (four in Dsim and Dsec sequences). The "surnumerous" nucleotides in *D. simulans* were not found to interrupt or to introduce putative binding sites. Interestingly, one DSXF binding site: consensus sequence (nT) (nG) (T/A) ACAATGT (A/T) (nC) C was present in seven sequences, at −272 to 299 for the three mel sequences, −328 for Dsim Sey and Dsim WUG and −213 for Dsec BI. The most strongly preferred core nucleotides (ACAATGT) in the DSX site were selected here.

A phylogenetic tree was constructed and showed that Dmel BDGP is closer to Dmel CS than Dmel Tai; likewise, Dsim Sey is closer to Dsim WUG than Dsim YB (Fig. 6).

4. Discussion

4.1. Role of DesatF in diene pheromone biosynthesis

desatF has previously been shown to be specifically expressed in females who produce dienes (in Canton-S and Tai strains from *D. melanogaster* and in *D. sechellia*); it is not expressed in *D. simulans*, which produces only monoenes. RNAi experiments have demonstrated a role for DesatF in female pheromone biosynthesis, through the desaturation of 7-monoenic fatty acids at the 11 position (Chertemps et al., 2006). The present data indicate that this locus is also capable of desaturating 5-monoenic fatty acids: *desatF* knock-down in Tai-Canton-S hybrids, resulted in a strong inhibition of 7,11- and 5,9-HC with a massive production of 7- and 5-monoenes. The same desaturase is therefore capable of desaturating monoenes with unsaturations in 7 or in 5 to produce dienes with another desaturation four carbons after the first double bond. Other animal desaturases have also been shown to act on different kinds of substrates (Castuma et al., 1977; Watts and Browse, 1999). The geometry of the substrate could be a substantial factor for the enzyme activity and the specificity of DesatF could thus be strict, regarding the position of the second desaturation relatively to the first one. In vitro, DesatF has no action on unsaturated substrates up to C20 and might use a longer (C22–C26) fatty acid substrate (Chertemps et al., 2007).

Several fatty acid desaturases from plants have been shown to produce fatty acids with conjugated double bonds (Cahoon et al., 1999, 2001; Iwabuchi et al., 2003). It could also be the case in insects: biosynthesis of the *Bombyx* sex pheromone bombykol, (*E*,*Z*)-10,12-hexadecadien-1-ol, occurs through two consecutive desaturation steps performed by a desaturase with activities of Z11 desaturation and delta10,12 desaturation (Moto et al., 2004). In *Drosophila*, the possibility of introducing two double bonds in positions 7 and 11 for Desat1 and 5 and 9 for Desat2 has been suggested (Marcillac et al., 2005a). However, no diene could be detected in functional expression of *desat1* and *desat2* in yeast (Dallerac et al., 2000). Moreover, these double bonds are not conjugated, separated by four carbons, and fatty acid desaturases with such activity have not yet been reported. The regulation of both genes is unknown; that of *desat2* seems simpler, with only one predicted transcript; for *desat1*, five different transcripts have been described. These transcripts are not equivalently represented and could have different tissue or

development expressions (Jallon and Wicker-Thomas, 2003). *desat2* transcripts are only found in 5,9-HD females (Dallerac et al., 2000), whereas the five *desat1* transcripts are present in both *D. melanogaster* sexes (Marcillac et al., 2005a). Both genes have pleiotropic effects, *desat1* involved in fatty acid and lipid metabolism (Ueyama et al., 2005) and in pheromone perception (Marcillac et al., 2005b) and *desat2* in resistance to desiccation (Greenberg et al., 2003).

We investigated the possibility that other desaturase(s) perform the same desaturation as DesatF. We constructed two sorts of hybrid females with one dose of *D. simulans* genome, and one dose of *D. melanogaster* genome, differing from each other by the absence or the presence of one copy of functional *desatF*, the rest of the genotype remaining identical. As no dienes were produced in hybrids deficient for *desatF*, we can conclude that *desatF* is the only enzyme involved in diene production in *D. melanogaster*.

D. melanogaster/*D. simulans* (Dmel/Dsim) hybrid females had about half of the dienes present in wild-type *D. melanogaster* females. However, the length of the dienes was very different: there were very few dienes in C29 and a large quantity of dienes in C25. This could come from a reduced activity of fatty acid elongation in these hybrid females. Actually, an elongase gene, *eloF*, expressed, like *desatF*, in *D. melanogaster* females and not in *D. simulans* is involved in the elongation of fatty acids to very long (up to C30) fatty acids, precursors of HC; RNAi knock-down of *eloF* gene resulted in the accumulation of C25 dienes at the place of C29 ones (Chertemps et al., 2007). Dmel/Dsim hybrid females have only one copy of functional *eloF* (brought by the parental *D. melanogaster* strain). This gene (like *desatF*) may be sensitive to gene dosage and one copy might be insufficient to produce C29 dienes.

4.2. Role of pheromones in courtship behavior

7-Monoenes with 27 ± 2C and 7,11-HC with 27 and 29C have been shown to induce male Canton-S wing vibration (Antony et al., 1985; Ferveur and Sureau, 1996; Marcillac and Ferveur, 2004). 7,11-Dienes have also been reported to affect the frequency and rapidity with which *D. melanogaster* males mate (Marcillac and Ferveur, 2004; Ueyama et al., 2005). The small level in 7,11-dienes in *lxd6* females was not compensated by the high level in 7-monoenes, concerning the effect on courtship behavior and accounted for the increased courtship and copulation latencies by Canton-S males. The number of copulation attempts was doubled but so was also copulation latency; these deficiency females elicited less copulation success with wild-type males than wild-type females.

The two *D. simulans* parental strains elicited different courtship behaviors by Canton-S males: YB and YB hybrid females induced more courtship from *D. melanogaster* males than Sey and Sey hybrids. Their HC profiles were though similar, especially for the hybrids, which differed mainly by fewer 7-T and more 7-P in YB. 7-T and 7-P are known to be inhibitory and excitatory for *D. melanogaster* males, respectively (Ferveur and Sureau, 1996; Antony et al., 1985).

The results obtained with deficiency and *TM3* hybrids were—at a first sight—surprising. Courtship latency was increased in hybrid *TM3* females (with dienes), compared with *lxd6* ones (without dienes) and copulation success was decreased. This could come from an inhibitory effect of dienes in C25 on male courtship behavior. A similar observation has been reported for *RNAi eloF* females, characterized by large quantities of C25 dienes, in which both courtship and copulation latencies were increased and copulation percentages decreased (Chertemps et al., 2007).

The most striking point concerning courtship behavior of *D. simulans* males is the very strong inhibitory effect of female dienes, even in small quantities, on male courtship. With *TM3* hybrids, copulation attempts were very rare or absent, depending on the parental strain and no copulation was observed. These female predominant hydrocarbons have been implicated in the inhibition of interspecific courtship (Coyne et al., 1994; Savarit et al., 1999). The *lxd6* hybrids led to more courtship, courtship parameters being very similar to the *D. simulans* parental strain. It resulted in mating success, which remained, however, two to four times lower than for the *D. simulans* parental strain.

These results emphasize the role of diene formation—and of *desatF*—in precopulatory courtship behavior.

4.3. Evolution of desatF

D. simulans and *D. melanogaster* diverged only 2.5 million years ago (Hey and Kliman, 1993). The two species differ by about 4–8% on average in DNA sequence (Caccone et al., 1988; Hey and Kliman, 1993). Several studies have estimated that they differ at the nucleotide level by 2–4%: sequences in the *Adh* gene region differ by 2.8% in the coding region and 3.1% in the 5′ non-coding region (Cohn et al., 1984), in the *rosy* region by 3.8% (Aquadro et al., 1988). In a more recent study on male-specific accessory proteins (Acps), Kohn et al. (2004) have found 10% divergence in the 5′-untranscribed regions. We found a similar divergence (11–14%) at the *desatF* locus and also the presence of numerous extra sequences in *D. simulans* promoter. In fact, genes with sex-specific expression are the most divergent between species (Proschel et al., 2006). As *desatF* shows female-biased expression in *D. melanogaster* it could have been submitted to strong selection in this species and as it is not expressed at all in *D. simulans* it might have evolved rapidly in this latter species without constraint. However, the two tests (relative rate test and McDonald–Kreitman test) used in this study failed to show a rapid evolution of *desatF*, but this may be due to the low number of sequences available.

The *D. melanogaster* subgroup consists of nine species. The phylogeny has been well characterized using analysis of chromosomal inversions and morphological and molecular traits (Lemeunier et al., 1986; Caccone et al., 1996;

Da Lage et al., 2007) and the cuticular hydrocarbons have been described (Jallon and David, 1987). The subgroup contains three major clades. Within the *melanogaster* clade, *D. melanogaster* diverged first followed by the *simulans–sechellia–mauritiana* triad. Two species of this clade (*D. melanogaster* and *D. sechellia*) are sexually dimorphic for their pheromones and express *desatF*, two are sexually monomorphic (*D. simulans* and *Drosophila mauritiana*) and do not express *desatF* (C. Wicker-Thomas, data not shown). The five species of the two other clades are monomorphic, with the exception of *D. erecta*, with females having 9,23-tritriacontadiene. The presence of dienes in this species, with double bonds at different relative positions suggests that a second desaturase with a specificity different from DesatF performs the second desaturation. The presence of *desatF* sequence in *D. simulans* genome suggests that the *D. melanogaster* and *D. sechellia* dimorphism is the retention of an ancestral condition, may be prior to *D. melanogaster* subgroup formation. One way to address this point would be through molecular analysis of *desatF* gene sequences inside and outside *D. melanogaster* subgroup.

In fact, gene silencing in *D. simulans* might be recent, as suggested by the relative conservation of codons in the coding sequences, compared to *D. melanogaster* and *D. sechellia* sequences, where *desatF* is expressed. The promoter sequences, on the other hand, seem to have diverged very rapidly and *D. simulans* promoter regions are about 10–15% longer than *D. melanogaster* ones. These extra-sequences might contain inhibitory elements which could down-regulate transcription.

Several transcription factor response elements were found in the 5′-flanking sequence of *desatF*, in particular Adf-1 and ADH-site d5 elements. Adf-1 was shown to bind to recognition elements in each of the two promoters of the Drosophila *alcohol dehydrogenase gene* (*Adh*), and binding of Adf-1 to the *Adh* distal promoter site activates transcription in adults (England et al., 1990). Adf-1 elements are also found in other genes, such as *antennapedia* and *dopa decarboxylase* (England et al., 1990). Remarkably, a DSXF motif was present in all *desatF* 5′-flanking sequences except YB one. This motif might explain the female-biased expression in *D. melanogaster* and *D. sechellia* species. In vitro and in vivo functional studies would be needed to further characterize the mode of action of these regulatory elements, but the absence of this DSXF motif in YB sequence would be in agreement with a rapid evolution following inactivation of *desatF* gene in *D. simulans*.

In conclusion, this study points out the importance of *desatF* for sexual behavior and precopulatory courtship. It shows that it is the only gene involved in diene pheromone biosynthesis in *D. melanogaster*. Three observations are particularly important: (1) the presence of dienes in small amounts in females is inhibitory for *D. simulans* male courtship behavior and prevents copulation; (2) toward hybrid females devoid of dienes, courtship parameters of *D. simulans* males are not much different from those toward *D. simulans* females, even if copulation is somewhat inhibited; and (3) *desatF* promoter region has evolved rapidly between the two species. These data confirm the importance of this gene—together with the *eloF* gene—in sexual isolation and speciation in this group.

Acknowledgments

We thank Catherine Montchamp-Moreau and Mikael Ritchie for advice and comments. Funding was provided by the French Ministry of Research and Education (for X-X M) and by the Centre National de la Recherche Scientifique (for C W-T and J-L D L).

Appendix A. Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibmb.2007.11.005.

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