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Evolution of insect pheromones and their role in reproductive isolation and speciation

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Abstract. The importance for reproductive isolation of a change in the pheromone biosynthetic pathway, resulting in a different pheromone blend, is discussed in Lepidoptera and Diptera. The different sites of pheromone production and the biosynthetic enzymes are briefly reviewed. Two examples of a modification in the pheromone blend leading to reproductive isolation in Lepidoptera are taken as examples: the first, in *Ostrinia nubilalis*, is involved in the formation of two different populations showing reproductive isolation; the second, in the genus *Ostrinia*, might be at the origin of the formation of two different species. In both examples, a modification in the function of a desaturase involved in pheromone biosynthesis brings about change in the pheromone blend. In the fruitfly, *Drosophila melanogaster*, a mutation at a desaturase locus leads to the formation of two populations which differ in their pheromone mixtures and have developed premating isolation. The closely related species, *D. melanogaster* and *D. simulans*, differ in their female pheromonal cuticular hydrocarbons. This pheromonal difference is due to two species- and female-specific genes, *desatF* and *eloF*. The activity of *desatF* could account for an effective barrier between these species. All these examples show that a birth and death process of desaturases is at the origin of major shifts in the pheromone blend leading to sexual isolation and speciation.

Résumé. L'évolution des phéromones d'insectes et leur rôle dans l'isolement reproducteur et la spéciation. Cette revue analyse l'effet d'une modification de la biosynthèse phéromonale sur l'isolement sexuel chez les Lépidoptères et Diptères. Nous rappelons brièvement les différents sites de production phéromonale et les enzymes de biosynthèse. Nous montrons deux exemples où une modification du bouquet phéromonal entraîne un isolement sexuel chez les Lépidoptères: chez l'espèce *Ostrinia nubilalis*, cette modification est à l'origine de la formation de deux populations montrant un isolement sexuel et dans le genre *Ostrinia* elle pourrait être à l'origine de la formation de deux espèces. Dans les deux cas, c'est une modification de l'activité d'un gène de biosynthèse, une désaturase, qui est à l'origine de la modification du bouquet phéromonal. Chez la mouche du vinaigre, *Drosophila melanogaster*, une mutation dans le locus d'une désaturase est à l'origine de la formation de deux populations différant par leurs phéromones et montrant un isolement reproducteur. Les deux espèces *D. melanogaster* et *D. simulans*, très proches génétiquement, diffèrent également entre elles par leurs hydrocarbures phéromonaux. Cette différence est due à deux gènes, exprimés de façon espèce et femelle spécifique, *desatF* and *eloF*. L'activité de *desatF* semble constituer une barrière effective entre les espèces. Ces exemples montrent qu'un phénomène de naissance-mort des gènes de désaturase semble être à l'origine de changements majeurs du bouquet phéromonal, entraînant un isolement reproducteur et une spéciation

Keywords: Biosynthetic pathways, desaturase, cuticular hydrocarbons, Lepidoptera, Diptera.

Mating in insects implies the search for a mate, and involves visual, acoustic and chemical signals, the combination of which attracts the sexual mate (Ewing 1983). In some insects, pheromones play an essential role in mate recognition. The pheromone blend can contain up to 30 different chemical compounds. In Lepidoptera and Drosophilidae the chemical signal is generally emitted by the female and is then detected by the male using sensory receptors located on the antenna and maxillary palps. When the male arrives

close to the pheromone source, he begins to pursue the female and performs a complex parade (courtship behaviour), which is often followed by mating (Hall 1994; Greenspan 1995). Pheromones determine a chemical signature. The nature of chemical compounds and the proportion of each compound of the pheromone blend contribute to species specific mate recognition. A change, even a subtle one, in the blend composition may result in reproductive isolation, the first step towards speciation. The characterisation of pheromone biosynthetic steps will thus lead to a better understanding of the evolution of pheromone systems and their role in sexual isolation and speciation.

Most pheromones, in spite of their apparent diversity, share similar biosynthetic steps. In this paper,

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we review several examples which indicate that a very small modification in a biosynthetic step can lead to the production of totally different pheromones, and thus be the origin of sexual isolation and speciation. In addition, a paragraph is devoted to the use of hydrocarbon analysis in taxonomy and identification of species of economic or medical importance.

Sites of pheromone biosynthesis

In most lepidopteran and some primitive dipteran

species, pheromone biosynthesis can occur in a specialized gland located at the end of the abdomen: the pheromone gland. Such pheromones are blend of volatile molecules with molecular weight around 200 to 300 (Roelofs & Cardé 1971). In most Diptera and cockroaches, pheromones are synthesised in large epidermal cells called oenocytes (Diehl 1975; Ferveur *et al.* 1997; Schal *et al.* 1998; Fan *et al.* 2003). These pheromones are generally blend of high molecular weight and less volatile compounds, acting at short

Main pheromone compounds in *Ostrinia nubilalis*

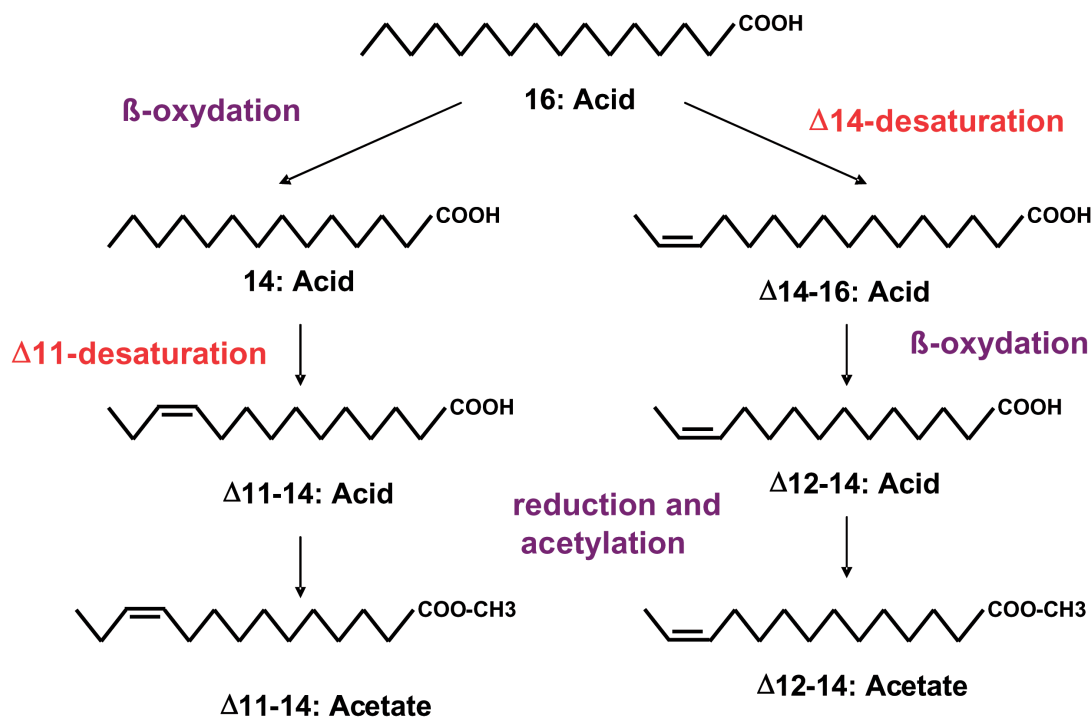


E11-14: Acetate



Z11-14: Acetate

Pheromone biosynthesis in *Ostrinia*



Ostrinia nubilalis

Ostrinia furnacalis

Figure 1

Main pheromone compounds in the two phenotypes of *Ostrinia nubilalis*, showing the E- and Z- conformation of the Δ11-14: acetate and biosynthesis of pheromones in *O. nubilalis* and *O. furnacalis*.

distance or by contact. Finally, other biosynthetic sites have been described, such as the gut in Scolytes or exocrine glands located on the legs in *Tribolium*.

Pheromones can contain between 8 (palm weevil) and 47 (cockroaches) carbon atoms and have very complex and diverse structures. Terpene pheromones (Scolytes) are synthesised from isoprene units (C₅H₈) (Titiger 2003). Most Lepidopteran pheromones originate from de novo biosynthesised fatty acids and have one to three unsaturations and an oxygenated functional group such as alcohol, aldehyde or acetate ester (Jurenka 2003). Hydrocarbons from Diptera and cockroaches also originate from very long fatty acids (Jallon & Wicker-Thomas 2003).

Biosynthesis enzymes

Pheromone biosynthesis from fatty acids involves a low number of enzymes: enzymes which elongate (elongases) or shorten (β -oxydases) the fatty acid chain, or introduce a double bond (desaturases). Finally, the acid function is reduced (reductase) and modified in alcohol, aldehyde, etc. or decarboxylated to hydrocarbon. Chain shortening has been evidenced only in lepidopteran species and has not yet been characterised at the enzymatic level. The combination and succession of the different steps give rise to a variety of compounds with different chemical properties.

The use of radio-labelled precursors up to the 1990s allowed the establishment of pheromone biosynthetic steps. However, the determination of the specificity of the biosynthetic enzymes has been made possible only with the development of molecular biology techniques.

One of the difficulties in the study of biosynthesis lies in the nature of these enzymes, bound to the reticulum membrane, and thus difficult to isolate and study in vitro. In insects, the first isolated desaturase gene was characterised thanks to the homology between rat and yeast desaturases (Thiede *et al.* 1986; Stuckey *et al.* 1990). This first sequence, obtained in *Drosophila* in 1997, was the fourth animal desaturase sequence (after rats, mice and carp) (Wicker-Thomas *et al.* 1997). This was followed by the characterisation of a desaturase in Lepidoptera in 1998, until today, over a hundred of desaturases have been described in insects (mostly in Lepidoptera) (Knipple *et al.* 1998). The study of their specificity is conducted using heterologous expression in a desaturase-deficient yeast: the yeast mutant cannot produce unsaturated fatty acids, but after expression of the foreign desaturase, unsaturated fatty acids are produced and can be analysed to study substrate and unsaturation specificity.

The study of elongases is even more recent due to the difficulty resulting from their very low homology (elongase protein sequences are only 20 to 40% identical). Elongases are very difficult to characterise. The first elongases characterised were those from yeast (Toke *et al.* 1996; Oh *et al.* 1997). The only elongases characterised in insects are from *Drosophila* (Chertemps *et al.* 2005 2007).

The use of molecular biology techniques has resulted in huge progress in the understanding of pheromone biosynthesis, and also the elucidation of biosynthetic gene evolution. In the following paragraphs, we describe how a change in a biosynthetic gene can be followed by a reproductive isolation, at the origin of speciation.

Evolution of pheromones in Lepidoptera (Fig. 1)

Sex pheromones show a remarkable activity on the mate of the opposite sex. The question of their evolution is particularly sensitive: when one population isolates from another, and then diverges to give rise to another species, one of the conditions that may favour its isolation is a modification in its pheromonal blend, so that the resulting population is prevented from mating with the former one. Both populations can then evolve independently. This change is possible for some species that use a complex mixture of a single chemical molecule but with different spatial conformations.

First example: the European corn borer, *Ostrinia nubilalis*

The species *O. nubilalis* is composed of two populations which differ in their pheromone blend (Takanashi *et al.* 2006): they produce the E and Z isomers of the 11-tetradecenyl acetate (Δ 11-14: Ac) in different proportions: in the Z population, females produce and males respond to a ratio Z11-14: Ac / E11-14: Ac of 97:3 (pherotype Z), whereas the E population produces and responds to the inverse ratio (pherotype E) (Roelofs *et al.* 1985). The difference in both the composition of female sex pheromone and in the timing of moth emergence might explain the isolation between the two races (Thomas *et al.* 2003).

Both populations also show different food preferences: in France, the Z population lives mainly on corn; the E population lives on *Artemisia* and hops (Pelozuelo *et al.* 2006). Host-plant preference might also favour the genetic differentiation between the two host races (Bethenod *et al.* 2005).

The evolution of pheromone production in females has occurred in parallel with pheromone reception in males, since males have to distinguish and respond positively to this change. The genes involved in changes

in pheromone production in females and pheromone receptors in males are not genetically linked, and are independently inherited (Löfstedt *et al.* 1989), but due to the selection pressure they evolve in a concerted way.

In nature, on the other hand, mating between E and Z moths is highly unlikely: in natural conditions the two populations do not share the same habitat. Insects expressing the hybrid phenotype are rare, less than 1% (Pelozuelo *et al.* 2006). Moreover, if hybrids are produced between both populations, males are unable to respond to any Z/E mixture and do not mate (Glover *et al.* 1991).

Roelofs *et al.* (1987) suggest that Z is the ancestral allele. As the genes that encode production and reception of pheromones are not linked, two mutations must be involved: a first mutation has resulted in a male response to larger pheromone ratios. These males could be maintained in the Z population because they were able to respond to the Z females. Another mutation, modifying the ratio Z/E, appeared later. Mutant females, richer in the E compound, could no longer attract the Z males and would have mated with E males. The selection pressure would then have resulted in the formation of an E population.

However, Z populations are found exclusively on corn, not on mugwort and hop, which are putative ancestral host plants. In contrast, E populations, which are mostly found on mugwort and hop, can also live on corn (Pelozuelo *et al.* 2006). The question on the conformation (Z or E) of the ancestral pheromone allele is still open.

The question then arises whether the change of Z to E (or E to Z) conformation was progressive or sudden. In other words, how has the pheromone blend evolved from the 97:3 Z/E ratio to the 3:97 Z/E ratio? The fact that almost no intermediary form exists suggests that the conformation modification was very sudden. Other examples are in agreement with this hypothesis. However, the distribution of pheromone races overlap in the eastern United States and intermingling of pheromone races has been described (Roelofs *et al.* 1985).

Evolution of a pheromonal system resulting in the differentiation of two species

In general, a modification of a pheromonal system is more difficult to conceive than first seems. Let us suppose that a female emits a different pheromone signal, due to a mutation. This signal has to attract males, because a male will mate with females emitting the most attractive signal for him. We thus have to consider a sudden change both in the pheromonal

blend and in perception. What is the chance of both these conditions coming together? A first response can be afforded by the study of two different moth species.

The two species, *Ostrinia nubilalis*, native to Europe, and *Ostrinia furnacalis*, native to Asia, have been separated for about one million years. The isolation mechanism that has led to the formation of both species has therefore evolved very rapidly. Both species use totally different pheromones. How has this difference been established?

The pheromone glands of Lepidoptera contain desaturases, which produce unsaturated fatty acids, precursors of the pheromones. All the *Ostrinia* species emit Z/E11-14:Ac mixtures except the Asian species *Ostrinia furnacalis* which emit Z/E12-14:Ac. These pheromones are specifically recognised by the species that produce them and their biosynthetic pathways differ: a desaturation with a $\Delta 11$ specificity is performed by the European corn borer species (Klun *et al.* 1980), and $\Delta 14$ specificity for the Asian species (Ma & Roelofs 2002). A further reaction of reduction and acetylation gives the Z/E11-14:Acetate in the European species whereas, in the Asian species, a supplementary reaction of chain shortening before the final reduction and acetylation gives the Z/E12-14:Acetate.

To complicate matters even further, an analysis of transcripts has shown that $\Delta 11$ and $\Delta 14$ desaturases are transcribed in the pheromone gland of both species (Ma & Roelofs 2002; Roelofs & Rooney 2003). These desaturases, after heterologous expression in yeast, produce a mixture of: Z11-16:Acid and Z/E11-14:Acid ($\Delta 11$ desaturase) and Z/E14-16:Acid ($\Delta 14$ desaturase). This implies that these desaturases are not necessarily translated in the pheromone gland and only the $\Delta 11$ desaturase is used by the European species, while the $\Delta 14$ one is used by the Asian species for pheromone biosynthesis.

The $\Delta 11$ desaturase is probably the ancestor form since $\Delta 11$ desaturases are more widespread in Lepidoptera, and reductase enzymes from both species have a small preference for acids issued from $\Delta 11$ and not from $\Delta 14$ desaturase. A duplication of the $\Delta 11$ desaturase may have occurred, and evolved to give a $\Delta 14$ desaturase, used for pheromone synthesis in *O. furnacalis* only. When this gene has become functional, the compounds emitted by the female completely change. The evolution of pheromones thus seems to have occurred not with small successive changes but with one major and sudden change.

In *O. nubilalis* populations, males respond to the pheromone synthesised by the $\Delta 11$ desaturase. However, one out of 200 males can also respond to the

Z/E12-14:Acetate produced by the $\Delta 14$ desaturase. If the $\Delta 14$ desaturase of a female becomes used for pheromone production, this female will mate with the rare males attracted by her pheromones. In time, the communication system between males and females will become stable and a new pheromonal population will appear and will be isolated.

Evolution of sex pheromones in *Drosophila* (Fig. 2)

The evolution of pheromones has appeared through major changes and not through a succession of minor changes. Another example involving *Drosophila* can also illustrate the proposed scheme.

Pheromone biosynthesis in cosmopolitan populations

In *Drosophila melanogaster*, interspecific differences in the composition of female cuticular hydrocarbons play an important role in male choice during courtship behaviour before mating. The mass of these cuticular

hydrocarbons is very large, 1 to 2 micrograms, representing about 1‰ of the whole fly mass. Their biosynthesis follows the scheme indicated in Figure 2. Saturated hydrocarbons do not have a behavioural role (but could act against desiccation). Unsaturated hydrocarbons with at least one unsaturation in position 7 contain more or less pheromonal properties. The most potent compounds are 7-tricosene (7-T) in males, which represents over 40% of male hydrocarbons (Jallon 1984; Antony *et al.* 1985), and which exerts an attraction on the females and repulsion on the males, and 7,11-heptacosadiene (7,11-HD) in females, which represents about 25% of total female hydrocarbons, and is very potent since alone it can induce male courtship resulting in mating. This diene is absent from males because the gene is not transcribed.

Pheromone biosynthesis involves a first desaturation by a desaturase called Desat1, which places a first double bond in position 7 (Wicker-Thomas *et al.* 1997; Dallerac *et al.* 2000). In females, a supplementary step,

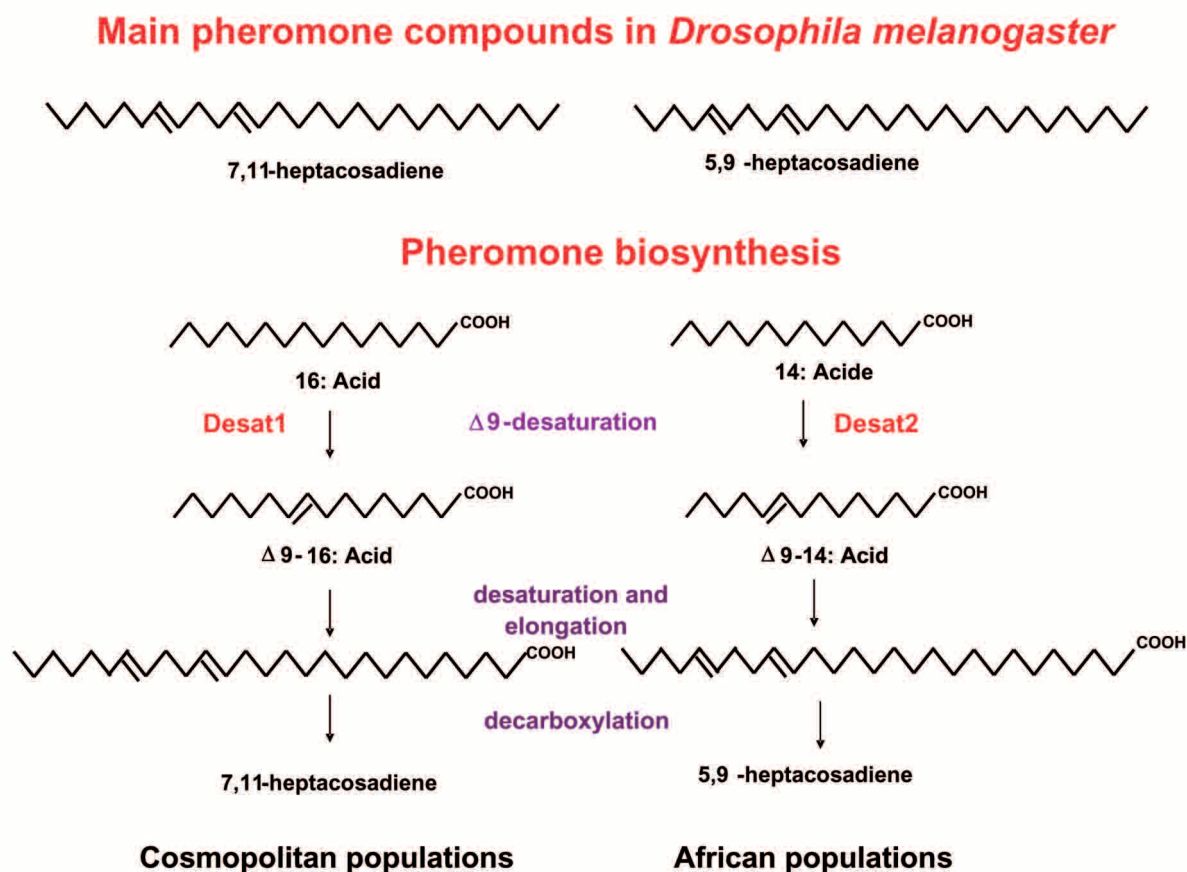


Figure 2

Main pheromone compounds in the two pherotypes of *Drosophila melanogaster* and biosynthesis pathway.

including a second desaturation and an elongation, is made by the female-specific enzymes *DesatF* and *EloF*, respectively, resulting in a second double bond in position 11 and an elongation of the dienic fatty acid up to C30 (Chertemps *et al.* 2006 2007).

Biosynthesis in African populations

Whereas most of the *Drosophila* females of cosmopolitan origin produce 7,11-HD in large quantities, some females originating from Africa and the Caribbean mainly synthesise an isomer of this pheromone, the 5,9- heptacosadiene (5,9-HD) and very little 7,11-HD (Jallon & Péchiné 1989; Ferveur *et al.* 1996). This pheromonal system does not seem to constitute an advantage for these populations, which mate and reproduce less rapidly than cosmopolitan populations (Ferveur *et al.* 1996; Fang *et al.* 2002). Actually, “5,9-HD” populations remain confined to some regions in Equatorial Africa and the Caribbean, whereas “7,11-HD” populations are present almost everywhere in the world. This pheromone polymorphism is due to the presence of two desaturase genes, *desat1*, transcribed in males and females from all the populations, and *desat2*, transcribed only in “5,9-HD” females, and responsible for the desaturation in position 5 (Dallerac *et al.* 2000). “5,9-HD” phenotype is probably the ancestral character because of the African origin of this phenotype. In these females, both desaturase genes are transcribed, but this may be due to a substrate competition: only 5,9-HD is produced in large amounts. During evolution, a mutation occurred (a 16 nt deletion in *desat2* promoter), which resulted in an inactivation of the *desat2* gene (pseudogene). Some “7,11-HD” populations also have a stop codon inside the open reading frame (Takahashi *et al.* 2001). Such females then produced large quantities of 7,11-HD, mated quicker and were able to colonise the entire globe. In this case, the loss of the function of a gene has resulted in a sudden change in the pheromone blend and in sexual isolation (Figure 4). This system is now widely studied because these two categories of populations are thought to be sub-species that could lead to the differentiation in two different species (Fang *et al.* 2002).

Do speciation genes exist in *Drosophila*?

In *Drosophila*, two species, *D. melanogaster* and *D. simulans*, diverged about 2.5 million years ago (Hey & Kliman 1993) and are considered as “twin species” because of their genome closeness (Caccone *et al.* 1988; Hey & Kliman 1993). However, they share different pheromonal types: the latter species is monomorphic concerning its hydrocarbons, with 7-T and 7-P present

in large quantities in both males and females (Jallon & David 1987). These hydrocarbons have been involved in homospecific recognition and heterospecific discrimination (Jallon 1984; Cobb & Jallon 1990; Coyne & Oyama 1995). The main pheromone of *D. melanogaster*, 7,11-HD, strongly represses mating in *D. simulans* males (Coyne *et al.* 1994; Coyne & Oyama 1995; Savarit *et al.* 1999). The pheromone difference is due to two genes: *desatF* and *eloF*, responsible for the desaturation of monoenes and elongation of the resulting dienes, respectively (Chertemps *et al.* 2006, 2007). We have shown that the genes were present in both genomes, but were only transcribed in *D. melanogaster*. The promoter region of *desatF* has evolved very rapidly, leading to a non-transcribed gene in *D. simulans*. We have also evidenced an inhibition of mating between *D. melanogaster* - *D. simulans* hybrids, removed when the *desatF* allele from *D. melanogaster* was not functional (Legendre *et al.* 2008). This shows the importance of *desatF* gene in sexual isolation and speciation in *Drosophila*.

Evolution of pheromones in other insects

All the data available for insect pheromones do not support the idea of pheromone evolution through small successive changes. Pheromone evolution is characterised by major changes that lead to completely different phenotypes between or even within species. This phenomenon, observed in Lepidoptera and Diptera, has also been described in Coleoptera in *Dendroctonus* and *Ips* genus (Symonds & Elgar 2004) and seems to be generalised to other orders. The study of pheromone biosynthetic steps shows that a single mutation can cause huge changes in the resulting pheromones, leading to a reproductive isolation of the mutated population and the formation of a new species.

Use of hydrocarbons in taxonomy

Chemical signatures have been shown to be important for mating or species recognition in a number of insect species. Differences in hydrocarbon phenotypes are sometimes, but not always, correlated with speciation and have been used as chemotaxonomic characters in insects (Carlson *et al.* 1978). Although some hydrocarbon variations can also be due to environmental conditions, hydrocarbons can be used –with other molecular markers– to discriminate close populations or species and numerous papers describe their importance in systematic and phylogeny analyses in insects belonging to various orders, such as Diptera, Lepidoptera, Hemiptera, Isoptera (see Bagnères & Wicker-Thomas 2010). They are especially important cues in termite species and caste recognition and more

generally in chemotaxonomy of insects of economic importance (Everaerts *et al.* 1997; Bagnères *et al.* 1998). Analysis of hydrocarbons has been essential for discrimination between closely related species of medical importance: it has allowed accurate and rapid identification of *Aedes* species (Pappas *et al.* 1994). Malaria-vector and non-vector forms of *Anopheles* populations can be discriminated based on hydrocarbon patterns (Kittayapong *et al.* 1990).

To be complete, we must also quote the use of hydrocarbon analysis in forensic entomology. Hydrocarbons are dependent on the developmental stage of some necrophagous Dipteran larvae and have thus been used to identify the age of the necrophagous larvae.

Conclusion

All studies have demonstrated the essential roles of hydrocarbons in insect speciation. Hydrocarbon analysis is also a prodigious tool for identifying insect species. Research is needed to understand the importance of hydrocarbons in some poorly studied insects as well as to elucidate the regulation of hydrocarbons.

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