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Evolution of male sex pheromone composition and diversification of the butterfly genus *Bicyclus*

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Chapter 1

General introduction

1.1 Speciation

Biodiversity is one of the most debated societal concerns. The International Convention on Biological Diversity (1992) defines biodiversity as 'the variability among living organisms $\left[...\right]$, this includes the diversity within species, between species and of ecosystems'. The concept of species is therefore central in order to quantify biodiversity. The best recognized definition of the species is the biological species concept, which defines species as groups of individuals that reproduce with each other and are genetically isolated from other similar groups (Mayr 1942). The criterion of genetic isolation is relevant because it emphasizes the evolutionary independence of the groups and defines a tangible frontier between species. While the biological species concept is simple in appearance, in practice it is much more challenging. In nature, it is difficult to visualize these reproductive events, let alone determine their success or failure. Moreover, the biological species concept suffers some limitations that are cause for controversy. For example, it cannot be applied to organisms that do not reproduce sexually, to many groups of plants in which unrelated species or even genera intensively hybridize with each other, and to extinct organisms, such as dinosaurs, that cannot reproduce anymore. In consequence, other so-called species concepts more useful in practice to delimitate species and derived from 'operational criteria' have been used and discussed (Hey 2006, de Queiroz 2007). Two examples are the phenetic species concept, in which a species is determined by common morphology, or the phylogenetic species concept, based on genealogy, for which the species comprises all the descendants of a common ancestor. Since species are the fundamental units of biodiversity, the process of separation of one species into two or more new species, termed speciation, is the basis for the expansion of biodiversity. Speciation is the evolution of reproductive barriers between groups of individuals of the same species, leading

to the cessation of gene exchange between them, consequently, giving birth to new species. The mechanisms of speciation, like the concept of species, is controversial and constitutes a central conundrum for evolutionary biology (Mallet 1995). This introduction on speciation will only deal with sexually reproducing organisms, for which the biological species concept is relevant.

1.1.1 History of the study of speciation

The book 'On the origin of species by means of natural selection' by Charles Darwin (1859) has long been recognized as a keystone for the study of speciation. However, it is only during the first half of the XIXth century that the Modern Evolutionary Synthesis, with the development of genetics in concert with evolutionary theory, made speciation a central focus of biology (Coyne & Orr 2004). One of the main actors during this period, Theodosius Dobzhansky (1937) emphasized the importance of barriers to gene flow and studied their genetic bases. Ernst Mayr, besides defining the biological species concept (Mayr 1942), also fostered the long lasting debate about the geography of speciation. Mayr strongly supported the hypothesis that species primarily arise in allopatry, that is by geographic isolation with the absence of gene flow (Coyne & Orr 2004). Since the 80s onward, advances in molecular biology have largely contributed to fruitful debate and tests of historical hypotheses (Coyne & Orr 2004). Central to this phase are studies about the mechanisms by which mate choice and sexual selection (Lande 1981, West-Eberhard 1983) or ecological adaptation (Mani & Clarke 1990, Schluter 2001) influence the development of reproductive isolation, beyond the geographical context. Finally, systematics has also improved in precision and robustness with molecular phylogenies allowing the development of the phylogenetic comparative method to test evolutionary hypotheses across large numbers of taxa (Felsenstein 1985).

1.1.2 Different barriers to gene flow

Two organisms can experience various forms of barriers preventing their successful reproduction. To achieve successful reproduction, two individuals of the same species must be sexually active at the same time and place. If their biology requires copulation (this is for example not the case of plants, echinoderms, or some molluscs), they must be able to find each other, to be reciprocally attracted, and to copulate. In turn, their gametes must meet to lead to fertilization, the egg must develop into a zygote able to survive in its environment and finally reproduce to restart the whole life cycle. Any obstacle recurrently interrupting this cycle between groups of individuals is a potential reproductive barrier. The different types of barriers are defined depending on their origin, intrinsic or extrinsic to the individuals, and relative to their timing during this cycle,

i.e. prezygotic or postzygotic. Common cases of reproductive barriers are mountain chains or seas separating populations during the reproductive period, termed geographic barriers, or adaptation to different host plant separating at the scale of a forest the reproduction of differentially adapted individuals, termed ecological barriers. Both ecological and geographic barriers are extrinsic and prezygotic. In contrast, intrinsic prezygotic barriers can result from a mismatch of male sexual traits and female preference preventing their mating, or the incompatibility of their sexual organs preventing successful copulation. Genetic incompatibilities between parental genomes that make the offspring unviable, unfertile, or maladapted to its environment and potential sexual partners, are postzygotic intrinsic barriers. This is not a comprehensive list of cases but it illustrates the variety of possible factors that can prevent sexual reproduction and stop the gene exchange between groups of individuals, leading to the formation of new species.

1.1.3 The geographical context of speciation

Based on the influential work of Ernst Mayr, geographic isolation or allopatry was historically considered the main factor of reproductive isolation between species. Therefore, the first classification for different types of speciation that emerged was based on the geographical context. Geographic separation is a good index of the ongoing gene flow between populations. The understanding of plate tectonics by Wegener, together with the discovery of increasing numbers of large fossils on the different continents during the XIXth century revealed the ubiquity and amplitude of speciation in allopatry. There are numerous examples of allopatric speciation, also termed vicariant speciation, with related species present on different continents, or separated by the same geographical barrier (Thomas *et al.* 2010). Similarly, the recurrent separation of sister species within various genera has been related to their temporary isolation in different glacial refugia during the Pleistocene (Hewitt 2004, Weir & Schluter 2004). In allopatry, even facing similar selection pressures on their respective ranges, the populations will necessarily accumulate genetic differences because migrations may not be sufficient to homogenize their genetic structure across the barrier. For example, the African fish *Pantodon buchholzi* (Pantodontidae) displays an extreme pattern of phenotypic stasis despite millions of years of geographic isolation between the populations in the lower Niger basin and the central Congo basin (Lavoué *et al.* 2011). Despite this striking phenotypic stasis, large genetic differentiation accumulated. On the contrary, if selection acts differentially between populations, reproductive isolation can arise very quickly. Experimental evolution has shown that only a few generations of differential selection could generate pre- or post-zygotic isolation between *Drosophila* populations (review in Coyne & Orr 2004). Differences in allele frequencies

among populations, in particular, can rapidly accumulate following a long-distance dispersal event, creating a founder effect (Mayr 1942). Indeed, when a small number of individuals colonise an allopatric zone, it may be subjected to strong genetic drift, accelerating its divergence from the source population and leading to speciation. Examples of founder effects are mostly described from rare dispersal events from the continent to small islands (Matzke 2014).

Sympatric speciation, as opposed to allopatric speciation, is the development of reproductive isolation in presence of gene flow between co-occurring populations. This process is difficult to demonstrate without ambiguity because of the apparently complex set of conditions required. For sympatric speciation to occur, evolution must favour the maintenance of different types of individuals within a population where individuals can freely meet with each other for reproduction. The second necessary step is the evolution of reproductive barriers between the types. This reproductive barrier necessitates that mating occur preferentially between individuals of the same type and is termed assortative mating. For example, in a phytophagous insect population, individuals adapted to a host plant A must also bear genes that make them preferentially mate with each other rather than with relatives adapted to plant B. This requires a non-random distribution of the genetic polymorphism, i.e. linkage disequilibrium, between genes of adaptation to host plants and genes inducing mate preference. A simple argument against the plausibility of sympatric speciation is that since assortative mating is not perfect at the beginning, gene flow between the groups of individuals induces recombination between the genes coding for adaptation to host plant and the genes responsible for mate choice. This recombination prevents the formation of linkage disequilibrium between alleles of local adaptation and mate choice, and consequently acts against the completion of assortative mating (Felsenstein 1981, Via 2001). Given these hostile conditions, a particular genetic architecture must be invoked to demonstrate the plausibility of speciation in sympatry (Via 2001). Pleiotropy, the effect of one gene on several traits, offers a solution of linking habitat preference or adaptation to mate choice without weakness facing recombination. This discovery was the first step for demonstrating that local adaptation and assortative mating could evolve in sympatry and ultimately lead to speciation (Via 2001). Traits involved in both local adaptation or habitat preference and mate choice were later termed 'magic traits' because by chance they achieve the two tasks and as such increase the plausibility of sympatric speciation (Servedio *et al.* 2011). Evidences from experimental evolution with artificial selection on *Drosophila melanogaster* confirmed that reproductive barriers could evolve pleiotropically in the presence of gene flow if the selective pressure for local adaptation was strong enough (Rice & Hostert 1993).

In addition to restrictive conditions of occurrence, sympatric speciation also suffers a stronger difficulty to be demonstrated in nature compared to

allopatric speciation. Indeed, it is a challenging task to determine if two incipient species were really sympatric and exchanging genes during the whole process of divergence, and not that they only met recently (Via 2001). As a consequence, it is still problematic to know definitively the relative frequency of sympatric and allopatric speciation; however, the current theory is that allopatric speciation occurs much more frequently than sympatric speciation (Jiggins 2006b). Importantly, the relative frequency of sympatric compared to allopatric speciation could vary between groups of taxa. For example, phytophagous insects would be more prone to ecological speciation in sympatry due to their close coevolutionary relationship with different host plants (Berlocher & Feder 2002), whereas freshwater fish would be more prone to allopatric speciation due to their low capacity to disperse out of their respective river bassins (Coyne & Orr 2004). Cichlid fishes in African lakes (Meyer *et al.* 1990, Schliewen *et al.* 1994), Howea palm trees on Lord Howe island (Savolainen *et al.* 2006), apple and hawthorn adapted races of *Rhagoletis pomonella* (Filchak *et al.* 2000), and brood parasitic indigobirds (Sorenson *et al.* 2003) represent scarce strong examples of sympatric speciation.

Parapatric speciation can be viewed as an intermediate type of speciation between the two extremes of allopatric and sympatric speciation. I will not elaborate on this topic further since it is now accepted that defining speciation on a geographic basis only is not sufficient (Butlin *et al.* 2008). Given the necessity of particular genetic architecture to explain sympatric speciation, and the role of natural selection in local adaptation, more relevant evolutionary questions needed to be addressed (Via 2001, Jiggins 2006b, Fitzpatrick *et al.* 2008). Those questions concern the role of the evolutionary forces, the traits involved in reproductive isolation and their underlying genetic architecture (Jiggins 2006b). The geographical context should now only be summarized as the amount of gene flow between individuals during the speciation process (Butlin *et al.* 2008). To conclude, it is important to keep in mind that speciation is a dynamic process that can take many generations, and that populations can undergo different geographic contexts in order to reach complete reproductive isolation (Butlin *et al.* 2008). As a particular illustration, we will see that the case of reinforcement speciation developed in the next section, is based on a postzygotic isolation that develops during an initial allopatric phase and ends in sympatry or parapatry where prezygotic reproductive barriers complete reproductive isolation.

1.1.4 Selection for premating isolation: the reinforcement process

Reinforcement for premating reproductive isolation is, at first sight, counter intuitive as selection effectively reduces the number of potential mating partners. This selection pressure occurs because of the reduced mating success of some

crossings between divergent populations of a species (Butlin 1987). This reduced success through poorly adapted or poorly fertile offspring comes from genetic incompatibilities between these populations during the preliminary phase of evolution (Butlin 1987). When the divergent populations meet during a so called 'secondary contact' phase, selection against hybrid crosses encourages the divergence of mating traits in order to accurately choose compatible partners (Butlin 1987). The pattern of divergence of the mating traits observed in the contact zone but not in allopatry is named reproductive character displacement (RCD, Ortiz-Barrientos *et al.* 2009, Vallin *et al.* 2012). RCD also includes the case, similar to reinforcement, when hybrids have null fitness either because they are not fertile or not viable such that the two lineages can be readily considered as two isolated species (Butlin 1987). Unless a study is able to attest that hybrids are viable and fertile, it is not possible to disentangle whether gene flow remains between the two lineages. The process of RCD needs to be distinguished from ecological character displacement (ECD), in which the divergence of the trait is driven by competitive interaction for other resources that mating partners (Slatkin 1980, Pfennig & Pfennig 2009).

After prezygotic isolation in the contact zone has developed, the divergence between the populations regarding the sexual trait and the preference can spread to zones of allopatry, such that the pattern of RCD eventually vanishes at the scale of the species (Ortiz-Barrientos *et al.* 2009). However, it is still possible to detect RCD at the interspecific level if sympatric species are more different than allopatric species (Lukhtanov *et al.* 2005). However, instead of spreading to zones of allopatry, the divergence in trait and preference can in turn initiate reproductive isolation between the population in the contact zone (displaced) and the allopatric ones (non-displaced). This scenario is termed the 'cascade' hypothesis (Ortiz-Barrientos *et al.* 2009), and is supported by studies using natural populations (Hoskin *et al.* 2005, Higgie & Blows 2007, Porretta & Urbanelli 2012).

There are two alternative outcomes for RCD when two lineages come into secondary contact and suffer poor hybrid fitness. The first is the local extinction of one of the lineages due to the cost of maladaptive mating. This is termed species sorting (Lukhtanov *et al.* 2005). The second is the fusion of the lineages that are not sufficiently differentiated, despite low hybrid fitness (Templeton 1981). Both species sorting and differential fusion can generate a pattern similar to RCD where species living in sympatry are more different than species living in allopatry. However, these two scenarios can be distinguished from RCD because they cause the disappearance, in sympatry, of the younger pairs of species, either by extinction or by merging (Jiggins 2006a).

Initially, speciation by reinforcement was popularized by Dobzhansky (1951, Butlin 1987). It remained controversial prior to the development of mathematical models that demonstrated its plausibility (Liou & Price 1994,

Kirkpatrick & Ravigné 2002). Seminal comparative analyses by Coyne & Orr (1989) on *Drosophila* species pairs showed that reinforcement was not an anecdotal process (Noor 1997). Further detailed studies of pairs of incipient species described convincing cases of reinforcement in nature. For example, in *Litoria* frogs (Hoskin *et al.* 2005), insects (*Drosophila*, Noor 1995; *Timema* walking stick, Nosil *et al.* 2003), fishes (*Gasterosteus* stickleback, Rundle & Schluter 1998), birds (*Ficedula* flycatchers, Sætre *et al.* 1997), plants (*Phlox drummondii*, Hopkins & Rausher 2012), or mammals (*Mus musculus*, Smadja & Ganem 2005). In *Drosophila*, a reinforcement process observed in nature could be experimentally produced after only nine generations (Higgie *et al.* 2000). Traditionally, the reinforcement of premating isolation was described as a process driven by natural selection acting on unfit hybrids. However, at the individual level, upon which selection actually acts on traits and preferences, the driving force is sexual selection for compatibility or good genes favouring the production of good quality offspring (Kirkpatrick & Ravigné 2002, Ritchie 2007, Shaw & Mendelson 2013). An unorthodox view of reinforcement would thus be to consider it as a process of speciation by sexual selection.

In their recent review, Ortiz-Barrientos *et al.* (2009) suggested that future research on reinforcement should involve a more detailed understanding of the genetic basis of traits and preferences and of the interaction of reinforcement with ecology and sexual selection. Ortiz-Barrientos *et al.* (2009) also suggested that further comparative analyses should be conducted to assess the frequency of this process in nature.

1.1.5 Sexual traits, sexual selection, and reproductive isolation

Sexual traits and sexual selection

Sexual traits and their evolution by sexual selection are most commonly mentioned as potent actors in the development of reproductive isolation (Panhuis *et al.* 2001, Ritchie 2007). Sexual traits can be grouped in three main types; the organs directly linked to copulation, the secondary sexual traits associated with mate choice, and the dimorphic traits linked to differences in life history traits between the sexes (Andersson 1994). The first and third types of sexual traits mostly evolve under natural selection since they are critical for intrinsic fecundity and survival. I will instead focus on the second type which evolves under sexual selection. Sexual selection is a part of natural selection resulting from the competition for the access to mating partners (Andersson 1994). While both sexual selection and natural selection favour individuals that achieve a better transmission of their genes, sexual selection can act together or opposed to natural selection (i.e. viability or fecundity). A simple illustration is that individuals competing for mates should better die young with many offspring

than grow old and healthy with reduced success. Sexual selection can thus lead to the evolution of amazing traits detrimental to survival if they improve access to mates. In sexually reproducing organisms, it is very common that reproduction is more costly for females given their postmating investment in offspring development and in the relatively smaller number of offspring they can produce compared to males. In addition, the production of offspring by females often requires a much larger time investment than in merely finding a mate, reducing the pool of females available to mate compared to the pool of males despite an even population sex ratio. Thus, females should be the 'choosy' sex, choosing better males to maximize offspring quality. On the other hand, males should mate with as many females as possible as they can afford to mate multiply due to the often limited investment they make in offspring production. Females are the limited resource for which males compete. This is where secondary sexual traits play a role. Competing males must display secondary sexual traits that closely match female preference and thus male secondary sexual traits can evolve under sexual selection exerted by females. Exceptions to this general rule where females compete for choosy males exist in species where male invests equally or more in parental effort than female. Andersson (1994) offers a review of insect, fish, and bird species with male responsible of brood care and sex reversal in the competition for mate.

Mechanisms of trait evolution under sexual selection

In a population with heritable female preference and male secondary sexual traits, females tendency to mate with their preferred males results in offspring inheriting the genes coding for both the preference and the corresponding male secondary sexual trait. Therefore a genetic correlation between the distribution of male traits and female preferences emerges in the population, a mechanism known as 'Fisherian' or 'Lande-Kirkpatrick' null model of sexual selection (Lande 1981, Kirkpatrick 1982, Kirkpatrick & Ryan 1991, Kokko *et al.* 2006, Prum 2010, Rodríguez *et al.* 2013). In this null model, there is an inherent advantage for the female to choose an attractive male which applies whenever there is genetic variability for trait and its preference. This is because the attractive male will sire attractive offspring more likely to mate at the next generation. This is the theory of the 'sexy son'. In this situation, the stronger the preference, the stronger the advantage, in turn encouraging the preference. This self-reinforcing loop is called the runaway process (Fisher 1930, Lande 1981) and could lead to infinite exaggeration of male trait and female preference unless stopped by natural selection or developmental constraints.

It is not only arbitrary sexual traits that are preferred. Since females gain an advantage when choosing the best quality males, the preferred sexual traits are often associated with the higher quality of the male. Direct benefit can be offered

to the female for reproduction in the form of nutritive nuptial gifts, territory, or parental care. Indirect benefits also exist when the males transmit better adapted genes to the offspring. For the display of the male to be honest and reliable for the females, the preferred traits attesting for the benefits are expected to be costly and are often exaggerated relatively to a naturally selected optimum (larger size, more conspicuous colour etc.). This cost insures that lower quality males (in lower condition as they are less adapted to the environment) cannot afford it (David *et al.* 2000). This mechanism is the good-gene viability indicator, or handicap model of sexual selection made popular by Zahavi (1975, 1977), and famously exemplified by the work of Hamilton & Zuk (1982).

Of note, in spite of the asymmetry in reproductive costs and the stronger sexual selection on male ornament, sexually selected traits are not necessarily expected to be sexually dimorphic (Kokko & Johnstone 2002). For example, this could be because of genetic or ecological constraint on the development of both sexes (Lande 1980). Similarly, apparent secondary sexual traits that are dimorphic are not necessarily under sexual selection in the males, but rather under natural selection in the females (Allen *et al.* 2011).

How can sexual traits diverge?

Different evolutionary forces can make the traits diverge between populations. The Lande-Kirkpatrick null model only predicts that traits match preferences in a line of equilibrium, but does not predict the direction in which both should evolve. Sexual traits can evolve simply by genetic drift (Uyeda *et al.* 2009). Since sexual selection is stronger than natural selection (Kingsolver & Pfennig 2007) and more conspicuous differences of secondary sexual traits have been observed between closely related species, it is tempting to think that sexual traits and preference diverge under the action of directional sexual selection. It is realistic when a quantitative trait is preferred ever more exaggerated as developed above. However different populations are expected to evolve in the same direction (larger value of the trait), and natural selection constraint should quickly stop the value of the trait at an upper boundary. A first mechanism lies in the theory of sexual conflict (Gavrilets 2000). Because of the strong competition for access to females, some males develop characters that are detrimental to females. Well known examples are traits linked to sperm competition. Males produce toxic seminal fluids that favour fertilization, or develop copulatory organs to remove the sperm of competitors from the females. These male traits improve their performance in sperm competition but decrease the reproductive success of the females. Situations where the reproductive advantage of one sex is detrimental to the other are termed sexual conflicts (Stockley 1997). Sexual conflicts induce an antagonist coevolution of the two sexes producing runaway evolution. This mechanism was supported by modelling (Gavrilets

2000) and would explain the very rapid evolution of fertilization traits and proteins between related taxa (Gavrilets 2014).

Another context of sexual conflict that can lead to divergent sexual selection on male secondary sexual traits occurs when these traits act as honest signals of good quality males (van Doorn & Weissing 2006). Indeed, the strength of competition for access to females can provoke the appearance of cheaters in the population. These are individuals able to display signals usually indicative of good quality and good genes while not possessing such quality, consequently giving poor reward to the choosy females. It is expected that female preference rapidly evolves toward another more honest male trait. This mechanism is equivalent to the rapid evolution by sexual conflict of traits linked to fertilization modelled by Gavrilets (2000), and could explain rapid divergence of sexual traits between populations by sexual selection.

When combined with ecological constraints, sexual selection can efficiently promote the divergence of sexual traits. The selection of a more efficient sexual signal by the environment is termed sensory drive. Famous examples of this mechanism are known in fishes. In sticklebacks or cichlid fishes for example, there is female preference for more conspicuous male colours in their light environment (Boughman 2001, Seehausen *et al.* 2008). Bluish or reddish signals evolved because they are more conspicuous depending on light availability at different depths in the water column, shallower or deeper water layer respectively. In cichlid fish there are repeated observations of adaptation of the visual system and male colouration to the light environment (Terai *et al.* 2006). This suggests that ecology can play a major role in shaping the direction of sexual preference. Another mechanism of evolution of male sexual traits by sexual selection, sensory bias, predicts that males may exploit a pre-existing attraction of the female toward some cues linked to food for example by producing comparable male traits (Andersson & Simmons 2006). In Guppies, this is the hypothesis explaining the preference of females for orange coloration of male sexual displays (Rodd *et al.* 2002). If food preference diverges between populations of a species, as it can be the case for host plant in insects, we can predict that the male traits of each population will be selected according to different ecology as a result of the sensitivity of the females. Sexual interference with species using the same communication channel can also displace the female preference or narrow its distribution to avoid overlap (Gröning & Hochkirch 2008). This is the process of sexual character displacement which is traditionally considered to be driven by natural selection (Butlin 1987). Reproductive character displacement or reinforcement usually concerns only a part of the species distribution range. This could consequently produce a divergence of the secondary sexual traits within species between populations affected or not by the presence of another species in the same area, as predicted by the 'cascade' hypothesis mentioned earlier (Hoskin *et al.* 2005, Higgie & Blows 2007, Ortiz-Barrientos *et al.* 2009,

Hoskin & Higgie 2010, Porretta & Urbanelli 2012). Finally, natural selection due to the presence of predators or parasites can shape the optimal preference of females differently depending on the ecosystem (Jiggins *et al.* 2001).

The importance of the evolution of sexual traits in speciation

The sexual traits and preference used for mate choice are often particular to one species and therefore individuals of a given species are not attracted to individuals belonging to another species. Therefore, if the mating preference diverges between two populations, the consequence is premating reproductive isolation and so speciation. Because of this potential for speciation, and since secondary sexual traits are often the most divergent between closely related species (Rodríguez *et al.* 2013), their evolution and divergence between populations is believed to be a key factor and of great interest for the study of speciation. Accordingly, since secondary sexual traits are under sexual selection, the role of sexual selection in speciation has long been hypothesized (Darwin 1871, Ritchie *et al.* 2007). A large meta-analysis of several comparative studies on large groups representing various taxa have recently demonstrated that proxies of stronger sexual selection (sexual dimorphism, mating system, testes or spermatophore size) were associated with more speciose groups (Kraaijeveld *et al.* 2011). Since the pattern is stronger for younger species pair comparisons, it further supports the role of sexual selection in promoting speciation.

As discussed above, it is not clear if sexual traits diverge between populations by sexual selection alone. Moreover, if two populations have recently diverged only in their female preferences, males that are attractive to both lineages of females will theoretically be at an advantage in terms of reproduction. They would rapidly invade the system and homogenize the two populations. It is then probable that speciation by sexual selection alone would require an absence of gene flow between populations (for example due to allopatry). One such example likely involves the divergence of the ornament of *Habronattus pugillis* jumping spiders in separated mountain ranges in south-eastern Arizona (Masta & Maddison 2002). Using a phylogenetic approach, they document a higher fixation rate of the display among populations compared to neutral genetic variability, indicating that the rapid evolution of the display is unlikely to be attributed to genetic drift but rather to selection. Since no obvious ecological divergence was detected that could have provoked the divergence of this mate choice trait, the most likely driver of this incipient speciation event is sexual selection.

It is generally accepted that the divergence of sexual traits by sexual selection in the presence of gene flow is made easier with the help of ecological factors. It has long been considered unlikely that sexually isolated species would be able to cohabitate without parallel ecological divergence (Weissing

et al. 2011). A recent model however suggests that a simple and likely common heterogeneity in the distribution of the resource would allow the long term coexistence of ecologically equivalent competitors (M'Gonigle *et al.* 2012). To date, the process of sensory drive described earlier for cichlid fishes represent one of the few example of sympatric speciation involving sexual selection (Seehausen *et al.* 2008). It is now commonly acknowledged that sexual selection is more efficient at promoting speciation alongside natural selection as a result of ecology (Ritchie 2007, Servedio & Kopp 2012). As a neat illustration, a recent analysis of 150 clades of cichlid fishes by Wagner *et al.* (2012) showed that both ecological factors and traits linked to sexual selection predict the diversity of these repeated radiations.

Since the discovery of the cichlid fish radiation system for the study of speciation, adaptive radiation and the ecological dimension of speciation have gained in interest (Schluter 2001, Leal & Losos 2010). Some species radiations, however, display weak or absent ecological differentiation between species compared to the divergence observed in their sexual traits (Mendelson & Shaw 2005, Arnegard *et al.* 2010a). Although ecological differences may be more difficult to detect than differences of sexual traits (Rundell & Price 2009), recent work by Arnegard *et al.* (2010a) revealed that the nature of the communication channel can also have a strong importance in speciation. Indeed modifications of the communication system that allows greater signal diversity seems to have repeatedly increased the rate of speciation (Arnegard *et al.* 2010b, Carlson *et al.* 2011). It appears that a mode of communication with a wide diversity of signals can have a same effect as ecosystems with wide variety of niches, reducing the competition between individuals and favouring the formation of new species by exploitation of new ecological or communication niches (Leal & Losos 2010). Identifying the traits responsible for reproductive isolation, and the context in which they evolved (for example ecological or non-ecological) are key questions to our understanding of the speciation process (Sobel *et al.* 2010).

1.2 Chemical communication

Until recently, visual and acoustic signals have been the phenotypic traits most investigated in the study of mate choice and reproductive isolation (Coleman 2009). This is probably due to a biased anthropomorphic viewpoint that other modes of communication are unlikely to be used in other organisms as they are not important in humans. Other modes of communication include chemical communication. The study of chemical traits and of chemical communication has lagged behind for several decades because the identification of chemical signals requires expensive and advanced techniques such as a strong knowledge

in organic chemistry rarely possessed by biologists, and chemical traits are often difficult to manipulate in behavioural experiments. Despite this, chemical communication is the ancestral and most widespread mode of exchanging information between individuals (Ache & Young 2005). Chemical signals used in mate choice, that is between individuals of a same species, are called pheromones (Karlson & Lüscher 1959). Chemical communication was described in algae, plants, yeast, ciliates, bacteria, and animals (Wyatt 2009, 2014, Chamberlain 2014). Across the animal kingdom, most groups transmit information through sex pheromones (e.g. nematodes: Choe *et al.* 2012; annelids: Zeeck *et al.* 1998, Novo *et al.* 2013; crustaceans: Sneddon *et al.* 2003; molluscs: Cummins *et al.* 2004; arachnids: Gaskett 2007; insects: Cardé & Minks 1997; reptiles and amphibians: Houck 2009; mammals: Burger 2005; fish: Plenderleith *et al.* 2005; birds: Bonadonna & Sanz-Aguilar 2012), and in most diverse ecosystems, from aerial to aquatic, in the soil, and from polar to tropical.

1.2.1 The role of chemical communication in speciation

It has previously been observed that differences in sex pheromones are responsible for reproductive isolation between species and sometimes between populations within species (Klun *et al.* 1975, Linn & Roelofs 1989). The attraction of males by female sex pheromones (FSP) in moths has been extensively studied for its application in biological control. Identifying the components of the FSP allowed the disruption of mate attraction of many crop pests through saturating the environment with FSP and therefore preventing the males to find females and reproduce (Witzgall *et al.* 2008). The European corn borer *Ostrinia nubilalis* (Lepidoptera, Crambidae) has become a model species in the study of chemical communication. The ratio of the two components of the FSP differs between two strains adapted to two different host plants. These opposing ratios make the cross attraction between strains quite rare (Malausa *et al.* 2005, Dopman *et al.* 2010) and is a textbook example of ongoing speciation due to divergence in olfactory communication (Smadja & Butlin 2009). Another compelling example is the reduced quantitative variation of FSP components when multiple species share the same habitat as described in *Yponomeutha* moths by Löfstedt *et al.* (1991). In this system, the ratio of two FSP components to which males are attracted are more different between three species sharing the same host plant than between three species living on different host plants. This pattern likely reflects the avoidance of cross attraction between closely related species in sympatry. In the oligophagous mustard leaf beetle *Phaedon cochleariae* (Chrysomelidae), Geiselhardt *et al.* (2012) demonstrated that males were more attracted to females that were fed on the same host plant species during the previous weeks, based on the plastic response of the cuticular hydrocarbon of the females. Since this assortative mating is based on a plastic preference for a

plastic trait that can both change at the scale of an individual life, it is difficult to draw conclusions on the real reproductive isolation it produces in nature. To conclude, as in many other fields of biology, *Drosophila* fruit flies are also a model system in chemical communication. On the Eastern coast of Australia, two *Drosophila* species share a part of their distribution range, *D. serrata* and *D. birchii*. *D. serrata* males present a reproductive character displacement of their cuticular hydrocarbons (CHC) composition when sympatric to *D. birchii*. Experimental exposition of allopatric populations of *D. serrata* to *D. birchii* caused a similar displacement of the CHC composition to the one observed in sympatric populations in nature (Higgie *et al.* 2000). This reproductive character displacement took place in only nine generations suggesting a strong selection for the avoidance of heterospecific matings (Higgie *et al.* 2000) and a high evolvability of the chemical signals. A subsequent study also demonstrated that females *D. serrata* from localities without *D. birchii* prefer the conspecific males of their own population compared to those having displaced CHC profiles due to sympatry with *D. birchii* (Higgie & Blows 2007). This example is striking in illustrating both the rapid evolution of male chemical signals and the associated female preference, and of the arising reproductive isolation between conspecific populations that experienced different selection pressures. These case studies are strongly consistent with the role of olfactory communication in the development of reproductive isolation further leading to speciation. Despite the ubiquity of chemical sexual signals in the animal kingdom, it remains unclear how frequently reproductive isolation evolves due to differentiation in chemical signals involved in mate choice in nature.

1.2.2 Molecular mechanisms of chemical communication in mate choice

The chemical signals used for intersex communication are sex pheromones. The chemical structure of molecules used as signals can vary a lot between organisms given the huge variety chemicals observed in nature. A large number of compounds have already been observed as participating to mate attraction and mate choice (El-Sayed 2011), from small carbon chains in moths (Zhu *et al.* 1995) to long amino-acid chains in salamanders (Palmer *et al.* 2010). The sex pheromones are either synthesized *de novo* (Figure 1.1), or recycled from compounds found in the diet (Honda *et al.* 2005). The biosynthesis of sex pheromone components has been thoroughly investigated in moths. In these species, the modification of fatty acid precursors are performed through a modular series of enzymatic reaction to shorten the carbon chain and add double bonds and functional groups (Figure 1.1, and see Jurenka 2004). The varying combinations of these enzymes and their differential expression lead to

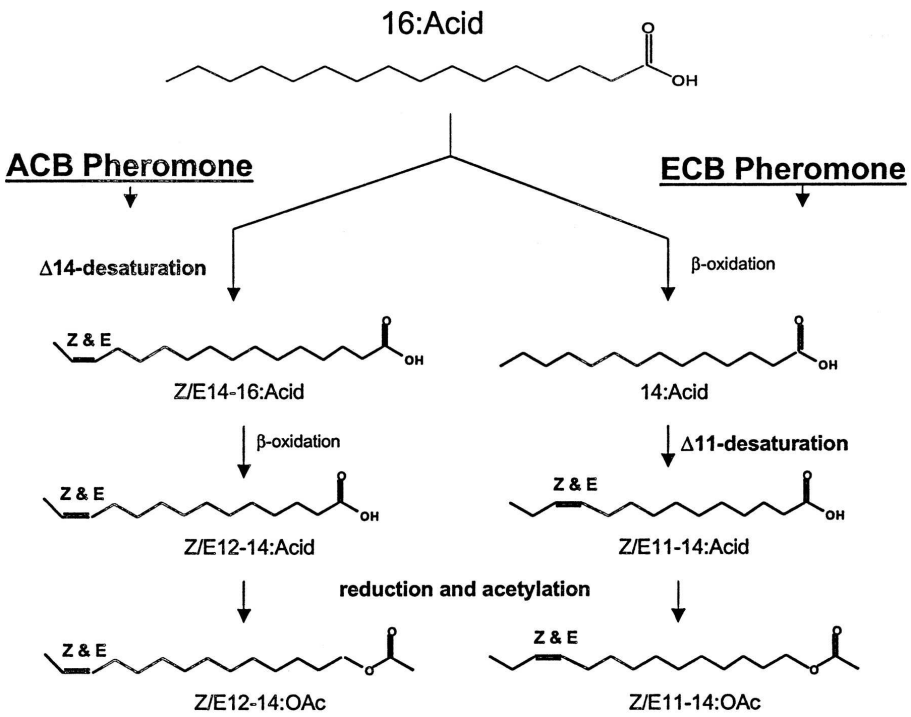


Figure 1.1: Biosynthesis of the female sex pheromone components of the European and the Asian corn borer (ECB and ACB) *Ostrinia nubilalis* and *O. scapulalis*.
As in many moth species, the FSP are composed of fatty acid derivatives. Different enzymes act during the biosynthesis. The desaturases add double bonds to the carbon chains, the β-oxidases shorten the chain by removing two carbon atoms, the reductases transform the acid functional group into an alcohol, and the acetyl transferases change the alcohol into an acetate. Figure from Roelofs *et al.* (2002).

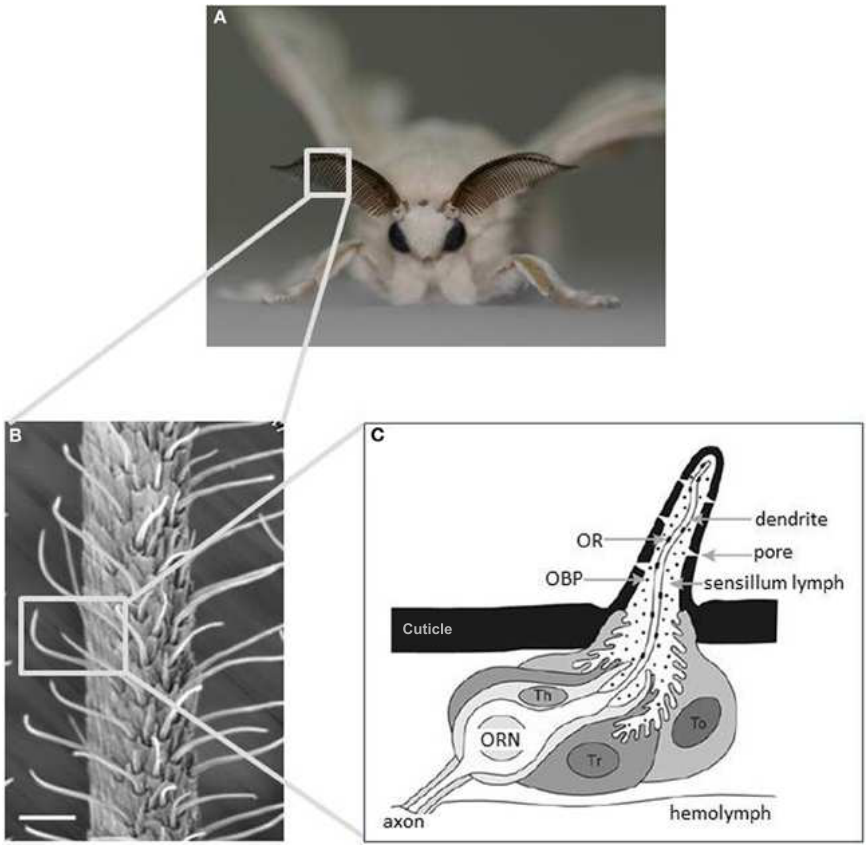


Figure 1.2: Olfactory organ of the silkmoth *Bombyx mori*.
(**A**) antenna of the male moth. (**B**) scanning electron micrograph of an antenna with numerous olfactory sensilla. (**C**) scheme of an olfactory sensillum with the olfactory receptor neuron (ORN) and the three accessory cells: tormogen (To), trichogen (Tr), and thecogen (Th). To and Tr cells secrete odorant-binding proteins into the sensillum lymph. Odorants are detected by ORs expressed on the dendritic membrane of ORNs. Figure from Jacquin-Joly & Merlin (2004) and Sakurai *et al.* (2014).

the observed interspecific differences of pheromone composition (Roelofs *et al.* 2002, Liénard *et al.* 2010, Albre *et al.* 2012).

Chemical compounds are detected by the receivers thanks to olfactory receptors (OR, Figure 1.2). In arthropods these are composed of olfactory neurons whose dendrites are bathed into lymph (Wyatt 2014, Figure 1.2). In insects, odorant compounds enter the lymph thanks to odorant binding proteins (OBP) allowing them to become soluble (Figure 1.2 and 1.3). Odorant-OBP complexes in turn bind to transmembrane proteins of the neuron dendrites to generate action potential and transmit the information to higher neural centres. The genes coding for the transmembrane protein that detects the chemical compounds (OR genes, Figure 1.3) represent large families, rapidly divergent, and highly specialized across species (López-Larrea 2012). The specificity of the OR genes to different compounds can vary (Hallem & Carlson 2006), but OR genes associated with sex pheromone detection can be very specialized to a target sex pheromone component and allow a strong discrimination with structurally close compounds present in related species (Leary *et al.* 2012). OBP may also confer specialized detection of sex pheromones as is observed in *Drosophila melanogaster* (Smith 2012); however it is not yet clear in other taxa.

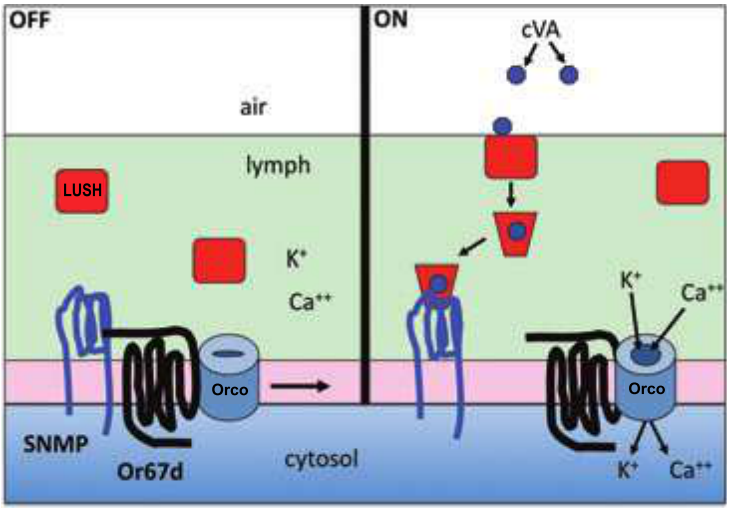


Figure 1.3: Current model for the olfactory detection of the sex pheromone component 11-cis vaccenyl acetate (cVA) in *Drosophila*.
Left, in the absence of cVA, LUSH (an OBP) is in the inactive state, and SNMP (a transmembrane protein) is bound to the complex formed by the OR (here Or67d) and the co-receptor ion channel (Orco), inhibiting ion fluxes. Right, in the presence of cVA, LUSH undergoes a conformational activation that allows it to interact with SNMP, releasing the Or67d/Orco complex and allowing ions to flow. Figure from Smith (2012).

1.2.3 Peculiarities compared to other modes of communication

Compared to visual or acoustic modes of communication, olfactory communication seems particularly prone to be used in sexual communication. This is likely to lead to reproductive isolation thanks to the large variety of signals that can be produced and to the privacy of the perception mechanisms that are associated with olfactory signals. One important feature of olfactory communication is the modular combination of families of enzymes involved in sex pheromone biosynthesis that can be combined in numerous ways to produce a seemingly endless number of compounds (Jurenka 2004, Byers 2006). This feature allows the olfactory communication system to evolve in endless dimensions. Another major feature of olfactory communication is the discrete nature of the chemical structure of the signals that require specific receptors. Whereas visual or acoustic sensory organs can detect a continuous range of signals, specific chemical compounds can only be detected by specific olfactory receptors. This feature allows the olfactory communication system to produce strong privacy regarding illicit receivers, such as predators or parasitoids (Zuk & Kolluru 1998, Haynes & Yeargan 1999, Steiger *et al.* 2011). It could be argued that some part of the continuous range of light and sound signals, like UV or ultrasounds, display the same pattern of private communication limited to individuals harbouring the appropriate visual or acoustic receptors. However, once an illicit receiver develops the ability to detect such visual or acoustic signals, no more sensory escape is available to the targeted emitter individual. In the case of olfactory receptors and signals, plenty of possible alternatives are available (Byers 2006) and an illicit receiver would have to be highly specialized to its prey to follow its evolution. Therefore the evolution of olfactory signals may suffer fewer constraints than public and more conspicuous sexual signals such as visual or acoustic ones.

How does such a private and strongly signal-receptor specific communication system evolve? One hypothesis is that many chemical compounds are inadvertently released by the individuals of a species and can be recycled as signals whenever receivers develop appropriate receptors (Steiger *et al.* 2011). Because the use of olfaction is widespread for various functions across animals, an important part of the genomes codes for OR proteins. For example in vertebrate and *Caenorhabditis elegans*, about 1000 genes are involved in olfactory detection of chemical compounds (1 to 5% of the coding proteins of the genome; Vosshall *et al.* 1999). Therefore a large number of OR can be recycled, duplicated and mutated for specialized use in sexual communication. To summarize, the availability of diverse chemical cues and of diverse OR to detect them likely allow rapid evolution of olfactory sexual communication, in a context of released ecological constraint thanks to high privacy of the signals.

Chapter 2

Objectives of the thesis and model organism

2.1 Objectives of the thesis

This work aimed at studying the evolution of a male sexual olfactory signal and its importance in the development of reproductive isolation between species. The underlying hypothesis was that the type of communication involved, olfactory communication, allows a less constrained evolution of the signal, which is in turn favourable to the development of reproductive isolation.

Using a group of closely related species, we aimed first at characterising the diversity of male sex pheromones across species and at understanding which evolutionary forces and ecological factors were shaping it. Our predictions were that, if the sexual olfactory signal was involved in reproductive isolation, it would display strong species-specificity, rapid evolution as well as a pattern of character displacement between sympatric, compared to allopatric, pairs of closely related species.

Secondly we wanted to characterize the diversity of the sexual olfactory signal at the intraspecific level, since it is the level at which reproductive isolation emerges to produce new species. We predicted that, if the sexual olfactory signal was involved in reproductive isolation, its differentiation would occur early, in a context of low genetic divergence.

2.2 *Bicyclus* as a study system

The butterfly genus *Bicyclus* (Kirby 1871, Nymphalidae: Satyrinae) is rich, with 88 described species that spread across sub-Saharan Africa (Condamin 1973, Sáfián *et al.* 2009, Figure 2.1). Distribution ranges widely vary in size depending on species, from highly localized (*B. amieti*) to ubiquitous across the

geographical region (*B. safitza*). Estimations based on molecular trees and the few Lepidoptera fossils place the period of diversification of this genus during the Miocene, and the more recent speciation events between 0.5 and 5 million years ago (Kodandaramaiah *et al.* 2010). Most *Bicyclus* species inhabit wet forest habitats and some are adapted to more open woodland savannah (Condamin 1973). Large number of species can be observed in sympatry (around 20 species in a single forest patch) which raises the question of the mechanism allowing their coexistence and reproductive isolation. Larval development of these species take place on Poaceae, with some clear exceptions (Larsen 2005). However, the biology of most of the species regarding resource use remains largely unknown. For example regarding host specialization, Satyrids are thought to be generalist on Poaceae in Europe (Bergman 2000), but tropical species could be different. Adult *Bicyclus* feed on fallen rotten fruits, which facilitates greatly their capture with fruit baited traps. A striking feature of the *Bicyclus* genus is the seasonal polyphenism displayed by most of the species, with wet and dry seasonal forms of wing pattern according to the climatic condition experienced during larval development. This is the ecological characteristic that initiated the scientific interest toward several species, especially *B. anynana* (Butler, 1879) (Brakefield *et al.* 2009). Brakefield and collaborators studied in detail the developmental biology of *B. anynana* in the lab, thanks to rearing under controlled conditions. Particular attention has been paid to the development of wing eyespots and its genetic bases. Similarly to evo-devo research on *Drosophila*, a series of mutant for different coloration patterns were isolated and advanced genetic tools were developed for examining these questions (Beldade & Brakefield 2002, Marcus *et al.* 2004). It was shown by artificial selection that various combinations of eyespot size could evolve (Beldade *et al.* 2002), raising the question of their biological significance. Behavioural experiments demonstrated the importance of male dorsal wing eyespots in mate choice, particularly the white UV reflective pupil (Robertson & Monteiro 2005, Costanzo & Monteiro 2007, Chapter 4 and Figure 4.1A). On the other hand, ventral eyespots seems to be linked to predator deflection (Olofsson *et al.* 2010). Comparative analysis of these wing eyespots across the genus also unravelled a difference in the mode of evolution of the two groups of eyespots, suggesting the effect of sexual selection on the dorsal forewing group (Oliver *et al.* 2009). The study of sexual traits involved in mate choice also revealed a role for male sex pheromones (Costanzo & Monteiro 2007, Nieberding *et al.* 2008, Chapter 4 and Figure 4.1A). Indeed three chemical compounds were related to male mating success, with females using these compounds to select older males and avoid inbred ones (Nieberding *et al.* 2012, van Bergen *et al.* 2013). A more thorough investigation of the chemical profile including less volatile cuticular hydrocarbons revealed that some compounds are associated with age or sex in various body parts and as such may convey information during courtship interactions and

contacts between partners (Heuskin *et al.* 2014). Finally, although *Bicyclus* can display seasonal polyphenism within species, the discrimination of closely related species in the field is not straightforward. The diagnostic trait generally used is the number and position of male wing structures, composed of modified wing scales and named androconia (Condamin 1973, Chapter 4 and Figure 4.1A). These androconia are assumed to produce the male chemical signal, confirmed by the reduced mating success after their ablation or inactivation in *B. anynana* (Costanzo & Monteiro 2007, Nieberding *et al.* 2008). Altogether, the large number of coexisting species, their wing pattern similarity, and some morphological differences putatively associated with male sex pheromones, suggest that the evolution of chemical sexual signals in this genus is a key factor to its diversification. The *Bicyclus* genus is therefore a well suited model for the study of the role of sexual olfactory communication in speciation.

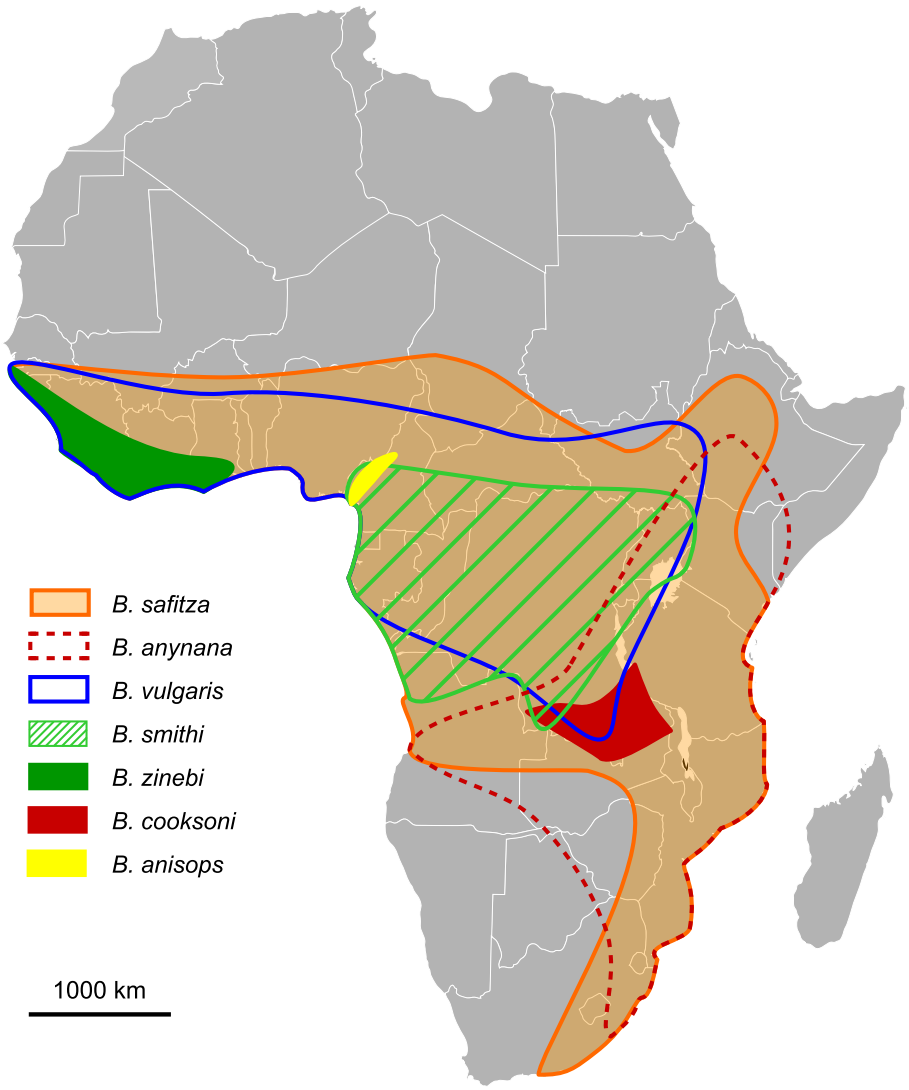


Figure 2.1: Distribution ranges of seven *Bicyclus* species.
B. safitza is the more widespread species of the genus. Distribution ranges are derived from Condamin (1973).

Chapter 3

Outline of the thesis

The first chapter of this thesis is a bibliographic review about sexual selection in *B. anynana* (**Chapter 4**). Since it was published in 2011, new results came out on this topic. Those concerning male sex pheromones are mentioned earlier in the description of the model organism.

Before this work, only preliminary sampling and analyses had been performed in an attempt to characterize the diversity of male sexual signals in different species of *Bicyclus*. The first necessary step was the investigation of interspecific diversity and was achieved by sampling males and females of as many species as possible and from different locations across the distribution range of the genus (**Chapter 5**). This large dataset involving 32 *Bicyclus* species revealed a wide diversity of chemicals with strong differences between species and allowed us to address several questions. The study of 32 species in concert made the use of conventional experimental methods inapplicable for the identification of male sex pheromone truly involved in mate choice. We therefore developed a standardize methodology to select major male compounds likely important for the male sex pheromone composition of each species ('pMSP' for putative male sex pheromone). This method was based on the knowledge on *B. anynana* male sex pheromone which is composed of three male-specific chemical compounds present at large concentration. Using the molecular phylogenetic tree of the genus, we performed a comparative analysis of these 32 species to investigate the mode of evolution of their pMSP. If there was a sexual signal, under sexual selection and suffering few extrinsic constraints, we expected to observe a large diversity and a rapid evolution. In addition if this signal plays a role in species recognition, we expected to observe stronger differences between pairs of species living in sympatry compared to allopatric ones. We compared the speed of evolution of the pMSP to that of other traits under sexual selection or natural selection, the different wing eyespots and to the androconial structures assumed to release the chemical signal. As expected, the pMSP

displayed a rapid evolution compared to the other traits. The selected pMSP compounds showed repeated and rapid appearance and disappearance along the phylogenetic tree. Moreover, we observed that species living in sympatry displayed a larger number of pMSP differences than species living in allopatry. This pattern of reproductive character displacement is coherent with the role of pMSP in premating isolation. We showed that alternative processes that could generate a similar displacement pattern were unlikely to have produced our data. The stronger difference of pMSP between species living in sympatry does not seem to be influenced by the environment. For example the compounds potentially derived from host plants do not display a higher species specificity than the compounds synthesized *de novo*.

A second step of this work (**Chapter 6**) was to investigate potential pMSP differences between populations within species. This more fine scale analysis allowed us to test whether chemical differences can arise before the complete reproductive isolation of the populations, i.e. the end of a potential speciation process. This is relevant because sexual trait differences can develop after the species have isolated and as such would not constitute a trigger to the development of reproductive isolation. We sampled five populations of the model species *B. anynana*, two of which more thoroughly to further investigate the pMSP variability within population. We observed quantitative and qualitative variation of pMSP composition among populations. Since the populations of *B. anynana* displayed a lower level of genetic differentiation than was found between species, and since we detected some evidence of hybridization, we could conclude that they are still able to exchange genes and thus are not separated species. Moreover, we observed large differences of pMSP composition within one population that were not associated with genetic differentiation between individuals. This further supports that differences in chemical sexual signals can appear in the presence of gene flow. Additionally, to evaluate the generality of the pattern that we observed in *B. anynana*, we sampled two geographically distant populations of each of three other *Bicyclus* species. Despite the small sample size, we could detect a chemical differentiation of the same magnitude in one of the three, *B. vulgaris*. This suggests that pMSP divergence within species is not restricted to exceptional cases, and that this trait could be a sexual signal involved in future reproductive isolation and speciation in the genus.

The results outlined above are all based on the developed method of selection of the pMSP in the 32 species. Therefore it is a natural follow up to verify some of them with experimental approaches. As a third part of this work (**Chapter 7**), we intended to experimentally investigate the activity of male sex pheromones of species other than *B. anynana*. We attempted to identify physiologically active compounds of the male blend of *B. smithi* using a GC-EAD protocol previously used for *B. anynana*. This produced no result which may be explained by the much lower sensitivity of *Bicyclus* female antennae compared

to the usually tested male moths antenna, as well as to the limited material that was sampled in Africa and directly used for the experiments in Lund University. The second experiment aimed to test if reproductive isolation between *Bicyclus* species is only due to the absence of conspecific MSP components or if some heterospecific compounds act as repulsive signals. We investigated the repulsive effect of a pMSP compound of *B. safitza* by adding it on the wing of *B. anynana* males. No decrease of the reproductive success was recorded compared to control males, and unexpectedly, the time until copulation was lower for *B. anynana* males perfumed with *B.safitza* pMSP than for the control males. It is unclear whether this heterospecific pMSP component acts on the motivation of the males during courtship or on the preference of the females.

This manuscript ends with a general discussion of the implication of the different results obtained and of future research perspectives that they generated (**Chapter 8**).

Chapter 4

Mate choice and sexual selection in a model butterfly species, *Bicyclus anynana*: state of the art*

Abstract: *Bicyclus anynana* (Lepidoptera: Nymphalidae) is a widely used model species in evolutionary and developmental biology. This species is part of a highly diversified subtribe (Mycalesina) resulting from a rapid species radiation in which sexual selection, in concert with adaptation to new environment may have played an important role. In this paper, we review the current knowledge about sexual selection in *Bicyclus anynana*. We discuss in an integrated view all traits identified so far as being relevant in mate choice: white pupil of fore wing dorsal eyespots, male sex pheromone and to a lesser extend wing size. We discuss also the potential functional roles of these traits for species and sex recognition and as information source on mate quality. We aim at highlighting the consequences of the large diversity of methodological approaches used so far and we briefly underline understudied traits (i.e. behavior) that could potentially be important for mate choice.

*This chapter was written by Gilles San Martin, Paul Bacquet and Caroline Nieberding and published in 2011 in the *Proceedings of the Netherlands Entomological Society Meeting* **22**: 9–22.

4.1 Introduction

A striking sexual dimorphism with males displaying secondary sexual structures, such as the feathery antennae of male moths, was described early in modern Evolutionary Biology (Darwin 1871). It caused astonishment because male exaggerated ornaments were likely to decrease individuals' survival and as such were in disagreement with the increasingly accepted theory of natural selection. Darwin himself proposed that sexual selection, rather than natural selection, could account for the presence and the evolution of these sex-specific traits (Darwin 1871). Sexual selection can be seen as a component of natural selection in which the reproductive success is determined by one sex in the population, not by the rest of the environment. Simply stated, sexual selection is a consequence of the fact that, in most species, females invest more in reproduction than do males. Females are therefore the limited resource in male-female sexual interactions: males usually compete for getting access to females and females play the role of the choosy sex. Female choice is based on some male traits which, if heritable, will become more common and potentially more remarkable in the population across generations. In species in which males invest a lot in parental care, such as some species of waders, sexual selection is reversed and males play then the role of the choosy sex (Andersson 1994).

Since *The Descent of Man, and Selection in Relation to Sex* (Darwin 1871), the original debate between Darwin and Wallace about the relative importance of sexual vs. natural selection in producing sexual dimorphism has taken the front scene again. The importance of natural selection in driving sexual dimorphism in the Lepidoptera was recently reviewed in details (Allen *et al.* 2011). Mating success depends on (1) the ability to find and (2) be chosen by mates (mate choice). Firstly, the ability to find a mate includes the steps of mate prospection efficiency, and pugnacity or motivation during courtship, which are likely to be partly under natural rather than sexual selection. Second, mate choice is also composed of several steps, some of which may also be under natural rather than sexual selection: intra-sex conflicts (e.g. male-male fights), pre-copulation mate choice (i.e. female choosing between males), and post-copulation mate choice (e.g. cryptic female choice between the sperm of several mates, Arnqvist & Rowe 2005).

Sexual selection is commonly explained by the following, nonexclusive, mechanisms (reviewed in Andersson & Simmons 2006):

- *Direct phenotypic effect or direct benefit*: male ornaments indicate their ability to provide a material advantage in case of reproduction (nuptial gifts, large territory or parental care). For example, many butterfly males transfer a spermatophore to the female during mating. Spermatophores may contain nutrients enhancing the female reproductive potential.

- *Indicator mechanism or indirect benefit*: male ornaments are linked to a genetic advantage it will transmit to the offspring.
- *Sensory bias*: female preference is here supposed to have initially evolved by natural selection (e. g. preference for a specific coulour indicative of food quality) and secondarily exploited in male ornaments.
- *Fisherian runaway, theory of the sexy sons*: Female preference is directed towards male ornaments preferred by the majority of females, so that male offspring will display the preferred ornaments and will thus be preferred too. If both male ornament and female preference are variable and heritable, a self-reinforcing correlation between trait and preference can appear (runaway mechanism). The ornament or signal targeted by the preference can be arbitrary (i.e. not linked to any direct or indirect advantages), yet the runaway mechanism can strengthen other mechanisms of sexual selection.
- *Genetic compatibility*: mate quality may depend on mutual genetic affinities in a one-to-one combination rather than associated to intrinsic absolute differences between individuals. For example, a selective advantage is provided by the maximization of the genetic diversity of the major histocompatibility complex (genes coding for immunity), as more genetically divergent individuals produce better offspring. Whereas aforementioned mechanisms allow for the most part females to assess male quality, in this last mechanism females may also assess whether the encountered individual is of the right sex or of the right species (sex and species recognition systems, Ryan & Rand 1993), in order to minimize genetic incompatibilities.

4.2 *Bicyclus* as a model species

Here we will summarize the accumulating information affecting mating success in the tropical butterfly *Bicyclus anynana* (Butler, 1879) (Nymphalidae: Satyrinae). *Bicyclus anynana* was at first brought to the laboratory in order to study phenotypic plasticity of multicoloured rings present on the wings (eyespots, Brakefield & Reitsma 1991). The most remarkable feature of this African species is indeed its seasonal polyphenism: a form typical of colder and drier seasonal environment (DSF) and a form representative of a hot and wet season (WSF; Figure 4.1; reviewed in Brakefield 2010). To fit the different ecological constraints of these alternating dry and wet seasons typical of the tropics, the differential expression of several traits is induced by the developmental temperature experienced by the larvae. Differences between the two forms concern

morphological differences in wing patterns, physiology and life history traits (Brakefield 2010). Since these early studies, *B. anynana* has become a model invertebrate for more fields of research including evo-devo of butterfly eyespots, and the study of life-history and ageing processes (Brakefield 2010). The relative convenience of its all-year-round rearing, its short generation time, its size allowing marking-recapture protocols and the species richness of the *Bicyclus* genus (over 80 subspecies described; Condamin 1973) as a generous source of comparative data have made this species a model well suited for a broad range of techniques to study ecology, evolution and development (Brakefield *et al.* 2009).

Several studies have investigated sexual selection issues in *B. anynana*, and recently the courtship behaviour of male *B. anynana* was described in details (Nieberding *et al.* 2008). Male courtship is composed of a ritualized succession of four steps (orientation, flickering, thrust and attempting copulation) which allow the male to display its sexual ornaments and the female to accept or reject it. Previous studies showed that the presence of the white pupils in the centre of the dorsal forewing eyespots (Robertson & Monteiro 2005) and the production of male sex pheromone (Nieberding *et al.* 2008) contribute to male mating success. Mating success is also affected by wing size (Frankino *et al.* 2005, 2007) but see the corresponding discussion. Additional traits likely affect mate choice and sexual selection but have not been tested so far: male vigour during 'fights' (flight display of several males that seem to discourage each other of approaching females) and courtship, and acoustic communication during courtship. We here discuss in an integrated view all traits identified so far as being relevant in mate choice and suggest the mechanism of sexual (or natural) selection acting on each trait. We also aim at highlighting the consequences of the large diversity of methodological approaches used so far in producing somehow biased or contradictory results across studies.

4.2.1 The experimental setup matters

Male mating success is assessed using behavioural experiments in which males are submitted to different treatments or put in different groups based on morphological differences and then competed in a 2:1 to 4:1 male-female sex ratio for mating success to wild-type virgin young females. Two main setups are used. The one most commonly used consists of a small cage (ca. 30 $\times$ 40 cm) in which a female is given the choice between 2–4 males, and couples are removed once a mating was initiated. Breuker & Brakefield (2002) add a second female which chooses between the remaining two males after the first couple has been removed. The second setup consists of releasing a large number of males and females in a tropical greenhouse to mimic their natural environment and densities (Frankino *et al.* 2005, 2007, Nieberding *et al.* 2008,

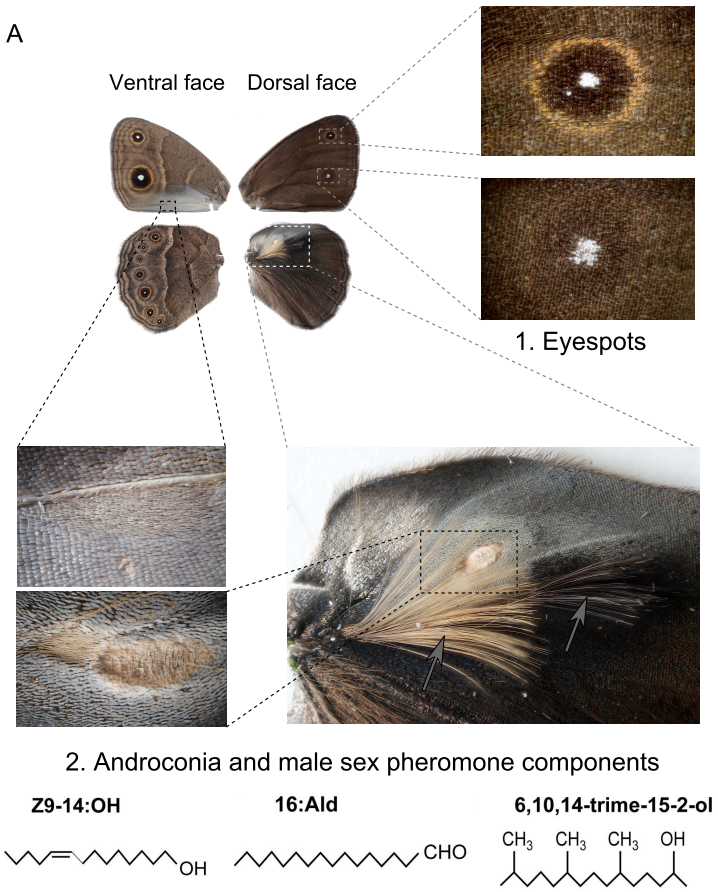
Fischer *et al.* 2008 used a large cage of about ca. 2 m^3). Male genitalia are then marked with coloured fluorescent powders which are transferred to the female genitalia during mating. The necessity for validations of behavioural data in semi-natural conditions was highlighted in *B. anynana* as the conditions of captivity may influence behavioural responses and affect the expression of key courtship traits (Joron & Brakefield 2003). In the large-scale greenhouse setup, both the ability of the male to locate and reach a potential mating partner and the close-range male courtship are assessed, so that the global male mating success is tested. This setup mimics the natural conditions of courtship better than small competition arenas, in which the first step of male mating success is not permitted. Additionally, mate availability is limited in small competition arenas, which is also likely to affect female choosiness.

4.2.2 Eyespots

Wing eyespots in *B. anynana* are composed of a white pupil surrounded by black and gold rings. They are present on the dorsal and ventral sides of the forewing and on the ventral hindwing of both sexes (Figure 4.1). So far, most of the work on mating success in *B. anynana* focused on several features of these eyespots, which were tested for their effect in mate choice and sexual selection. Features include global size of eyespots, relative size of gold and black rings, and size, shape and UV reflectance of white pupils. Eyespot size treatments are based on the choice of extreme morphologies naturally occurring in the lab population (Robertson & Monteiro 2005), or produced by artificial selection (Beldade 2002), or by wing injury performed during wing development. In the latter case, injury of the future position of the eyespot or ectopic injury produces smaller than normal eyespots if the injury is done shortly after pupation, whereas later injury induces bigger than normal eyespots (French & Brakefield 1995, Brakefield & French 1995, Breuker & Brakefield 2002). Males with contrasting sizes of black and gold rings and of white pupils were obtained using artificial selection (Beldade & Brakefield 2002, Allen *et al.* 2008). Eyespot pupils can be suppressed by applying black paint or a transparent solution that decreases UV reflectivity (Robertson & Monteiro 2005, Costanzo & Monteiro 2007, Prudic *et al.* 2011).

Size and brightness of the ultraviolet reflecting pupils present on the dorsal forewing appear to be the only eyespot feature that matters to females. Pupil absence is strongly selected against, as are artificially enlarged pupils, whereas small to intermediate (normal sized) pupils seem to function equally well (Robertson & Monteiro 2005). To our knowledge, the relative importance of upper and lower dorsal forewing pupils has not been tested so far.

The other eyespots features (ventral forewing and hindwing eyespots, black and gold rings, eyespot global size) are not under direct mate choice. However,



correlations between these features and white pupils (R = 0.8–0.86 for pupil and eyespot diameters, Nieberding *et al.*, in preparation) could lead to apparent sexual preference on the other traits. Moreover, contradictory results found by Breuker & Brakefield (2002), in which females preferred normal-sized black rings of the dorsal forewing eyespots compared to smaller ones, may arise from their methodological protocol. Breuker & Brakefield (2002) produced smaller than normal eyespots by damaging the cells positioned on the future eyespots early during development, and it is likely that such smaller eyespots had no pupil left (French & Brakefield 1995). As such, the absence of white pupils rather than eyespot size was likely counter-selected by females in their experiment.

Interestingly, although it is generally admitted that there is no sexual dimorphism in *B. anynana* eyespots (Brakefield *et al.* 1998, Breuker & Brakefield 2002), it was recently shown that pupils display seasonal, but also sexual, cryptic dimorphism: female pupil size is stable across the wet and dry seasons, but

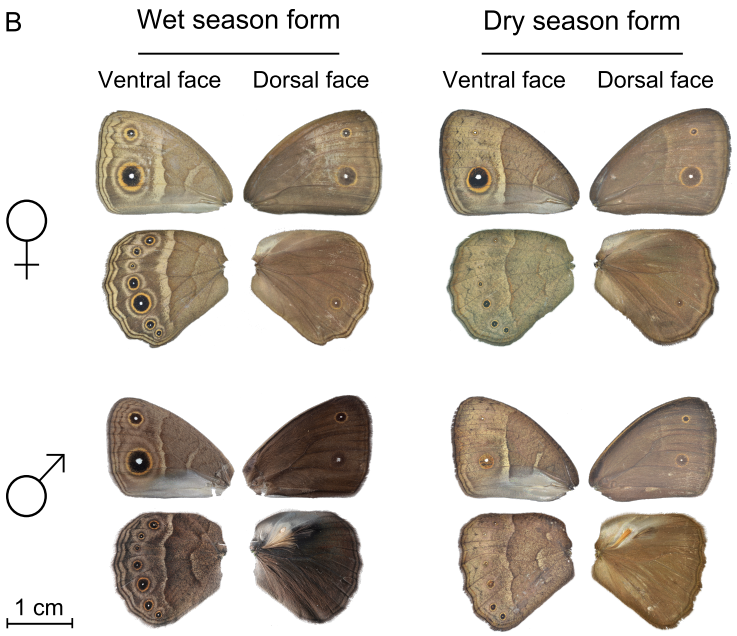


Figure 4.1: (**A**) Traits known to be under sexual selection in *Bicyclus anynana* illustrated for a male of the wet season form: 1) UV reflectance of the white pupil on the forewing dorsal face eyespots; 2) Male sex pheromone components produced by the androconia situated on the ventral face of the forewing and the dorsal face of the hindwing (arrows point the hairpencils). (**B**) Ventral and dorsal coloration of the wet and dry season forms in males and females.

male pupils are approximately 3–6 times smaller than female pupils in the wet or dry seasons, respectively (Prudic *et al.* 2011). UV-reflectance of these white pupils varies significantly between seasons for both sexes (Prudic *et al.* 2011). Such size differences are biologically meaningful as females display marked mate preferences for males with pupils within this range of size differences (4 times larger pupils for 'large' males compared to 'smaller' ones; Robertson & Monteiro 2005). The existence of sexual dimorphism in this eyespot feature may matter for sex recognition: pupil size may indicate sex status as females avoid males with larger than normal pupils, a female characteristic (Breuker & Brakefield 2002, Robertson & Monteiro 2005). The presence of a male-specific sex pheromone (see below) is likely an additional signal for sex recognition but we cannot exclude multiple cues for sex recognition.

So far there is little experimental evidence about the information conveyed by eyespot features to females, which prevent us from predicting the long-term evolution of the traits across *Bicyclus* species. Oliver *et al.* (2009) suggest that the function of eyespots depends on the wing surface considered: dorsal forewing is under sexual selection, whereas ventral hindwings are associated

to predator escape, a function often invoked for butterfly eyespots. Across *Bicyclus* species, dorsal forewing eyespots evolve more rapidly than eyespots from other wing surfaces in agreement with the higher rate of evolution usually displayed by sexually selected traits (Oliver *et al.* 2009). Moreover, dorsal forewing eyespots, together with three other traits involved in mate choice, evolve at different rates between males and females, further supporting their hypothesis.

Ventral hindwing eyespots would thus only be under natural selection pressures. However, the literature gives little evidence that *B. anynana* eyespots are associated to predator escape (Lyytinen *et al.* 2003, 2004, Vlieger & Brakefield 2007), but the efficiency of eyespots as predator escape features could depend on the context, e.g. the light conditions during the experiment (Olofsson *et al.* 2010). Regarding dorsal forewing eyespots, varying levels of fluctuating asymmetry of eyespot pupils, a feature usually associated to male quality, was not associated to female mate choice (Breuker & Brakefield 2002, 2003). The left-right symmetry of the black rings and not of the white pupil size was here measured but it is likely that the treatment had an impact on both features of the eyespots (French & Brakefield 1995).

Thus, so far there is little information about the role of eyespots in conveying information about male quality to females. In addition to the aforementioned potential role of eyespots in sex recognition, eyespots may also play a role in species recognition, i.e. in reproductive isolation, as a large variation is observed in the size of forewing dorsal eyespot size and of the black and gold rings across *Bicyclus* species (Brakefield & Roskam 2006, Brakefield 2010). Oliver *et al.* (2009) found also that several pairs of *Bicyclus* sister species differ in the composition of dorsal wing ornaments. However, these studies do not focus on the specific features of the eyespots (pupils) that play a role in female mate choice in *B. anynana*. Part of the interspecific variation of eyespots is probably correlated to pupil variation, but the range of phenotypic variation for the pupils (presence or absence of the two pupils, limited range of size) is limited compared to the variability of the other features of eyespots. We propose (but this remains totally speculative) that the simple presence or absence or size of the pupils is not very species-specific but that the combination of their presence and of the male courtship behaviour (e.g. frequency of wing flickering) acts as a speciesspecific visual 'Morse code' to the female. It would also be timely to test whether females from species other than *B. anynana* use additional eyespot features in mate choice.

4.2.3 Wing size

Fore- and hindwing sizes are strongly correlated in *B. anynana* (Frankino *et al.* 2005). The first behavioural experiments were performed in small cages

using the variation in size naturally occurring in the lab-population. These experiments concluded that mate choice is not influenced by differences in wing size (Breuker & Brakefield 2002, Robertson & Monteiro 2005). In contrast, 'normal-sized' males displayed a higher mating success than large- or small-winged males obtained by artificial selection (fore- and hindwings selected in the same direction) and competed in large-scale, more natural-like, conditions (greenhouse experiments, Frankino *et al.* 2005). The allometric relationship between foreand hindwings is also important as the mating success of 'normal'-sized males is much higher than males with artificially modified allometries (fore- and hindwings selected in the opposite directions, producing 'large forewing and small hindwing' males, and vice-versa, Frankino *et al.* 2007). Alternatively, artificial selection on wing size could have modified correlated traits known to affect mate choice: pupil and androconia size (see below), which was not checked. Lastly, these results may be either due to female mate choice (and corresponding stabilizing sexual selection on wing size or correlated traits) or to the male aptitude to reach the female (natural selection on male flight ability).

4.2.4 Male sex pheromone (MSP)

Sex pheromone production is suppressed by applying a 'scent-blocking' nail solution on the androconia of young males (Robertson & Monteiro 2005, Nieberding *et al.* 2008), or by removing the androconia with fine scissors (Nieberding *et al.* 2008), before they start pheromone production. Costanzo & Monteiro (2007) recently showed using a small cage set-up that the androconia are important to *B. anynana* male mating success. Males for which the putative pheromone production of the fore- and hindwing androconial structures was blocked mated less often than control males in male-male competition experiments. They found that the chemicals emitted by both androconia were equally important for female choice. Moreover, they showed that the chemicals are likely to be perceived by the female antenna, because when the latter were blocked, females were no longer able to discriminate between scented and unscented males. Costanzo & Monteiro (2007) suggested that the MSP in *B. anynana* could be involved in sex recognition, because they observed that males would often attempt to court males that had their androconial structures blocked.

Recently, we have shown that the transfer of pheromone from males to females in *B. anynana* is probably associated with the flickering and thrust phases of courtship (Nieberding *et al.* 2008). We identified by gas chromatography coupled to electro-antennograms the male components that induced a female antennal response, characterized their chemical structure, namely (Z)-9-tetradecenol (Z9-14:OH), hexadecanal (16:Ald) and 6,10,14-

trimethylpentadecan-2-ol (6,10,14-trime-15-2-ol), hereafter MSP1, MSP2 and MSP3, respectively. We confirmed that the androconia are involved in their production which occurs after adult emergence. We also assessed the behavioural activity of these components in semi-natural conditions (Nieberding *et al.* 2008). Taken together, the absence of supplementary secondary sexual structures on the body of males (Condamin 1973), the absence of novel synthesized components by males reared on the native food diet, and the recovery of male mating success of perfumed operated males, suggest that the three identified components form the full MSP blend of *B. anynana* (Nieberding *et al.* 2008).

These results together highlight that the production of a MSP is important for mate choice. Yet, there is so far no published information about the existence of sexual selection acting on the MSP blend. Accumulating observations suggest that sexual selection on MSP composition is likely: MSP titres and ratios display a high variability in stock males of *B. anynana* providing an opportunity for sexual selection (Nieberding *et al.* 2008). Our analysis of courtship also revealed that virgin females frequently reject one or more males before accepting a mate, and that female rejection usually occurs directly after the flickering and thrust phases when MSP transfer probably occurs suggesting that females could use MSP to assess the quality of individual males. If sexual selection is acting on MSP composition, variation in MSP blend may be associated with variation in male 'quality' (i.e. direct or indirect genetic benefits). The toolkit developed for *B. anynana* (e.g. methodology of micro-surgical manipulations, synthetic pheromone components and experimental competition assays) enables the relevant experiments to be performed to investigate the type of information that is conveyed by *B. anynana* MSP. Finally, ongoing work on MSP diversity in the whole *Bicyclus* genus reveals large differences in MSP composition between species. Rapid evolution of the MSP signal suggests Fisherian runaway dynamics, and the differences between species could allow females to use MSP as a species recognition tool.

4.2.5 Sexual (and natural?) selection on the male side

Cost of reproduction in females is well known and studied, and one generally expects to observe trade-offs between life-history traits related to reproduction. For example, larval and adult diets affect egg size, a trait correlated to female fitness in most insect species (Bauerfeind & Fischer 2009). It is now generally accepted that reproduction is costly for males as well, thus males too may be subjected to trade-offs between investment in reproduction and other life-history traits such as longevity. We start to understand at which level male cost for reproduction may be positioned. *B. anynana* males produce a spermatophore that contains both fertile and non-fertile sperm. Both types are transferred to the female during copulation and non-fertile sperm can constitute up to 95% of

an ejaculate (Lewis & Wedell 2007). Spermatophores in *B. anynana* are small in comparison to other Lepidoptera and do not contain large amounts of nuptial gift (Ferkau & Fischer 2006). Yet, spermatophores decrease in size between first and subsequent matings, and later spermatophores have a decreased dry mass and a higher water content suggesting that spermatophore production is indeed physiologically costly (Ferkau & Fischer 2006). Similarly, in *Bicyclus* butterflies, which feed on rotten fruits as adults, adult diet did not affect male mating rate or sperm production except that the first transmitted spermatophore was bigger for males fed on fruit than with sugar. Bigger males produce bigger spermatophores with both more non-fertile and fertile sperm (Lewis & Wedell 2007). Lastly, variation in the number of previous successful copulations or the sodium composition of the male diet did not affect female reproduction in terms of number of eggs, sodium contents of eggs, but may slightly affect egg hatchability (Molleman *et al.* 2004).

It remains generally unclear how life-history factors have shaped lifetime investment into male sexually selected traits (Fischer *et al.* 2008). Fischer *et al.* (2008) investigated male reproductive success in relation to age and nutritional status, condition and wing damage. They propose that older males should be more willing to invest in potentially costly behaviour (e.g. courtship) as they age, as older males stand to lose substantially less in trials than younger ones ('residual reproductive value' hypothesis). In agreement with this hypothesis, older males had a considerably higher mating success than younger males in small cage experiments. Yet, the higher mating success of older males may be either due to male motivation for courtship increasing with age (Fischer *et al.* 2008), or to females preferring older males (Nieberding *et al.*, unpublished data), or most likely to a combination of the two factors. The higher mating success of older males faded away in experiments using larger cages in which male flight to locate and reach females is also tested. This suggests that the potential differences in male motivation may be masked under more natural conditions. This outcome was expected, as mating success does not only depend on male motivation but also on male physiological factors, such as flight endurance, manoeuvrability, energy reserves, responsiveness, (e.g. Joron & Brakefield 2003), all of which are likely to diminish with age (Fischer *et al.* 2008) and may be under natural rather than sexual selection pressure. Noticeably, there is a positive correlation between male age at first mating and both size of the first spermatophore and proportion of non-fertile sperm (Z. Lewis, unpublished data, in Lewis *et al.* 2010).

Additional support in favour of the importance of male behaviour in female mate choice was recently provided (Janowitz & Fischer 2010). Ample mating opportunities during male lifetime is correlated to a reduced mating success (Janowitz & Fischer 2010). Courtship and flight activity tended to directly affect male mating success, with more active males gaining more matings, and

courtship activity was reduced in males having had regular access to females during their life. Male access to mates did not affect the longevity, fecundity, egg hatching rate of mated females. There was no evidence either for a cost of mating on male survival or fertility (Janowitz & Fischer 2010).

Other male-specific traits under natural selection likely affect their mating success; one of them being developmental time. Many animal species display sexual dimorphism in developmental time: the mean adult emergence is usually shorter in males compared to females (protandry). Protandry can be advantageous for males because it increases their probability of mating (Zwaan *et al.* 2008). Early emerged males are more likely to encounter virgin females, which is important as only approximately one third of *B. anynana* females remate in the field (Brakefield & Reitsma 1991; one quarter in the lab; Brakefield *et al.* 2001). After 30 generations of artificial selection on protandry, lines selected for lower protandry (males emerging later and females emerging earlier than in the stock population) had lower egg-to-adult survival and lower rates of hatching, which suggests that interactions with fertility might constrain certain directions of change in patterns of protandry (Zwaan *et al.* 2008). The proportion of non-fertile and fertile sperm produced in spermatophores, as well as the time necessary to mate were also affected in artificially selected lines for lower or higher protandry, which suggests that there might be a trade-off between developmental time and sperm production (Lewis *et al.* 2010).

4.3 Perspectives

Recently, it was shown that the alternative male (competition) and female (choosiness) roles typical of sexual selection are not necessarily fixed within a species (Prudic *et al.* 2011). Plasticity in sexual roles arises within *B. anynana* in relation to temperature experienced during larval development and reversed sexual selection takes place (male choosiness and female competition for accessing males, Prudic *et al.* 2011). This switch is correlated with a change in direct benefits to females and mating costs in males. Thus, the discrete seasonal environments known to produce these two developmental forms impose alternating, symmetrical patterns of sexual selection, one season on male ornaments, the following season on female ornaments. Studied ornaments are the dorsal eyespot pupil size and UV reflectance, which are shown here to be dimorphic between seasonal forms (Prudic *et al.* 2011), but also between sexes (see above). This symmetry through time in male and female sexual roles results in the maintenance of sexual ornaments in both sexes. It leads to the question of which phenotypic traits are used by choosy males to recognize females of their own species. So far, the only traits likely involved in species recognition are the dorsal forewing pupils which provide limited variability, as

discussed above. Courtship behavioural differences across species—currently understudied—may provide an answer to this question. Lastly, this recent study challenges our ideas about the long-term evolution of the previously discussed male traits under the pressures of sexual, or natural, selection in such a dynamic process.

Chapter 5

Selection on male sex pheromone composition contributes to butterfly reproductive isolation*

Abstract: Selection can facilitate diversification by inducing character displacement in mate choice traits that reduces the probability of maladaptive mating between lineages. Although reproductive character displacement (RCD) has been demonstrated in two-taxa case studies, the frequency of this process in nature is still debated. Moreover, studies have focused primarily on visual and acoustic traits, despite the fact that chemical communication is probably the most common means of species recognition. Here we showed in a large, mostly sympatric, butterfly genus, a strong pattern of recurrent RCD for predicted male sex pheromone composition but not for visual mate choice traits. Our results suggest that RCD is not anecdotal, and that selection for divergence in male sex pheromone composition contributed to reproductive isolation within the *Bicyclus* genus. We propose that selection may target olfactory mate choice traits as a more common sensory modality to ensure reproductive isolation among diverging lineages than previously envisaged.

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5.1 Introduction

Species diversification is typically a by-product of drift or ecological adaptation of populations evolving in allopatry. Sometimes, when formerly separated lineages come into secondary contact, selection can increase divergence in their mate choice traits to reduce the risk of maladaptive mating. This process is termed reproductive character displacement (RCD) and includes reinforcement, a special case where gene flow is ongoing between lineages (Dobzhansky 1937, Butlin 1987). Once controversial, RCD has received theoretical support (Liou & Price 1994). Empirical evidence for RCD has also accumulated mostly from studies comparing a pair of species (e.g. Sætre *et al.* 1997, Rundle & Schluter 1998, Higgie *et al.* 2000, Nosil *et al.* 2003, Hoskin *et al.* 2005, Hopkins & Rausher 2012, Lambert *et al.* 2013) while the frequency of RCD in nature is best addressed using large assemblages of recently diverged species (Coyne & Orr 1997, Lukhtanov *et al.* 2005, Seddon 2005, Le Gac & Giraud 2008, Ortiz-Barrientos *et al.* 2009, Hopkins 2013). Moreover, these studies have focused primarily on visual and acoustic mate choice traits, despite the fact that chemical communication is ubiquitous and probably the most common means of species recognition across living taxa worldwide (Smadja & Butlin 2009, Wyatt 2014). Compared to visual, acoustic or electric signals, chemical signals such as sex pheromones are unique in that their perception requires specific combinations of chemical receptors (Su *et al.* 2009, Sakurai *et al.* 2011, Leary *et al.* 2012). Therefore, chemical signals may ensure a high level of privacy in communication between interacting organisms, and their evolution may be less dependent on selective forces other than mate choice. Thus, while these studies demonstrate that RCD has occurred, the assessment of how frequently RCD occurs in nature (Noor 1997) should thus focus on large assemblages of closely related species and on mate choice traits most commonly used in species recognition—i.e., chemical mate choice traits (Smadja & Butlin 2009, Wyatt 2014). In this study, we tested for the presence of reproductive character displacement in a chemical mate choice trait in contributing to the evolution of recurrent reproductive isolation under natural selection.

The more than 80 species of the Sub-Saharan butterfly genus *Bicyclus* (Lepidoptera, Nymphalidae) are strikingly similar morphologically. This speciose group diversified during the Miocene and pairs of sister species differentiated between 0.5 and 5 million years ago (Kodandaramaiah *et al.* 2010). Up to 20 species can be captured in a day in a single forest patch, where males pursue and court females from various *Bicyclus* species, and rare heterospecific matings have been observed in captivity and in nature. This suggests that interspecific interactions are frequent and it raises the question of how reproductive isolation occurs between these sympatric and morphologically similar species (Figure 5.1). Interestingly, the key taxonomic trait used to distinguish *Bicyclus* species

is the number and position of androconia (Condamin 1973), the wing structures thought to be involved in the production and emission of male sex pheromone. Furthermore, it has been demonstrated that male sex pheromone (MSP) plays a key role in mate choice in the model species *Bicyclus anynana* (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013). After landing behind a female, *B. anynana* males flicker their wings and erect their androconial hair, likely favouring the dissemination of MSP at short range (Nieberding *et al.* 2008). Therefore, we postulated that selection on differentiation in the sex pheromone composition has produced recurrent RCD in this butterfly genus. Building on knowledge from the model species *B. anynana* (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013), we first developed a methodology to select from complex chemical profiles the components most likely to form the male sex pheromone for over 30 *Bicyclus* species. Accounting for the potential effect of the environment, we then assessed the presence of a recurrent pattern of character displacement in the predicted sex pheromone composition across the genus. We next compared the rate of evolution of the predicted sex pheromones to that of other, morphological, *Bicyclus* mate choice traits (the wing eyespots, Oliver *et al.* 2009, Kodandaramaiah 2011). Finally, we examined the extent to which the evolution of composition of the predicted sex pheromone and of androconia are coupled.

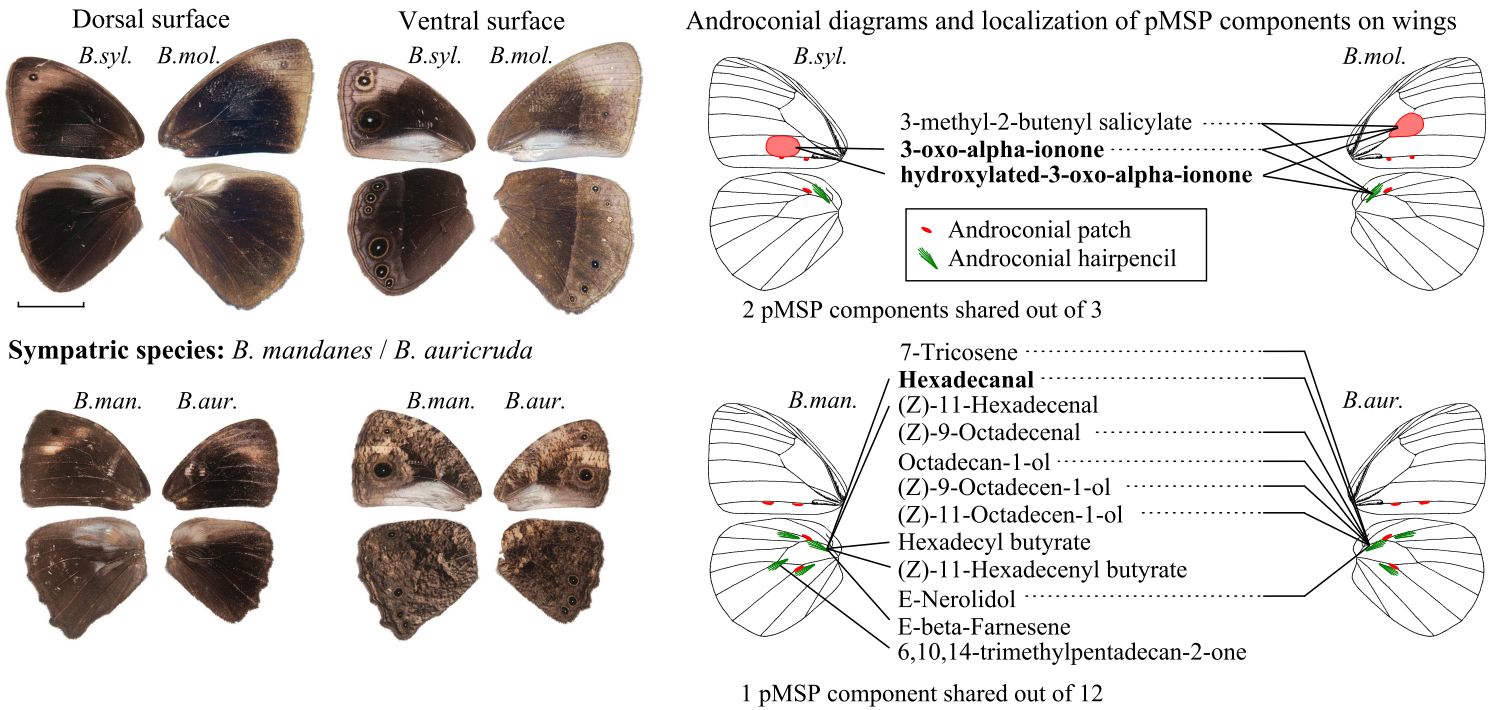


Figure 5.1: Morphological (left) and chemical (right) diversity of males of an allopatric (upper panels) and a sympatric (lower panels) pair of sister species.
The scale bar for the pictures represents one centimetre. Names of shared pMSP components are in bold.

5.2 Materials and Methods

5.2.1 Sampling

We sampled three males and two females of 32 *Bicyclus* species in four African countries (Figure 5.S1) using fruit baited traps. The different androconia and the remaining part of the wings of freshly killed individuals were stored in separate glass vials filled with n-heptane as a solvent for chemical compounds extraction and further gas-chromatography and mass-spectrometry analyses (GC-MS, Appendix 5.5.1).

5.2.2 Selection of the predicted MSP dataset

We developed a standardized routine to select the compounds most likely composing the MSP among the more than 40 compounds identified per species. We based this method on data collected for *B. anynana*, for which the behaviourally active chemicals forming the MSP have been identified using GC-EAD and behavioural assays (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013). We called the selected compounds the 'predicted male sex pheromone' (pMSP) components. We first removed any peak smaller than 10 ng, to homogenize the detectability of compounds between samples and to permit the identification of all selected peaks. Despite the ability of GC-EAD to detect the presence of olfactory receptors for chemicals present in minute amounts, the *B. anynana* MSP was shown to be formed by the most abundant, male-specific and repeatable compounds (Nieberding *et al.* 2008). We thus selected the compounds on average more than five times more abundant in males than in females and present in at least two out of three sampled males. This first selection still resulted in more compounds than known from data obtained using GC-EAD and behavioural assays in *B. anynana*, in which the MSP components are the top third of the most abundant compounds (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013). Moreover, MSP components that experience directional sexual selection are usually more abundant than other compounds (see Steiger *et al.* 2011 and examples in Lepidoptera: Iyengar *et al.* 2001, Andersson *et al.* 2007, Hymenoptera: Ruther *et al.* 2009, lizards: Martín & López 2010, or elephants: Greenwood *et al.* 2005). Thus, from the first selection, we kept the top third most abundant compounds for each *Bicyclus* species, forming the dataset of pMSP components used in all subsequent analyses (Table 5.S1). Of note, we did not include the Malawian lab stock of *B. anynana* from which the selection procedure was designed but rather a field-caught Ugandan *B. anynana* population (Appendix 5.5.1).

5.2.3 Scoring of morphological traits

We coded the presence and absence of 20 androconial structures grouped according to their position in relation to the wing veins and dorsal or ventral wing surface (Appendix 5.5.1). We coded presence or absence of the 17 eyespots and 2 wing bands (Appendix 5.5.1) separated in two groups according to their presence on the ventral or dorsal wing surfaces because they evolve under different selection pressures: the ventral eyespots likely evolve under natural selection while the dorsal characters evolve under sexual selection (Oliver *et al.* 2009, Kodandaramaiah 2011).

5.2.4 Test of reproductive character displacement (RCD)

First, we coded each trait (pMSP, androconia, dorsal forewing eyespots as well as ventral fore- and hindwing eyespots) as a series of binary characters (present or absent). We compared the pMSP composition between species taken pair by pair. Because most *Bicyclus* species do not share any pMSP component (see Results), in 85% of the species pairs the commonly used Jaccard distance (proportion of pMSP differences) has a value of 1. Yet, pairs of *Bicyclus* species differ widely in their number of pMSP differences. Therefore, we partitioned the pMSP composition into two complementary variables: the number of pMSP differences and the number of shared pMSP components, per pair of species. There was no correlation between the numbers of pMSP components shared and different between species, supporting our partitioning approach (Mantel test with 9999 permutations, $n = 496$ pairs of species, $\rho = -0.11$, $P = 0.15$). To allow meaningful comparison across traits, we also scored the other, morphological, traits similarly (shared versus different) although these matrices showed significant correlations for these morphological traits (androconia $\rho = -0.52$, $P < 0.01$, ventral eyespots $\rho = -0.35$, $P < 0.01$, and dorsal eyespots $\rho = -0.64$, $P < 0.01$). Yet, using the Jaccard distance for these morphological traits did not change our results (not shown). Second, pMSP were also compared between species based on their amounts, and using the Euclidian distance on log-transformed amounts of pMSP components.

We tested whether the variability of pMSP composition between pairs of species could be explained by the role of this trait in reproductive isolation. We expected larger differences in pMSP composition or amounts between sympatric than between allopatric pairs of species (Lukhtanov *et al.* 2005). Since androconia (via pMSP) and dorsal eyespots are also linked to mate choice, we also expected a RCD pattern for these traits. We defined sympatry and allopatry solely based on our field data such that sympatric pairs of species had been caught simultaneously in the same fruit-baited trap. Extensive work in museums has however confirmed that our assignation of sympatry and allopatry was

relevant (O. Brattström, personal observations, 2014). We performed multiple regressions on the pMSP matrices using linear models in the ecodist R package (Goslee & Urban 2007, Legendre *et al.* 1994, R Development Core Team 2011) to test whether sympatry or allopatry explained the variation in pMSP composition between pairs of species. This is a non-parametric method which creates a null distribution of the estimated parameters by permuting the data and does not rely on the assumption of independence of each observation (each pair of species). We included the following control factors in the model: phylogenetic distance between species within the pairs (phylogenetic tree[†] based on COI, COII, and EF1α genes; Appendix 5.5.2); and forest versus savannah habitats, as humid environments such as rainforests may affect olfactory communication (Symonds & Elgar 2008). We did not include an interaction term between the phylogenetic distance and sympatry because this statistical method has not been validated for models with an interaction term between variables (P. Legendre, personal communication, 2012).

5.2.5 Estimation of the rates of evolution of the chemical and morphological traits

We mapped each series of binary characters (pMSP components, androconia, dorsal forewing eyespots and ventral fore- and hindwing eyespots) on the phylogenetic tree (Appendix 5.5.2). We measured the rate of evolution as a turnover of character presence and absence across the *Bicyclus* phylogeny. For this, we fitted a symmetrical (equal rates of gain and loss) continuous time Markov model to each trait using a Bayesian framework in the program BayesTraits Multistate (Pagel *et al.* 2004). Although an asymmetrical model better fitted the androconia and ventral eyespot traits than a symmetrical model, both yielded similar results for comparing the rate of evolution between all traits (Figure 5.S5).

5.3 Results

5.3.1 High specificity of predicted sex pheromone composition

The 32 sampled *Bicyclus* species displayed on average 42 ± 17 chemical volatile compounds (mean $\pm$ s.d.). Chemical profiles were found to be repeatable between conspecifics when three males and two females were analysed using

[†]We used the non-transformed branch lengths, as opposed to an ultrametric tree, since we assumed that they were proportional to the neutral divergence. We choose to use neutral divergence—which depends on the generation time, the population effective size, and the mutation rate—instead of the time of divergence because we believe that it is a biologically more appropriate control for the study of trait evolution.

GC-MS analyses (Appendix 5.5.1 and 5.5.4). For the 32 *Bicyclus* species, we selected a total of 75 compounds that likely formed the composition of their respective MSP (3.7 $\pm$ 2.1 pMSP components per species, mean $\pm$ s.d.; Figure 5.2, Table 5.S1 and Figure 5.S2a). These pMSP components showed a wide diversity of chemical structures, and for some of them have been previously identified as sex pheromone components in moths (El-Sayed, 2011). Of the *Bicyclus* pMSP components, 51% were fatty acid derivatives, 20% terpenes and terpenoids, 2% aromatics, 6% carotene derivatives and the remaining 21% miscellaneous or of unknown origin (Figure 5.2 and Table 5.S1). Although the behavioural activity of the male odours has yet to be demonstrated in bioassays, the low within-species variability of chemical profiles (Spearman rank correlation R (mean $\pm$ s.d.) = 0.81 $\pm$ 0.15 in males and 0.74 $\pm$ 0.18 in females; Figure 5.S7) contrasted with the high between-species variability of male chemical profiles (R = 0.18 $\pm$ 0.16; Wilcoxon rank test $P < 2.2 \times 10^{-16}$, Figure 5.S7; 71% of pMSP components were species-specific and each pMSP component was shared by 1.6 species on average out of 32; Figure 5.2, Table 5.S1 and Figure 5.S2b) and is consistent with a role of the pMSP in species recognition. This pattern of high species-specificity remained true when we considered the additional *Bicyclus* lineages in which the 75 pMSP components were found as trace compounds but had not been selected as pMSP components in these latter species (grey cells in Figure 5.2b); each pMSP component or its trace was shared by 4.5 species on average out of 32. Moreover, we observed that most observed changes in pMSP composition arose recently in the evolution of the *Bicyclus* genus (Figure 5.2 and Appendix 5.5.2) and some pairs of sympatric species can display a remarkably large number of pMSP differences (Figure 5.1).

5.3.2 Reproductive isolation by olfactory communication

These results suggest that pMSP composition could be involved in premating reproductive isolation between *Bicyclus* species. To test this hypothesis, we next compared pMSP composition between sympatric and allopatric pairs of species while controlling for phylogenetic distance within pairs. The number of pMSP differences was greater between species in sympatric pairs, compared to allopatric pairs (permutation test; 9999 permutations; n = 496 pairs of species; effect size = 0.66; P = 0.009; Figure 5.3a). In contrast, the number of shared pMSP components was solely determined by phylogenetic distance (permutation test; 9999 permutations; n = 496 pairs of species; effect size = -2.49; $P < 0.001$; Figure 5.S8a and Appendix 5.5.6). Therefore, RCD appears to develop by the recurrent accumulation of new, distinct, chemical components and not by a reduction in the number of shared pMSP components. Several factors contribute to the robustness of this result. First, we used a conservative

test, defining as sympatric only those pairs of species caught simultaneously in the same fruit-baited trap. Since we expected sympatric pairs to be chemically more different than allopatric pairs, mistakes in the assignation of sympatry or allopatry would homogenize the differences in pMSP composition expected between the sympatric and the allopatric groups of species and as such decrease the observed RCD pattern. Second, the number of pMSP differences tended to decrease with phylogenetic distance within pairs of sympatric species (Figure 5.3a). Although we could not test the significance of this effect (P. Legendre, personal communication, 2012), it suggests that the pMSP divergence is due to interactions between closely related species in sympatry, which is expected for RCD since less closely related species are less likely to hybridize (Jiggins 2006a). In this regard, considering only the most recently diverged pairs of species tripled the effect size of sympatry (model using only species pairs separated by less than 0.15 of phylogenetic distance in Figure 5.3a; $n = 38$ pairs, $R^2 = 0.31$; effect size = 1.77 and $P = 0.02$ for 'sympatry'; effect size = 2.03 and $P = 0.07$ for 'habitat'; effect size = 5.48 and $P = 0.59$ for 'phylogeny', Table 5.S2). Third, the significantly higher number of pMSP component differences between sympatric compared to allopatric species pairs was robust to the deletion of three pairs of allopatric species which could be considered as outliers in our dataset (Appendix 5.5.5 and Figure 5.S10). Fourth, the RCD pattern was also found when we tested for the variation in amount, and not simply in the presence or absence, of the pMSP components across the *Bicyclus* phylogeny: sympatric pairs of species displayed significantly higher differences in amount in pMSP components than allopatric pairs of species (Figure 5.3b; $P = 0.05$). Fifth, the RCD pattern remained when we included the pMSP components found as traces in the additional *Bicyclus* lineages, either considering their presence or absence (Figure 5.3c; $P = 0.04$) or as a trend when considering their variation in amount (Figure 5.3d; $P = 0.08$).

As controls, we tested for the presence of RCD in other adaptive, morphological, traits: dorsal forewing eyespots (important in mate choice, Kodandaramaiah 2011), ventral wing eyespots (important in predator avoidance, Kodandaramaiah 2011), and androconia (putatively important for pheromone production). For these traits, the numbers of shared characters and of differences are comparable for sympatric and allopatric species pairs, and both depend on phylogenetic distance (Figure 5.3e-g and Figure 5.S8 and Appendix 5.5.6). In contrast to the olfactory trait, neither of these morphological traits evolved character displacement between sympatric closely related lineages. The evolution of androconia in particular, seems not to be linked to the signal they release, as specific structures are not associated with specific pMSP components (Appendix 5.5.7).

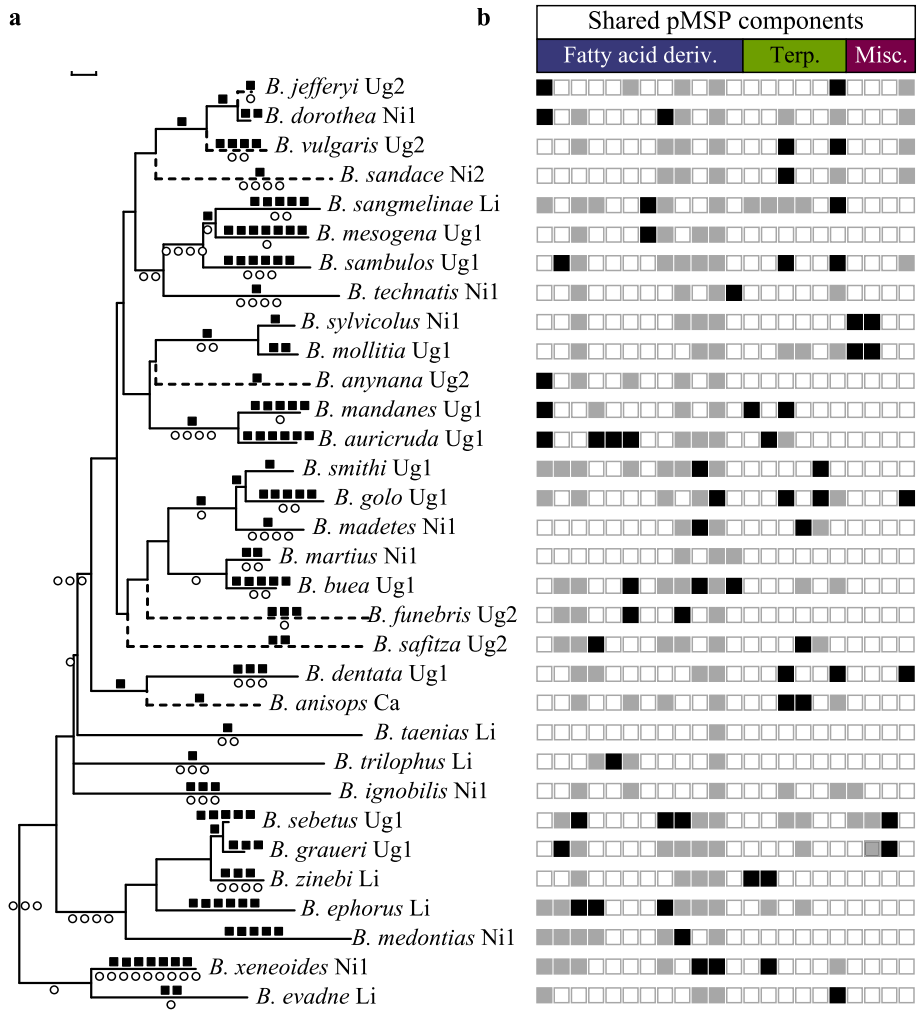


Figure 5.2: Wide diversity and evolution of the pMSP composition and of androconia across the phylogeny of *Bicyclus*.
(**a**) Bayesian consensus tree of the sampled species based on mitochondrial genes COI and COII, and nuclear gene EF1α. Filled squares and open circles represent changes in presence and absence for pMSP components and androconia, respectively (Appendix 5.5.2). Plain and dotted tree branches represent forest and savannah habitats respectively. The scale bar above the tree represents 1% of genetic divergence. Codes following species names correspond to sampling locations: Li for Liberia (Sapo); Ni for Nigeria (1: Afi mountains and 2: Yankari); Ca for Cameroon (Fossong Ellelem); and Ug for Uganda (1: Kibale and 2: Mburo, see Figure 5.S1).

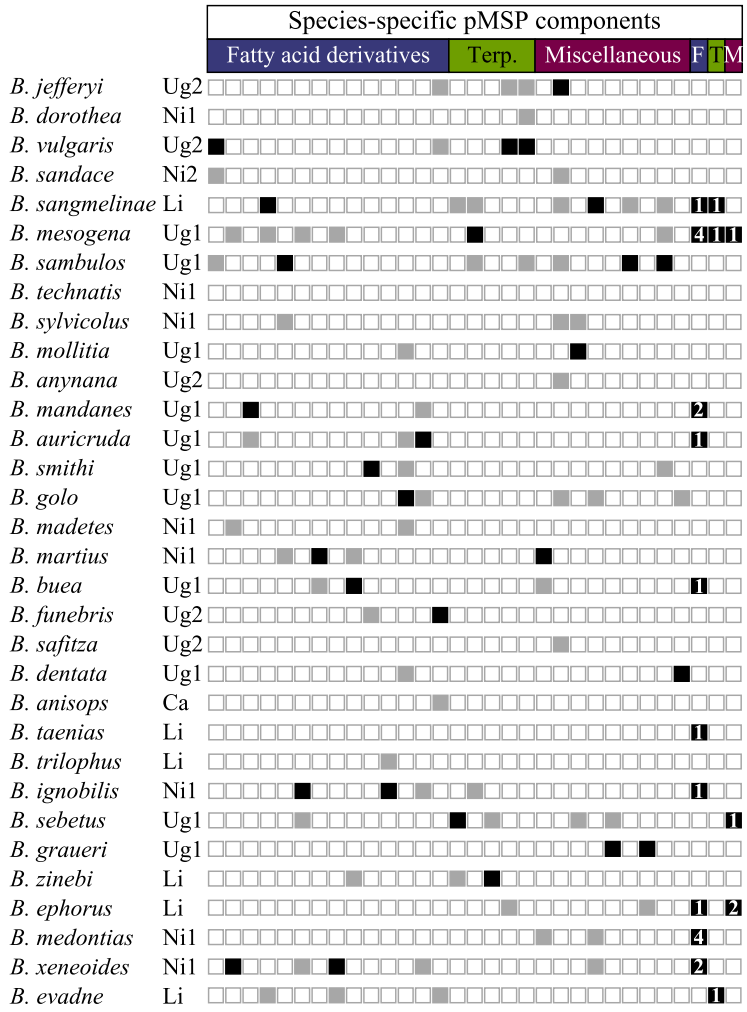
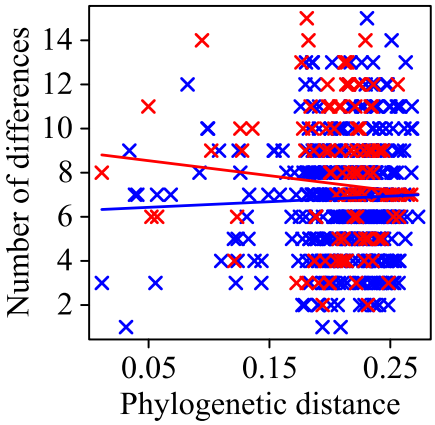


Figure 5.2 (continued):

(**b**) The left part of the table shows the pMSP components (black cells) shared by two or more *Bicyclus* species and the right part of the table shows the pMSP components specific to one *Bicyclus* species. Grey cells represent pMSP components found in additional *Bicyclus* species as trace compounds but not selected as pMSP components in these latter species. Black cells in the three last columns represent numbers of additional strictly specific pMSP components. The pMSP components are classified in three chemical types: fatty acid derivatives, terpenes and terpenoids, and miscellaneous (see the identification of individual compounds in Table 5.S1).

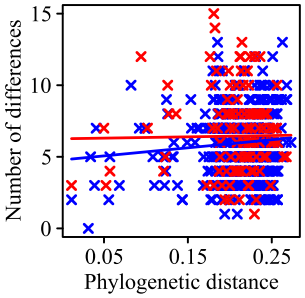
a pMSP

Factors	Effects	*P*
Intercept	4.33	
Phylogeny	0.15	0.97
Sympatry	**0.66**	**0.01**
Habitat	1.58	0.06
R^2	0.13	0.04



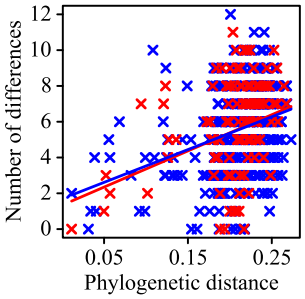
c pMSP and their traces

Factors	Effects	*P*
Intercept	2.80	
Phylogeny	4.44	0.35
Sympatry	**0.50**	**0.04**
Habitat	**1.43**	**0.05**
R^2	0.13	0.03



e Androconia

Factors	Effects	*P*
Intercept	-0.81	
Phylogeny	**18.81**	**<0.01**
Sympatry	0.03	0.89
Habitat	**1.54**	**<0.01**
R^2	0.24	<0.01



g Ventral eyespots

Factors	Effects	*P*
Intercept	1.68	
Phylogeny	-0.29	0.91
Sympatry	0.01	0.96
Habitat	0.51	0.12
R^2	0.04	0.17

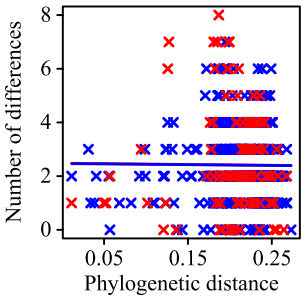
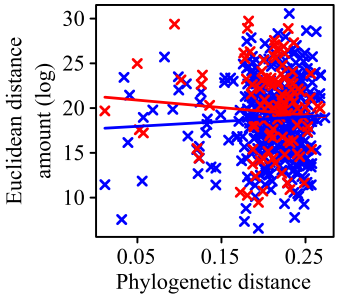


Figure 5.3: Reproductive character displacement for pMSP composition but not for other, morphological, adaptive traits.
The following traits were tested for RCD: (**a**–**d**) pMSP composition, (**e**) androconia, (**f**) dorsal eyespots, and (**g**) ventral eyespots.

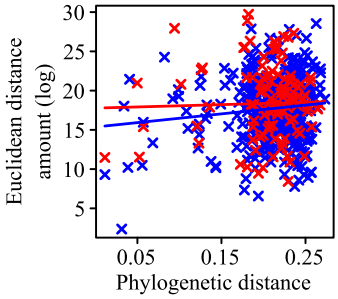
b pMSP

Factors	Effects	*P*
Intercept	15.33	
Phylogeny	1.88	0.84
Sympatry	**0.79**	**0.05**
Habitat	1.99	0.12
R^2	0.08	0.11



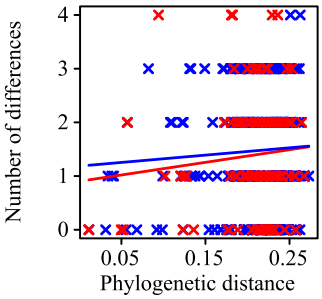
d pMSP and their traces

Factors	Effects	*P*
Intercept	12.91	
Phylogeny	9.06	0.27
Sympatry	0.72	0.08
Habitat	1.85	0.11
R^2	0.09	0.08



f Dorsal eyespots

Factors	Effects	*P*
Intercept	1.27	
Phylogeny	1.62	0.22
Sympatry	-0.08	0.47
Habitat	-0.09	0.57
R^2	0.01	0.51



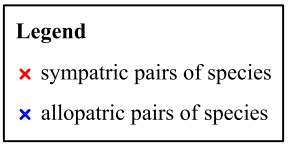


Figure 5.3 (continued):
Panels **a** and **c** represent the number of differences in presence and absence of pMSP components; panels **b** and **d** represent the Euclidean distances on amount of pMSP component; panels **a** and **b** involve our selection of 75 pMSP components only, while panels **c** and **d** also include the pMSP components when found as traces in additional *Bicyclus* species ('grey cells' in Figure 5.2b). On the left of each panel, the statistical output of the following model is given in a table for each trait: *phenotypic difference between species* $\sim$ *intercept + phylogeny + sympatry + habitat*. Significant effects are represented in bold. On the right of each panel, graphs show the phenotypic differences between species against their phylogenetic distance, using the following model: *phenotypic difference between species* $\sim$ *intercept + phylogeny + sympatry + habitat + phylogeny : sympatry*. These graphs thus include the interaction term for illustration sake and the effect of the habitat is averaged. Of note, the results for differences in pMSP composition (**a**) are robust to the removal of the three bottom left allopatric species pairs (Figure 5.S10).

5.3.3 Limited habitat-driven evolution of pMSP composition

In addition to RCD, ecological character displacement can also drive divergence of mate choice traits when natural selection occurs to reduce ecological niche competition (Slatkin 1980, Jiggins 2006a). We considered two ecologically significant factors that could affect pMSP evolution: habitat type (forest versus savannah) and host plant specificity (Landolt & Phillips 1997). Habitat type had indeed a marginally significant effect on pMSP differentiation (effect size = 1.58 and $P = 0.06$; Figure 5.3a, Figure 5.S9 and 5.S10), but was not responsible for the pattern of pMSP character displacement observed between closely related sympatric species pairs. The effect of habitat type was due to a higher number of pMSP components in forest than in savannah species (generalized estimated equation; $n = 32$ species; effect size = 4.52; $P < 0.01$; Appendix 5.5.8), which suggests that a higher diversity of olfactory signals may compensate for the potential impairment of olfactory communication in humid environments like rainforests (Symonds & Elgar 2008). The number of species (higher in forests) could not be separated from the effect of habitat due the reduced number of sampled localities ($n = 6$; Appendix 5.5.8). Of note, forest species also displayed a larger number of androconia, but not of eyespots, compared to savannah species (as shown indirectly by Figure 5.S9).

Ecological character displacement could also result from adaptation to different host plants at the larval stage to avoid competition, which could induce modification of MSP composition and reproductive isolation at the adult stage (Stennett & Etges 1997). Although host plant specificity remains unknown for most *Bicyclus* species, 56% of pMSP components are fatty acid derivatives which can be produced *de novo* by insects (Liénard *et al.* 2014, Wang *et al.* 2014). The remaining 44% of pMSP components may be, but are not necessarily, derived from host plant use (e.g. Schulz *et al.* 2011, Figure 5.2). Moreover, these potentially host-plant derived components did not show higher species-specificity than fatty-acid derived pMSP components (shared respectively by 1.65 ± 1.43 and 1.48 ± 0.93 species; mean $\pm$ s.d.). In the laboratory, rearing *B. anynana* larvae on grasses closely related to its assumed natural host plant (*Oplismenus sp.*) or on maize (*Zea mays*), the usual lab host plant for this butterfly, did not affect the chemical profiles of adults (Nieberding *et al.* 2008). These results suggest that host plant adaptation may have only a limited effect on pMSP composition or differentiation across the *Bicyclus* genus.

5.3.4 Differential fusion or species sorting do not explain the pattern of pMSP evolution

Two other alternative processes to RCD (species sorting, and differential fusion Templeton 1981, Lukhtanov *et al.* 2005) might explain the greater divergence of pMSP composition in sympatric compared to allopatric species pairs. These processes occur if, after the differentiation of pMSP composition between species evolved in allopatry, secondary contact results in the merging of insufficiently differentiated lineages (differential fusion) or the local extinction of one (species sorting), while those lineages that have evolved sufficiently strong reproductive isolation are able to coexist. We found, however, that allopatric pairs of *Bicyclus* species were not younger than sympatric ones (Appendix 5.5.9) which would be expected if a subset of the sympatric species (the youngest) had merged or went extinct in sympatry during secondary contact due to weak reproductive isolation (Jiggins 2006a). Thus, our data suggest that neither differential fusion nor species sorting explain the recurrent pattern of pMSP diversification across the genus.

5.3.5 Rapid evolution of sex pheromone composition

In the few documented case studies, species interactions caused the evolution of RCD over very short evolutionary time scales in nature, i.e. in a small number of generations (Higgie *et al.* 2000) or a few thousand years (Hoskin *et al.* 2005). Thus we expect that any mating traits which produce reproductive isolation by RCD should evolve rapidly. Although the actual rates of change must be treated with caution (Appendix 5.5.3), the results of the comparison suggest that pMSP have diverged over four times more rapidly than dorsal eyespots, another mate choice trait in *B. anynana* (Oliver *et al.* 2009), which in turn have evolved more than twice as fast as androconia (Figure 5.4). This relatively high turnover of pMSP composition across species is in agreement with the involvement of this trait in premating reproductive isolation (Higgie *et al.* 2000, Hoskin *et al.* 2005, Arnegard *et al.* 2010a).

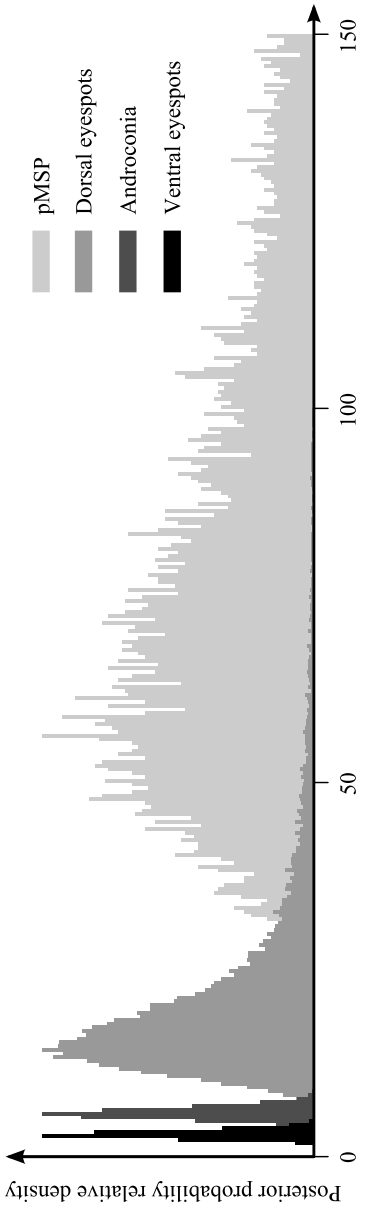


Figure 5.4: Higher rate of character change for pMSP composition compared to morphological traits. The distribution of evolutionary rates for, from left to right, ventral eyespots, androconia, dorsal eyespots, and pMSP based on Bayesian estimation. The most probable rates of both gains and losses (number of change relative to genetic evolution) are 2.9, 5.7, 14.4, and 59.7, respectively. Similar results were found when gains and losses were estimated as two independent parameters (Figure 5.S5).

5.4 Discussion

Our results correlate the local co-occurrence of closely related *Bicyclus* species to the larger differences in composition, and in amount, of their major male volatile compounds. This suggests a link between the establishment, or increase, of reproductive isolation between *Bicyclus* species and the recurrent differentiation of their major male volatile compounds.

Our results are based on a selection method targeting the volatile wing compounds most likely acting as male secondary sexual traits. We based our selection method on criteria established in the field of sexual selection and widely supported across taxa and across phenotypic traits: we picked male-specific, repeatable and most abundant compounds as these are most likely under directional selection for largest possible amounts by females. These characteristics are indeed the ones of the male sex pheromone identified using electro-physiological and behavioural experiments in the model species *B. anynana* (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013). The use of a selection method also has the advantage to avoid circular reasoning, as we show that the compounds chosen on one basis (male sex specificity, repeatability and amount in males) show a biological pattern of species-specificity and reproductive character displacement (RCD hereafter). However, our selection of pMSP components contains likely some chemicals not playing the role of sex pheromone, and we likely missed some sex pheromone components in our pMSP selection. We may also have included as pMSP, compounds that have other function in reproduction, such as anti-aphrodisiacs. Yet, the behaviour of *B. anynana* does not suggest the existence of anti-aphrodisiacs (e.g. no pupal mating like in *Heliconius*, Estrada *et al.* 2011). Anti-aphrodisiacs are transferred between male and female abdomens, which were not analysed here. Moreover, traits involved in male-male competition for accessing females, such as anti-aphrodisiacs, are unlikely to be under selection for increased differentiation in sympatry compared to allopatry. Altogether, our criteria maximized the probability of selecting compounds acting as male secondary sexual traits under sexual selection by females, and the inclusion of compounds with other functions in our pMSP selection, such as anti-aphrodisiacs, would have blurred the RCD pattern. Future behavioural studies using synthetic sex pheromone perfumes as in Nieberding *et al.* (2012) are needed to confirm the role of the selected compounds in sexual communication and reproductive isolation in field caught populations of *Bicyclus* sharing some pMSP components.

Importantly, the presence of a recurrent RCD pattern was stronger for the chemical trait when we use their pattern of presence and absence (Figure 5.3a), rather than their variation in amount across species (Figure 5.3b). In addition, the RCD pattern held true for the chemical trait when we included the pMSP components found scattered as trace compounds in other *Bicyclus*

lineages ('grey cells' in Figure 5.2b). This was expected as, notwithstanding our selection method, the majority of male major compounds are simply absent in related species. The traces of pMSP components found in other *Bicyclus* species are either present: i) at low amount, in which case we expect the female to reject the male, as shown in *B. anynana* in which mate choice is based on maximal amounts of MSP (Nieberding *et al.* 2012, van Bergen *et al.* 2013); ii) in a female, in which case the chemical cue is not going to be a problem as females rarely court other females in *B. anynana*. Thus, in *Bicyclus* butterflies, the presence or absence of pMSP components likely allows species recognition. Our results do not exclude that, as is found in moths (Smadja & Butlin 2009, Wyatt 2014), variations in ratios between compounds or the presence of minor chemical compounds may participate to reproductive isolation in this genus, together with the presence of abundant pMSP components.

Our results show that incipient *Bicyclus* species initially share some compounds (Figure 5.S8a) and achieve reproductive isolation by accumulating more rapidly pMSP differences in sympatry than in allopatry (Figure 5.3). With time, differences in pMSP components continue to accumulate between species and shared pMSP components are lost because pMSP turnover is high (Figure 5.4), which is why most *Bicyclus* species currently do not share any pMSP component anymore. Importantly, it does not imply that all pMSP differences appeared to achieve reproductive isolation in sympatry, as allopatric pairs of species also display a substantial number of pMSP differences (Figure 5.3a).

Experimental evolution of rapid reproductive isolation by RCD of sex pheromones in a pair of *Drosophila* species (Higgie *et al.* 2000) supports the hypothesis that the evolution of chemical communication may contribute to reproductive isolation by RCD. Direct experimental evidence that links sex pheromone composition and reproductive isolation was repeatedly found in Lepidoptera in which the presence of a heterospecific sex pheromone compound typical of a closely related sympatric species, decreases attraction to traps baited with female pheromones (e.g. Löfstedt *et al.* 1991). In *B. anynana*, the absence of the MSP decreases the mating success of males (Nieberding *et al.* 2008). This suggests that differences in MSP composition may have an effect on species recognition in addition to mate choice since males of other *Bicyclus* species lacking the MSP typical of *B. anynana* males would have a low mating success when courting *B. anynana* females (Mendelson & Shaw 2012).

Natural selection is traditionally flagged for RCD as a response to the production of maladaptive hybrids (historical explanation, Butlin 1987). Yet, the avoidance of maladaptive mating by females can be considered as promoted by sexual selection for good genes (Shaw & Mendelson 2013). Moreover, divergent sexual selection produces rapid diversification of secondary sexual traits (Rodríguez *et al.* 2013) such as observed for pMSP composition. It is notoriously difficult to separate natural and sexual selection in driving the

evolution of secondary sexual traits and many authors see the two processes as a continuum from species recognition to mate choice (Ryan & Rand 1993, Mendelson & Shaw 2012). Here, we cannot exclude natural or sexual selection in the evolution of pMSP composition as the two processes can produce the same RCD pattern.

Together, our data provide large-scale evidence of recurrent RCD in an olfactory mate choice trait. We propose that chemical communication displays unique properties compared to visual, acoustic, or electric (e.g. Arnegard *et al.* 2010a) modes of communication, which may facilitate its role in the evolution of premating reproductive isolation. First, we found similar pMSP components in distantly related *Bicyclus* lineages and most pMSP components in one *Bicyclus* species could be found scattered as trace compounds in a few other *Bicyclus* lineages (in 14% of the species on average, noted by grey cells in Figure 5.2b). This large phenotypic standing variation, as found in other species (El-Sayed 2011, Niehuis *et al.* 2013), suggests that each *Bicyclus* species has access to most of the enzymes involved in the biosynthesis of the pMSP components. Thus, the wide diversity and the rapid evolution (Figures 5.2 and 5.4) of sex pheromone composition in *Bicyclus* may be explained by the lack of intrinsic biosynthetic constraints, as has been found in *Drosophila* and some moths (Takahashi *et al.* 2001, Shirangi *et al.* 2009, Albre *et al.* 2012). Second, sex pheromone components are qualitative, discrete, signals that require specific one-to-one corresponding chemical receptors to allow communication to take place (Su *et al.* 2009, Sakurai *et al.* 2011, Leary *et al.* 2012), which gives rise to a unique 'lock-and-key' privacy in communication between courting individuals. Interestingly, the stimulation of the sex pheromone-specific olfactory receptor is sufficient to trigger complete courtship behaviour in the silk moth *Bombyx mori* (Sakurai *et al.* 2011) and *Ostrinia* Lepidoptera (Leary *et al.* 2012), and the replacement of a sex pheromone receptor by that of another species is sufficient to induce male courtship towards heterospecific females (Sakurai *et al.* 2011). Thus, we expect that the turnover of sex pheromone receptors expressed in specific olfactory neurons can elicit the same adaptive courtship behaviour in response to newly produced sex pheromone components without additional modification of hard-wired circuits that link the olfactory neurons to higher neuronal centres. This provides a mechanism by which such qualitative chemical signals can undergo rapid evolution. We propose that, relative to other modes, chemical communication is: i) an *open* niche as the diversity of potential new chemical compounds is seemingly unlimited; ii) *private* provided the 'lock-and-key' nature of olfactory receptors and sex pheromone components, and as such evolves under a limited number of selective forces (i.e. illicit receivers such as predators or parasitoids); iii) *labile* given that a turnover in sex pheromone signals and receptors can take place without disrupting the subsequent adaptive behavioural courtship steps. As such, this ubiquitous mode

of communication may act as a more general driver of reproductive isolation by RCD than previously envisaged.

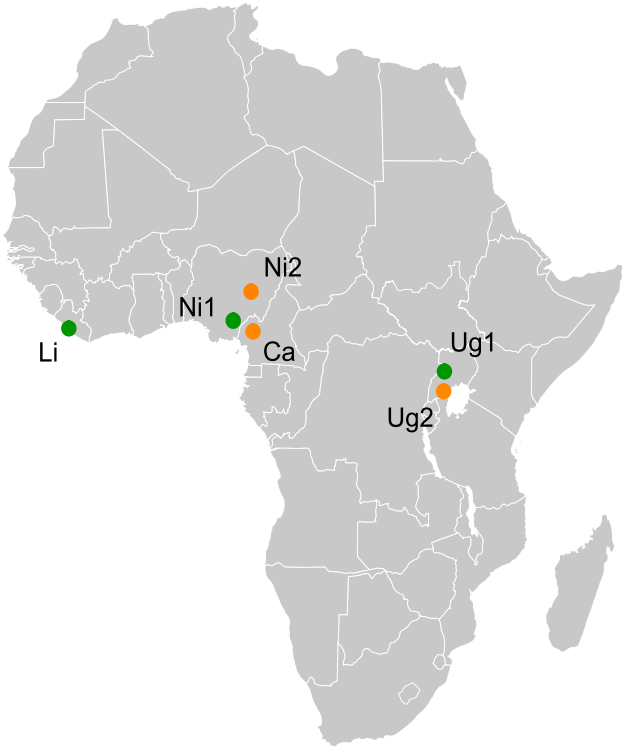


Figure 5.S1: Africa map with the localization of the sampling sites.
The site abbreviations correspond to the tree in Figure 5.2 (main text): Li for Liberia (Sapo); Ni for Nigeria (1: Afi mountains and 2: Yankari); Ca for Cameroon (Fossong Ellelem); Ug for Uganda (1: Kibale and 2: Mburo). Green and orange dots represent tropical forest and savannah sites, respectively.

5.5 Supporting Information of the manuscript

5.5.1 Sampling and phenotypic scoring

Species Sampling

We trapped *Bicyclus* butterflies at a total of six sites located in four African countries (Figure 5.S1). At each site, we trapped butterflies during a two-week period, and all but five species were sampled from a single site. For each site, we used 10-20 traps and placed them at least 30 m apart. We checked traps daily and moved them regularly around the site, to maximize the captures. At some sites, we placed groups of traps at two locations, never more than 2 km apart. At the scale we sampled, we did not observe any differences in species composition across traps for the species included in our study. Although our two-week field sampling scheme might bias our chemical analysis, since the relative amounts of some MSP components can change with age (Nieberding *et al.* 2012), our sampling reflects the actual MSP diversity encountered by interacting individuals in the field.

Our species sampling does not include the Malawian stock population of *B. anynana* from which the selection procedure was designed, because it is not living in sympatry with any of the species sampled for this study and it was reared in the lab for more than thirty years (Brakefield *et al.* 2009). The *B. anynana* population used in this study was caught in Uganda and is part of a different subspecies, *B. anynana centralis* (Condamin 1968), which does not produce MSP1 and MSP3 found in *B. anynana anynana* from Malawi (Nieberding *et al.* 2008).

Scoring pMSP components

We conducted chemical analyses using three males and two females per species. We removed both the forewing and hindwing from one side (right or left) of each freshly killed butterfly, (males only) dissected each androconium from each of the wings, and extracted each androconium in a separate 1.5 ml screw-cap vial containing 100 µl redistilled n-heptane with 1 ng/µl of (Z)-8-tridecenyl acetate as an internal standard. We placed the remaining tissue of each dissected wing (and wings of females) in a vial of 300 µl n-heptane with 0.33 ng/µl of the internal standard. We stored the body in 100% ethanol for genetic analysis and the undissected wings in glassine envelopes for analysis of morphological traits.

We analyzed wing extracts on an Agilent 5975 mass-selective detector coupled to an Agilent 6890 gas chromatograph, equipped with a HP-5MS capillary column (30 m $\times$ 0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific, USA). The oven temperature was programmed from 80 °C for 3 min, then

to 210 °C at 10 °C/min, hold for 12 min and finally to 270 °C at 10 °C/min, hold for 5 min. Inlet and transfer line temperatures were 250 °C and 280 °C, respectively, and helium was used as the carrier gas. We only analyzed those compounds with a retention time under 31.8 min (retention time of pentacosane using our method); we expect that those compounds with retention time > 31.8 min are gustatory chemicals with low volatility. We identified chemical compounds by comparing gas chromatography retention times and mass spectra with authentic standards acquired commercially or prepared by synthesis on both non-polar (HP-5MS) and polar (INNOWax, 30 m $\times$ 0.25 mm i.d., and 0.25 μm film thickness; J&W Scientific, USA) columns. The position of double bonds was localized by the DMDS microreaction (Dunkelblum *et al.* 1985). Tentative structures were assigned for compounds not fully identified (Table 5.S1 in a separate excel file). All 873 GC-MS files are available on Dryad Digital Repository[‡].

If different species tend to share the same sets of structurally related pMSP components, pseudoreplication could occur in our data. To assess the risk for pseudoreplication, we tested whether structurally related components in the pMSP tended to be shared across species, using Fisher's exact tests for all possible pairs of such structurally-related components. Each contingency table contained the number of species for which: both compound a and structurally-related compound b are present, only compound a is present, only compound b is present, or, both compound a and compound b are absent. Out of 231 possible pairs of shared, structurally related components, the test was marginally significant for only one pair (n = 32, or = 12.86, P = 0.53). In this case, the closely related species *B. mollitia* and *B. sylvicolus* both possess the same pair of structurally related components (Figure 5.1). This is probably caused by phylogenetic inertia, and not biosynthetic constraints, since these two compounds are each present alone (but not selected as pMSP components) in two other species, *B. ignobilis* and *B. graueri* (Figure 5.2). For all other possible pairs, the presence of shared, structurally related components in one species was not significantly correlated with the presence of this pair of compounds in other species, eliminating the potential for pseudoreplication in our dataset.

[‡]see `http://dx.doi.org/10.5061/dryad.768sd`

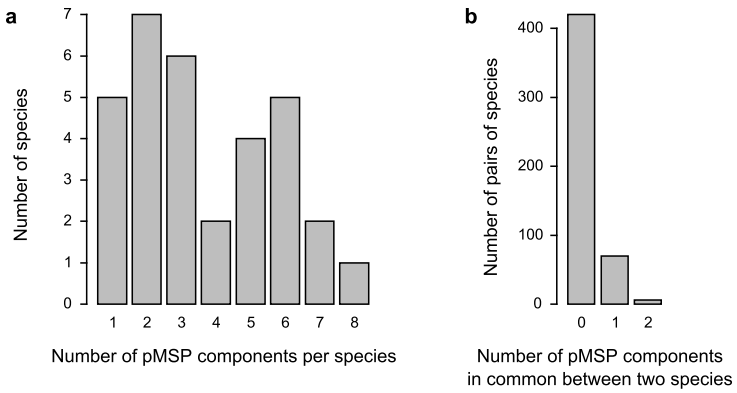


Figure 5.S2: Number of pMSP components per species (**a**) and shared per pair of species (**b**).

Table 5.S1: List of the chemical compounds detected for our sample of 32 *Bicyclus* species and final selection of 75 pMSP components (separate excel file).
This table lists all compounds that were present above 10 ng in samples of homologous wing parts in at least two out of three males of the same species. The 75 compounds selected as both male-specific and abundant (pMSP) are highlighted in Column 1. We investigated the chemical structure of all pMSP components; the table details the chemical analyses performed for each compound. Compounds were generally 'fully identified' only in one species (using reference compounds and DMDS reactions) and then recognized in the other species based on similarity of spectra and retention times. The characteristics of the mass spectra of the few remaining unidentified pMSP components are given. Homologous compounds (based on identical mass spectrum and retention time) to the above listed ones that were observed in additional species were also added to the table. All these homologous compounds were assigned the same reference number to facilitate comparison between species ('compound number' Column in the table). Some compounds identified by Wang *et al.* (2014) are not included here because either a) the compound fell out of the window of retention time considered here as a criterion for inclusion as a pMSP component, b) the compound was not male-specific, or c) the compound was not among the most abundant or was not a repeatable compound in the species where it occurred.

Scoring androconia

Androconia are wing structures consisting of modified scales arranged as 'patches' or 'brushes'. We identified androconia by their location in relation to the wing veins on a given wing surface (Figure 5.S3). In total, we identified 20 different structures. Distribution of the androconia on wing surfaces is similar across most *Bicyclus* species; we verified the patterns in our sample by cross-checking 90 currently recognized *Bicyclus* species in museum collections and determined that distribution patterns are robust and not biased by the selection of species included in the pheromone sampling. The ventral hindwing surface lacks androconia in all *Bicyclus* species known. On the dorsal hindwing, androconia lie close to vein intersections; in species with atypical wing venation (e.g., *B. buea*, *B. maritus*), the position of the androconia corresponds to changes in vein position. On the ventral forewing, some androconia are located posterior to the most posterior vein (Vein 1 in Figure 5.S3), with no interconnecting veins. We were able to reliably identify these posterior forewing androconia and determine their homology across species by taking advantage of the fact that each of these forewing androconia overlaps one of the hindwing androconia when the wings are held in a resting position. Also on the ventral forewing, the androconium located anterior to Vein 1 and the androconium crossing Vein 1 were always scored as separate characters.

Scoring eyespots

Eyespots are located on all wing surfaces in *Bicyclus*. However, eyespots on different wing surfaces have likely evolved under different selection pressures, and behavioural studies in the model species *B. anynana* demonstrate that although dorsal forewing eyespots function in mate choice (Robertson & Monteiro 2005, Costanzo & Monteiro 2007, Oliver *et al.* 2009), ventral hindwing eyespots do not, and instead appear to decrease the efficiency of predator attacks (Olofsson *et al.* 2010). In addition, many *Bicyclus* species exhibit seasonal polyphenism, which affects the size and presence of eyespots in wet season versus dry season forms. Ventral eyespots appear to be most sensitive to changes in temperature (the cue which determines seasonal morphology). To standardize our dataset, we scored eyespot presence or absence for wet season forms only—this corresponds as well to the season in which most reproduction occurs. In cases where our samples were wet-season forms collected from the same locality as those used by Oliver *et al.* (2009; which used wet-season forms only), we coded any missing eyespots and bands as 'present' when they were missing from the dry season form, but present in the wet-season form.

5.5.2 Phylogenetic reconstructions and character mapping

Monteiro & Pierce (2001) and Oliver *et al.* (2009) reconstructed the phylogeny of 58 *Bicyclus* species based on Maximum Likelihood and Bayesian methods, using partial sequences of the mitochondrial genes cytochrome oxidase I and II (COI, COII) and of the nuclear gene elongation factor 1α (EF-1α). Our work added two taxa (*B. ephorus* and *B. sylvicolus*) and completed data for a third (*B. sangmelinae*).

Sequencing: Following an adjusted protocol from Monteiro & Pierce (2001), we sequenced a part of the mitochondrial gene cytochrome oxidase I (COI) and the nuclear gene elongation factor 1α (EF-1α). In *B. sangmelinae*, we failed to obtain the first half of the COI sequence, therefore this part was kept from the dataset of Monteiro & Pierce (2001). The resulting sequences are available on GenBank with accession numbers KC786271 to KC786277.

Model of substitution: We used the AICc and BIC criteria in the program jModeltest 0.1.1 (Guindon & Gascuel 2003, Posada 2008) to select of the model of sequence evolution to use in our phylogenetic reconstruction. The GTR+G model was found to be the most suitable across three different methods of data partitioning: one partition with the three genes; two partitions to separate mitochondrial genes from the nuclear gene; and three partitions with one separate model for each of the three genes.

Bayesian reconstruction with the different partitions: We first conducted a Bayesian reconstruction of the tree using the parallel version of the program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003, Altekar *et al.* 2004). The Metropolis-coupled, Markov chain Monte Carlo (MCMCMC) process included four chains, three heated and one cold with a temperature parameter of 0.15. Starting from random trees, we simultaneously performed two independent runs for 20 million generations each, and sampled the chains every 2000 generations yielding a total of 10,000 samples for each run. The convergence of the two runs was assessed visually with the program Tracer 1.5 (Drummond *et al.* 2006); the standard deviation of the split frequencies stabilized around 0.003 halfway through the run time. Therefore we obtained the 50% majority-rule consensus from the latter 10 million generations (10000 samples in total). Among the three methods of data partitioning, the model with two partitions (nuclear and mitochondrial sequences) received very strong support from the posterior probabilities (Bayes factors of 503 and 30 when compared to the models with one or three partitions, respectively). The Bayes factors are twice the difference of the logarithm of the harmonic mean of the likelihoods of the two models . A value greater than 2 is taken as 'positive' evidence that the model is better, greater than 5 as 'strong' and greater than 10 as 'very strong' evidence.

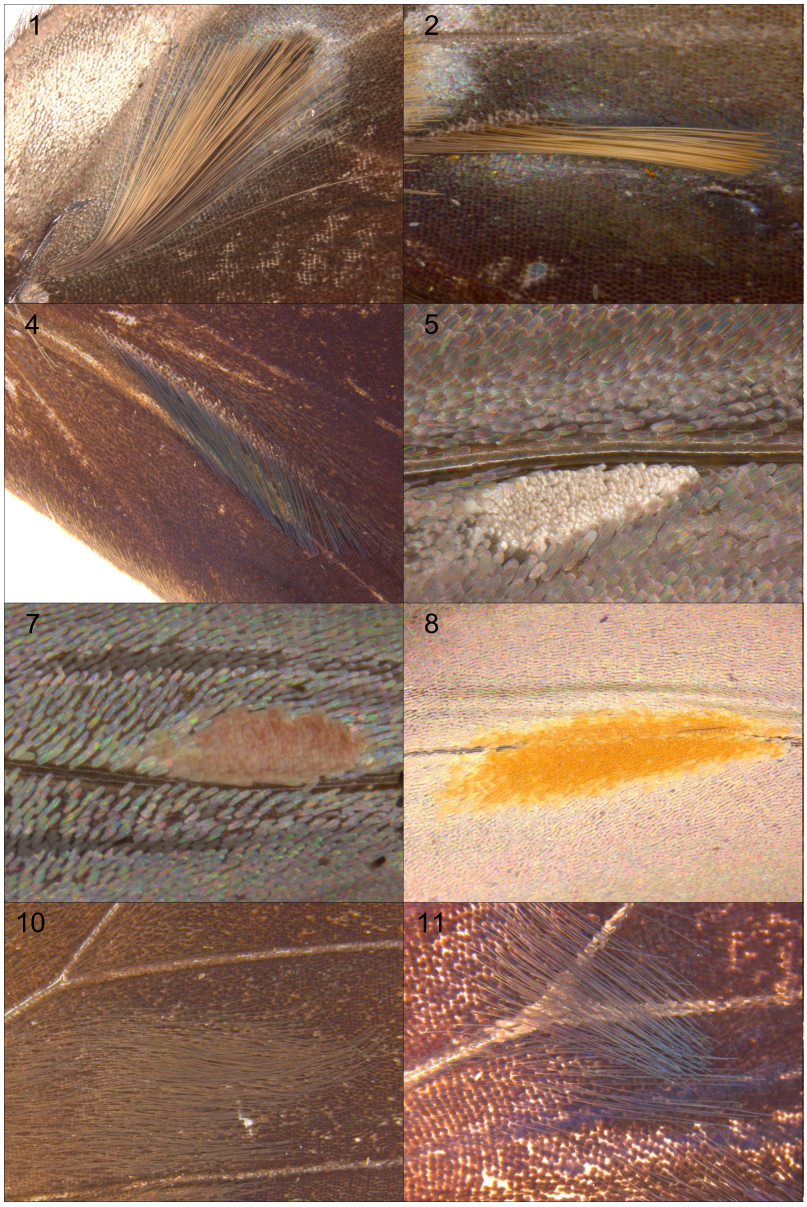


Figure 5.S3: Example picture of each androconial unit and scheme of androconia positions.
Numbers correspond to Figure 5.S12. Vein and space numbering follows the simplified 'English' numerical system (Miller 1969). Dorsal hindwing: (1a) cell brush and (1b) patch in space 7; (2a) brush and (2b) patch in space 6; (3a, 3b) brushes and (3c) patch located around distal part of wing cell; (4a, 4c) brushes and (4b, 4d) patches along space 1c and vein 1b. Ventral forewing: (5) patch under vein 1 covering androconia type 1;

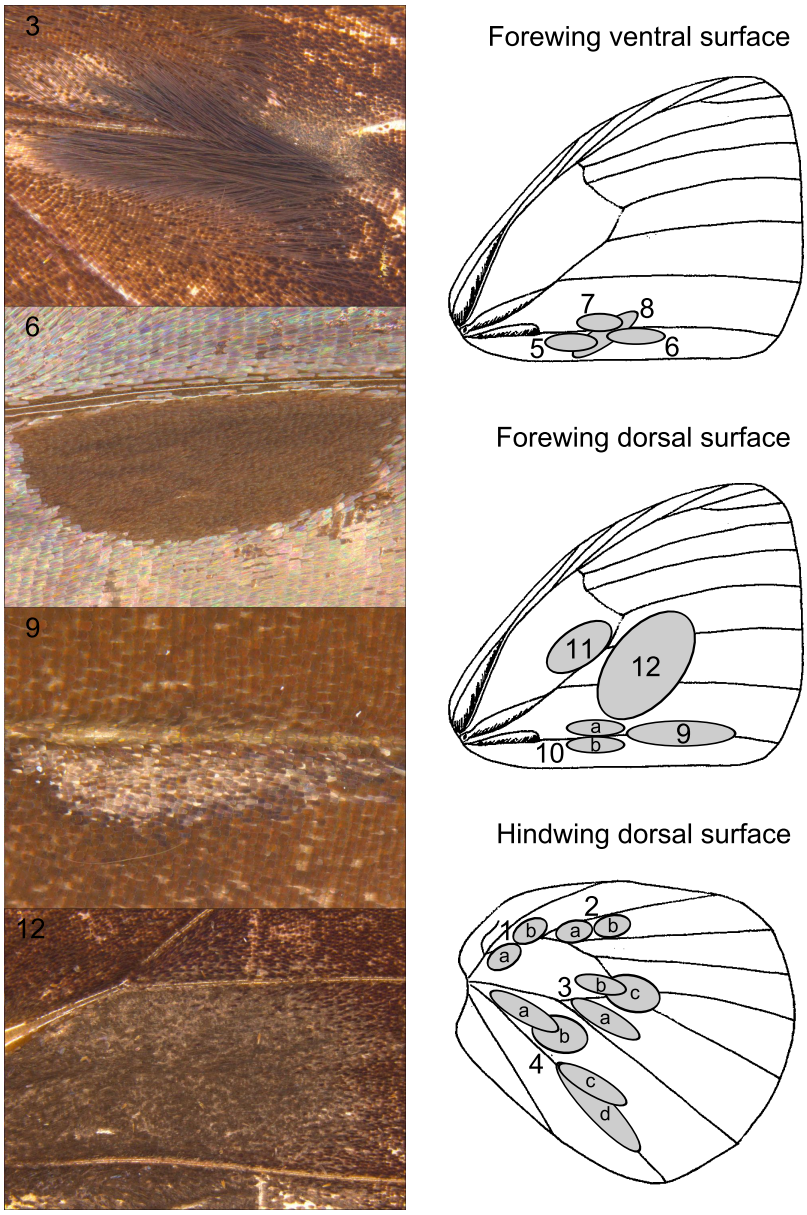


Figure 5.S3 (continued):

(6) patch under vein 1 covering androconia type 2; (7) patch above vein 1; (8) patch stretching across vein 1. Dorsal forewing: (9) hairs along vein 1; (10a, b) patch along vein 1; (11) brush in wing cell; (12) patch of discal scales. These samples belong to the following species: 1) *B. madetes*; 2) *B. golo*; 3) *B. xeneoides*; 4) *B. ignobilis*; 5) *B. anisops*; 6) *B. safitza*; 7) *B. taenias*; 8) *B. medontias*; 9) *B. dentata*; 10) *B. sambulos*; 11) *B. martius*; and 12) *B. mollitia*.

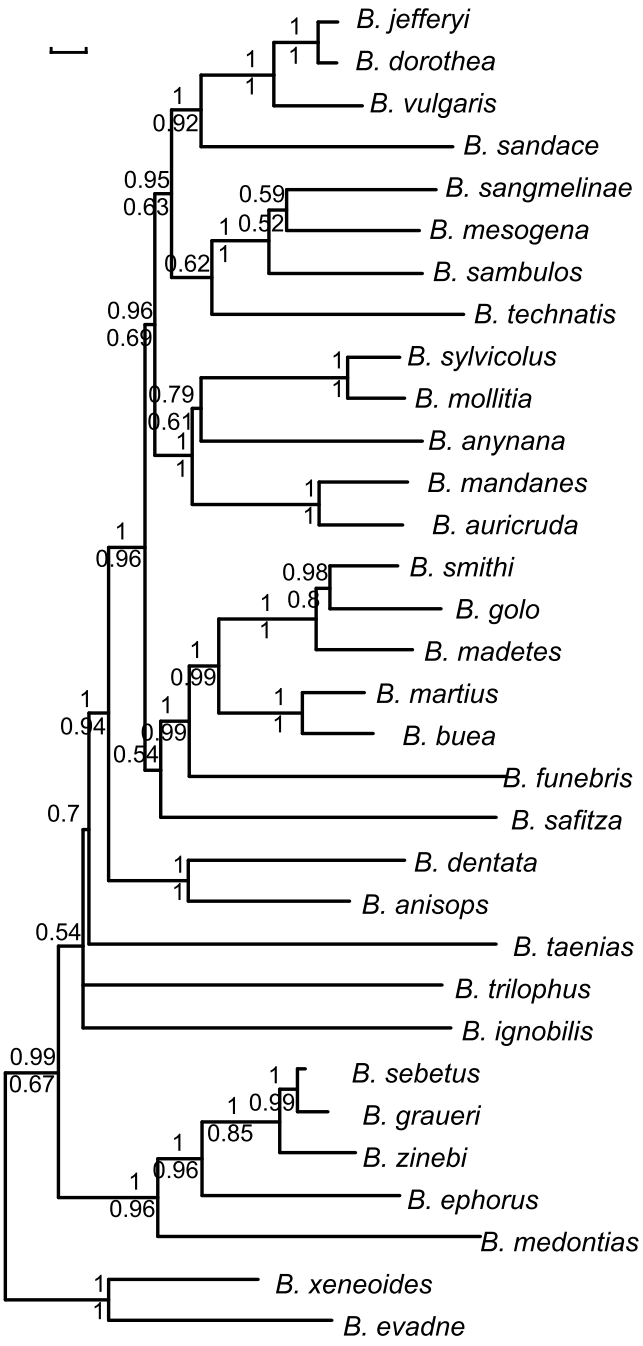


Figure 5.S4: Pruned Bayesian consensus tree.
Numbers represent posterior probabilities (above) and bootstrap values (below) when higher than 50%. The scale bar above the tree represents 1% of genetic divergence.

Maximum likelihood reconstruction and bootstrap: Since posterior probabilities can overestimate the node support (Cummings *et al.* 2003), nonparametric bootstrapping (n = 2000 pseudoreplications) of the Bayesian consensus tree was performed using the online version of the program GARLI (Genetic Algorithm for Rapid Likelihood Inference, Zwickl 2006, Bazinet & Cummings 2011). The Bayesian and Maximum Likelihood methods gave compatible results, although some nodes remain unresolved with the Maximum Likelihood method. As expected, the tree we obtained showed strong similarity to the recent Bayesian reconstruction (Oliver *et al.* 2009). For all our analyses, we therefore used the Bayesian trees, pruned to only contain the taxa with available data for the pMSP. Resolution of the tree and bootstrap support of the topology were strong (BT and PP shown in Figure 5.S4).

Character mapping: to represent the changes of presence and absence of pMSP components and androconia along the phylogenetic tree, we used the maximum likelihood approach with the package StochChar v1.1 in Mesquite 2.75 (Maddison & Maddison 2011) with a symmetrical continuous time Markov model of evolution (Figure 5.2).

5.5.3 Estimating rate of evolution for chemical and morphological traits

One versus two rates of evolution for gains and losses

Estimated rates are similar when we estimate gains and losses as a single parameter (Figure 5.4), or two separate parameters (Figure 5.S5). For the ventral eyespots and androconia, the model containing different rates of gains and losses has stronger support than the model containing a single, common rate (Bayes factors of 10.47 and 3.32, respectively; see Appendix 5.5.2 for definition of Bayes factor). The rate values indicate that the frequency of gains is higher than the frequency of losses, meaning that both traits have diversified during the radiation of *Bicyclus*. In contrast, for dorsal eyespots and pMSP components, the model with a common value for the rates of trait gains and losses is better supported than with different rates of gains and losses (Bayes factors of 2.40 and 2.18, respectively).

Comparing rates between traits

Our results show that, across species, pMSP composition turns over four times faster than dorsal eyespot patterns, which in turn occurs twice as fast as androconial characters (Figure 5.4). However, it is important to interpret these results with caution. The way we coded the morphological traits, distinguishing them only on the basis of their position on the wing (presence, absence), may account in part for their lower rate of evolution compared to pMSP. Indeed, androconia

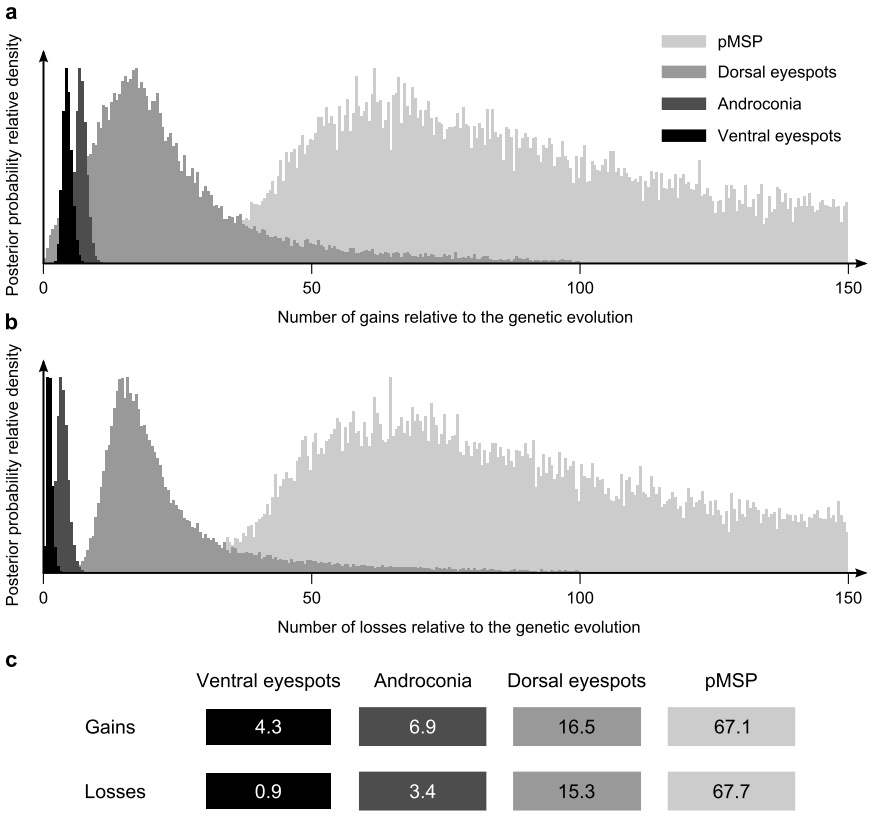


Figure 5.S5: Posterior distribution of evolutionary rates when gains and losses are estimated as two independent parameters.
The traits (from left to right) are: Ventral eyespots, androconia, dorsal eyespots, and pMSP components. The most probable rates of gains and losses (**a** and **b**, respectively; number of change relative to genetic evolution) are presented in panel **c**.

and eyespots can differ in many features in addition to their position on the wing, including shape or UV-reflectance, the latter being a target of sexual selection for *B. anynana* eyespots (Robertson & Monteiro 2005). Moreover, the way we coded the pMSP diversity may also account for their observed higher rate of evolution: the pMSP components were distinguished on the basis of their unique chemical structure. Yet, the same pMSP chemical precursor can lead to different pMSP components that are considered different traits (here present or absent given our coding) rather than different character states. This increased the number of scored traits compared to morphological traits, potentially inflating the real rate of evolution of pMSP compared to morphological traits. These limitations are inherent to the analysis of rates of evolution of independent traits. We considered the chemical distinctiveness of each pMSP component biologically meaningful since the interaction between sex pheromones and their olfactory receptors are highly specific (Sakurai *et al.* 2011).

5.5.4 Within- and among-species variability in chemical profiles

Between individuals of the same species

We used Spearman-rank correlations to estimate the similarity of whole chemical profiles between different individuals within each species. We estimated the correlation for the amount of each chemical in the profile for a) the three males sampled in each species, and b) the two females sampled in each species, accounting for where each was localized on the wings (androconia, rest of wing). Results therefore include four correlation estimates per species (a total of 128 for the 32 species). We performed this analysis using: 1) the complete list of chemicals; 2) the 50% and 10% most abundant chemicals per species for males only; and, 3) the pMSP components for males only (Figure 5.S6). Chemical profiles were found to be repeatable between conspecifics and this similarity increases when the most abundant compounds are considered separately (Figure 5.S6). For some species, the similarity was low between conspecifics but we considered this as natural variability (potentially due to age differences between individuals, Nieberding *et al.* 2012) and kept these species in the data. This is conservative since the confidence on their assigned MSP composition is weaker than for other species and risk to obscure the observed effect of RCD.

Between individuals of different species

We also used Spearman-rank correlations to compare the similarity of chemical profiles when a) pairs of individuals were drawn from the same species, versus b) pairs of individuals were drawn from different species. For males, the correlation was significantly higher when males were conspecifics (Figure 5.S7a), versus when males belonged to different species (Figure 5.S7b). For

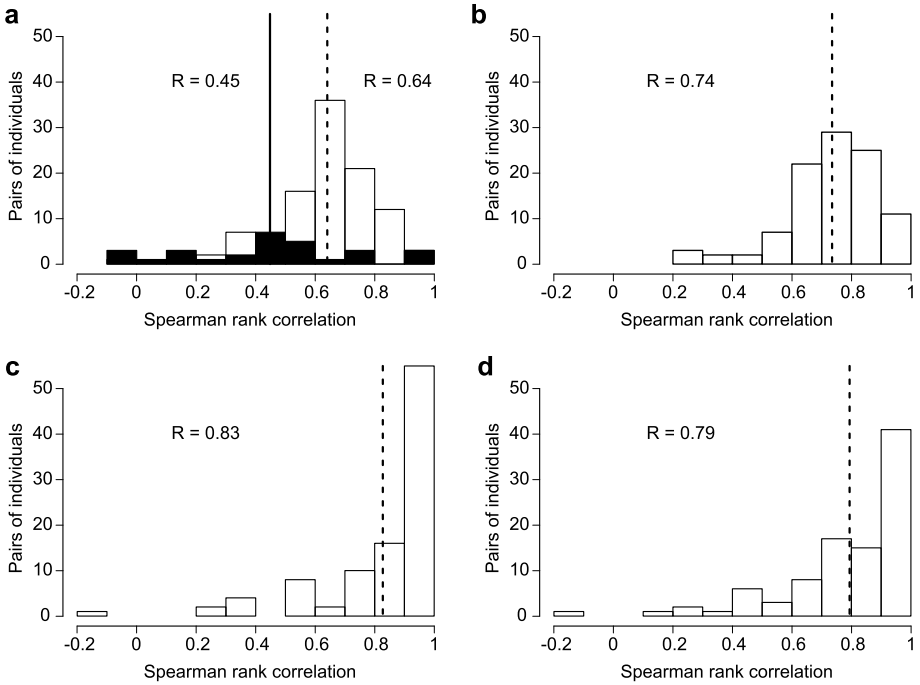


Figure 5.S6: Similarity of chemical profiles among conspecific individuals.
Distribution of the Spearman-rank correlations (R) between individuals of the same sex for different subsets of compounds (mean values of R represented by vertical bars). (**a**) all compounds (males and females in white and black, respectively); (**b** and **c**) the 50% and 10% most abundant male compounds (males only); (**d**) the selection of 75 pMSP components (males only).

females, chemical profiles were more similar across species compared with males (Figures 5.S7e and f). We conducted additional analyses to determine whether this result (more differences in heterospecific profiles among males than among females) was due to the larger number of chemical compounds identified in males (as most pMSP compounds are strictly male-specific). To do this, we resampled a subset of male chemical compounds to equal the average number of chemical compounds typical of female chemical profiles and showed that the gap between the distributions of conspecific and heterospecific correlations in males persisted (Figure 5.S7c and d). Moreover, Wilcoxon rank test is more significant for males (W = 423006, $P < 2.2 \times 10^{-16}$ and W = 421267, $P < 2.2 \times 10^{-16}$ for all pMSP components and for the resampled data, respectively) than for females (W = 28793, $P = 2.5 \times 10^{-5}$). These results suggest that the large differentiation of male chemical profiles between species could be used in species recognition.

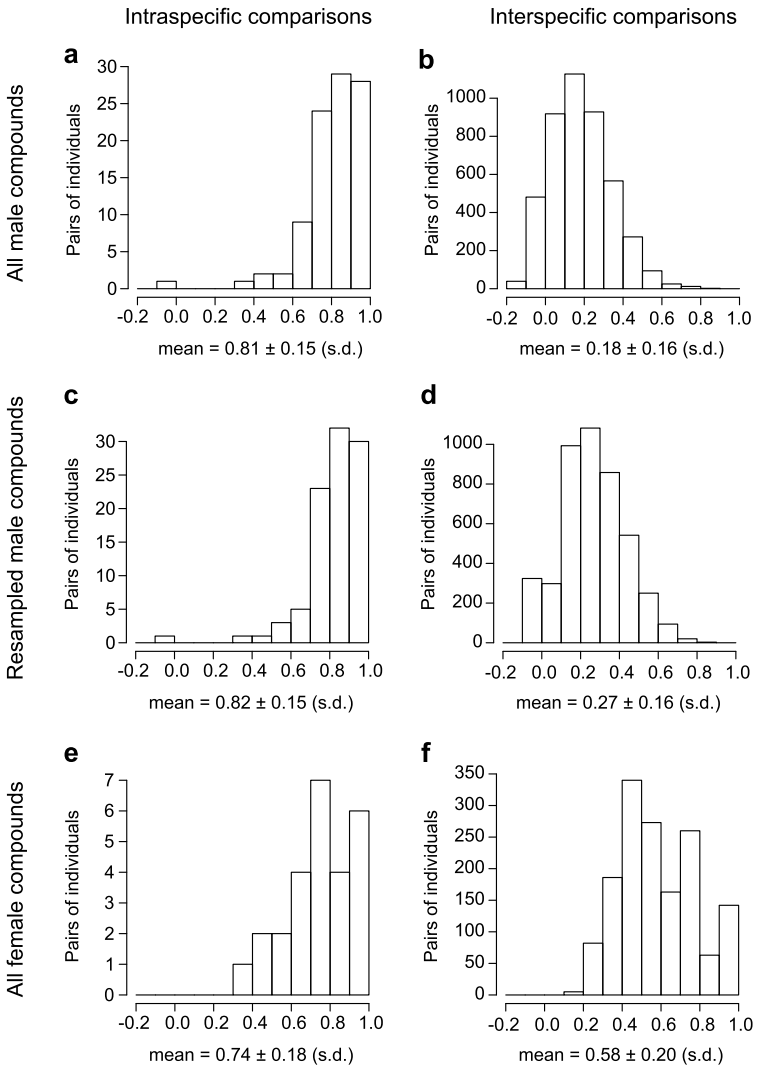


Figure 5.S7: Similarity of chemical profiles among individuals of the same and of different species.
Spearman rank correlations on the amounts of all pMSP components between pairs of conspecific (right), or heterospecific (left) individuals. Of note, panel **a** is different from Figure 5.S6d because in the latter, the localization of the compounds on the wing is taken into account while here only the total amount per individual is used.

5.5.5 Test for Reproductive Character Displacement

Test for RCD in the number of shared traits across species

The number of shared pMSP components was determined only by phylogenetic distance (permutation test; 9999 permutations; n = 496 pairs of species; effect size = -2.49; $P < 0.01$; Figure 5.S8). We observed a similar pattern for the androconia (effect size = -14.02; $P < 0.01$) but not for dorsal and ventral eyespots (P = 0.52 and 0.60, respectively; but see Appendix 5.5.6).

'Young' versus 'old' species pairs

Figure 5.3a shows that the effect of sympatry on differentiation of pMSP composition is strongest when species pairs are closely related phylogenetically, compared to older species pairs. Therefore, we recalculated the model coefficients after splitting the data between 'young' and 'old' pairs of species around the phylogenetic distance of 0.15 (Figure 5.3a). Significance of the model parameters was obtained after permutation of the whole dataset, and fitting again the model on the two groups of pairs (Table 5.S2). Results show that the effect size of sympatry is much larger when only young pairs of species are included in the analysis (1.77), compared to when either all species pairs (effect size of 0.66, Figure 5.3a) or only older pairs of species (0.53) are considered. Separate permutation of the two partial datasets gave equivalent results (results not shown).

Table 5.S2: Effect of the phylogenetic distance, the sympatry, and the habitat type on the number of pMSP differences for the young and old pairs of species.

Factors	Young pairs (n = 38)		Old pairs (n = 458)	
	Effects	P	Effects	P
Intercept	2.50		5.62	
Phylogeny	5.48	0.59	-5.57	0.70
Sympatry	1.77	0.02	0.53	0.04
Habitat	2.03	0.07	1.59	0.06
R^2	0.32	0.22	0.12	0.07

Robustness of RCD to potential outliers

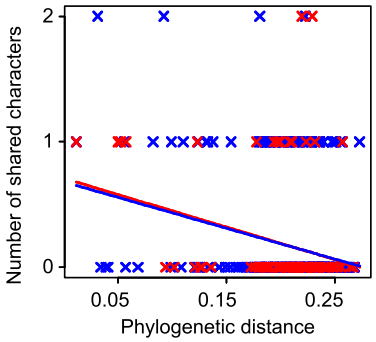
We found a greater number of pMSP component differences across sympatric species compared to allopatric species, and this result is robust even when three potential outlier pairs of species are deleted (Figure 5.S10). It is important to note that these three pairs of species are not phylogenetically close—in fact, they represent independent evolutionary events at the scale of the genus and should not be discarded from the analysis. It is interesting to note, however, that the effect of forest habitat on the number of pMSP differences between species is significant with the 3 pairs removed from the data.

Effect of habitat on RCD

Species inhabiting forest possessed greater numbers of pMSP components and more androconia. While this affects the number of differences we found in these traits (Figure 5.S9a,b), it does not alter the effect of sympatry on the number of differences in traits.

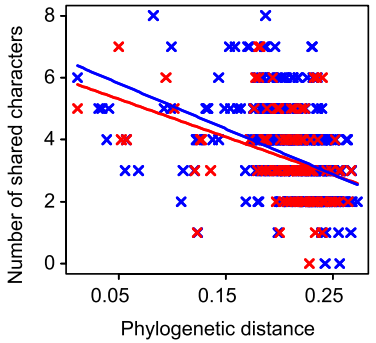
a pMSP

Factors	Effects	*P*
Intercept	0.77	
Phylogeny	**-2.49**	**<0.01**
Sympatry	0.00	0.96
Habitat	-0.06	0.29
R^2	0.07	0.00



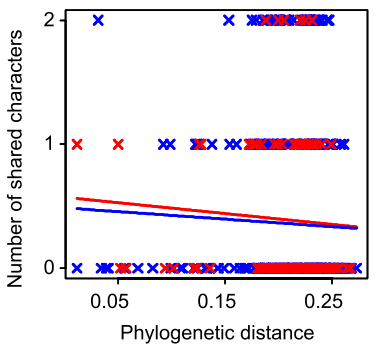
b Androconia

Factors	Effects	*P*
Intercept	6.78	
Phylogeny	**-14.02**	**<0.01**
Sympatry	-0.12	0.35
Habitat	-0.23	0.45
R^2	0.19	<0.01



c Dorsal eyespots

Factors	Effects	*P*
Intercept	0.77	
Phylogeny	-0.67	0.52
Sympatry	0.03	0.64
Habitat	-0.17	0.23
R^2	0.03	0.31



d Ventral eyespots

Factors	Effects	*P*
Intercept	9.45	
Phylogeny	-1.24	0.60
Sympatry	0.08	0.51
Habitat	0.02	0.96
R^2	0.00	0.94

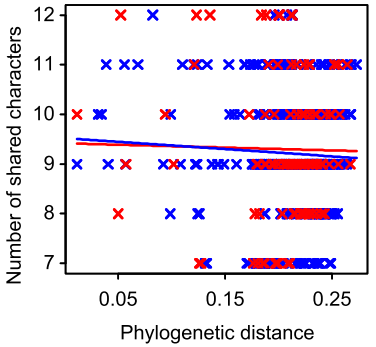
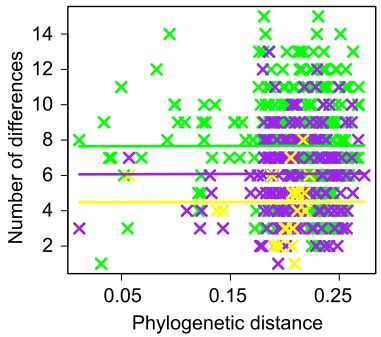


Figure 5.S8: Sympatry, phylogeny and habitat effects on the number of shared characters between pairs of species.
For each trait, (**a**) pMSP composition, (**b**) androconia, (**c**) dorsal eyespots, and (**d**) ventral eyespots, statistical output of the following model is given in a table on the left: *number of shared characters per pair of species* ~ *intercept + phylogeny + sympatry + habitat* (Methods). Significant effects are represented in bold. On the right, graphs show the numbers of shared characters between pairs of species against their phylogenetic distance, using the following model: *number of shared characters per pair of species* ~ *intercept + phylogeny + sympatry + habitat + phylogeny : sympatry*. Sympatric and allopatric pairs of species are represented using red and blue crosses, respectively. These graphs thus include the interaction term for illustration sake and the effect of the habitat is averaged.

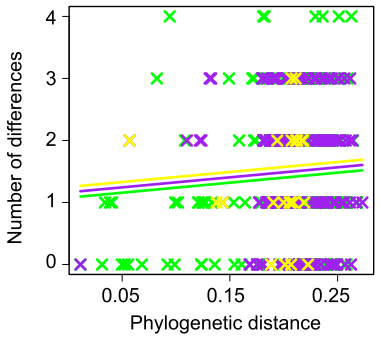
a pMSP

Factors	Effects	*P*
Intercept	4.33	
Phylogeny	0.15	0.97
Sympatry	**0.66**	**0.01**
Habitat	1.58	0.06
R^2	0.13	0.04



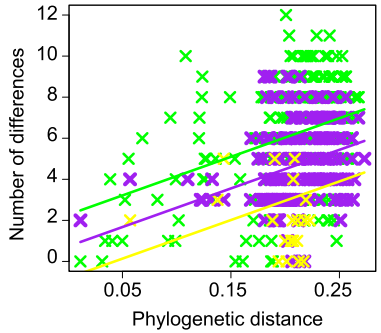
c Dorsal eyespots

Factors	Effects	*P*
Intercept	1.27	
Phylogeny	1.62	0.22
Sympatry	-0.08	0.47
Habitat	-0.09	0.57
R^2	0.01	0.51



b Androconia

Factors	Effects	*P*
Intercept	-0.81	
Phylogeny	**18.81**	**<0.01**
Sympatry	0.03	0.89
Habitat	**1.54**	**<0.01**
R^2	0.24	<0.01



d Ventral eyespots

Factors	Effects	*P*
Intercept	1.68	
Phylogeny	-0.29	0.91
Sympatry	0.01	0.96
Habitat	0.51	0.12
R^2	0.04	0.17

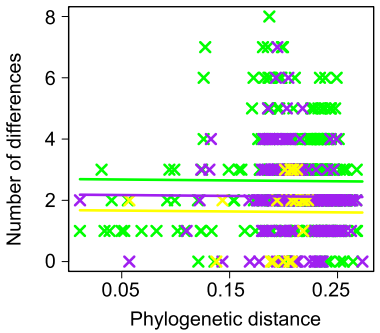


Figure 5.S9: Effect of the habitat type on the number of different characters between species.
For each trait (**a**) pMSP composition, (**b**) androconia, (**c**) dorsal eyespots, and (**d**) ventral eyespots, the statistical output of the following model is given on the left: *number of differences per pair of species* ∼ *intercept + phylogeny + sympatry + habitat* (Methods; same model as for Figure 5.3). Significant effects are represented in bold. On the right, graphs show the numbers of trait differences between pairs of species against their phylogenetic distance for the same model. Here the effect of sympatry is averaged and the effect of habitat is represented in yellow, green and purple for species pairs from savannah, from forest or from mixed habitats, respectively.

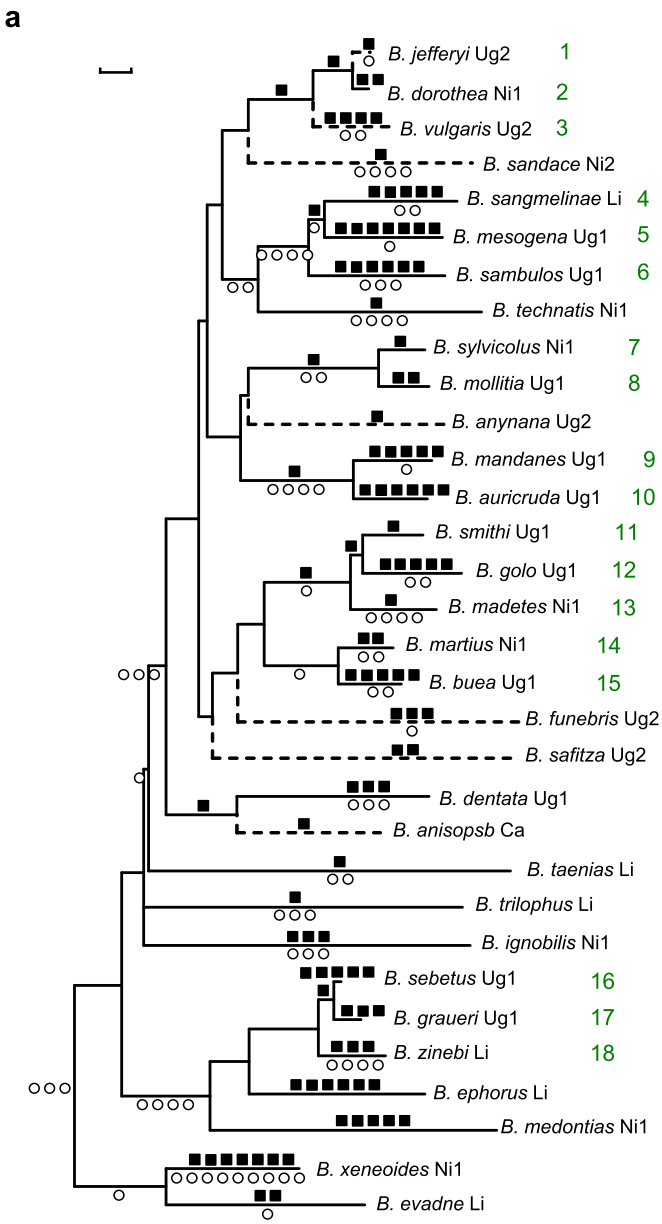
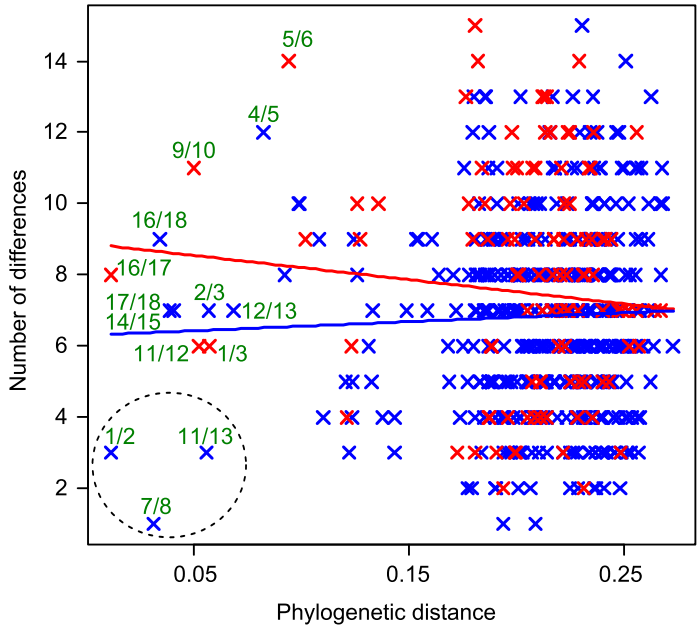


Figure 5.S10: Composition of the youngest pairs of species and robustness of the RCD pattern to the exclusion of potential outlier pairs.
(**a**) Pruned Bayesian consensus tree with inferred changes for pMSP components (filled squares) and androconia (open circles; Appendix 5.5.2). The scale bar above the tree represents 1% of genetic divergence. Plain and dotted tree branches represent forest and savannah habitats respectively.

b



c

Factors	Effects	*P*
Intercept	5.00	
Phylogeny	-3.13	0.29
Sympatry	**0.58**	**0.05**
Habitat	**1.63**	**<0.01**
R^2	0.14	<0.01

Figure 5.S10 (continued):
(**b**) Numbers of different pMSP components between pairs of species against their phylogenetic distance. Sympatric and allopatric pairs of species are represented by red and blue crosses. Lines represent the prediction of the following linear model: *number of differences per pair of species* $\sim$ *intercept + phylogeny + sympatry + habitat + phylogeny : sympatry*; for representation sake the effect of the habitat is averaged. Green numbers indicate the position of the youngest pairs of species in the phylogenetic tree. (**c**) Results of the corresponding regression on distance matrices without the three potential outlier pairs surrounded in panel **b** by the dotted circle.

5.5.6 Test for the presence of a phylogenetic signal for chemical and morphological traits

We tested whether a phylogenetic signal was present for all chemical and morphological traits using two methods. First, for each trait, we compared the fit of a symmetrical Markov model of evolution on two different phylogenetic trees following Mooers *et al.* (1999). The traits were allowed to evolve either along the branches of the real phylogenetic tree, or along a star-like tree (equivalent to a scenario of no shared history between species). When a phylogenetic signal exists for the trait, the real tree should explain the trait evolution better than the star-like tree. We compared the performance of the two trees using Bayes factors in the program BayesTraits (see the definition of Bayes factor in Appendix 5.5.2). For all the traits, the real phylogenetic tree allowed a better fit of the model than the star-like tree (symmetrical Markov model, Bayes factors: 5.36, 100.89, 5.09 and 33.61 for pMSP, androconia, dorsal and ventral eyespots, respectively), showing that more closely related species tend to share more characters than more distantly related ones.

Second, we measured the consistency (CI, Kluge & Farris 1969) and retention indices (RI, Farris 1989) which assess the congruence between the data and the tree under the hypothesis of maximum parsimony. This analysis was run with R (version 2.13.1 for windows, R Development Core Team 2011) and the package Phangorn (version1.4-1, Schliep 2011) and Picante (version 1.3-0, Kembel *et al.* 2010). Since the CI is the ratio between the minimum possible number of character changes in the phylogeny and the observed number, it is sensitive to autapomorphies (characters present in only one species) which inflates its value (Sanderson & Donoghue 1989). The number of autapomorphies has no effect on RI. We therefore only considered the shared characters in the analysis. To test for the significance of these measures, we compared them to a null distribution obtained by repeatedly computing the indices after permutation (9999 replicates) of the associations between sequences of characters and species. This method allows testing for the phylogenetic signal without disturbing the potential association of characters in each blend or combination of androconia or eyespots. It thus avoids the production of unrealistic datasets with, for example, some species having no androconia or too many chemical compounds. Because of the difference between the datasets in terms of percentage of shared characters, the expected values of CI and RI under the null hypothesis of no congruence between tree and trait are different (see the mean of the null distributions on Figure 5.S11). Since the P values were very small for both traits, we measured the distance of the observed value to the null distribution relative to the standard deviation of the null distribution. These indices showed that the congruence between the characters and the phylogeny is stronger in androconia than in pMSP, and stronger in ventral than in dorsal

eyespots (no signal in the latter; Figure 5.S11). The strength of the phylogenetic signal measured with CI and RI followed the same ranking as the sizes of the Bayes factors obtained with the model comparison.

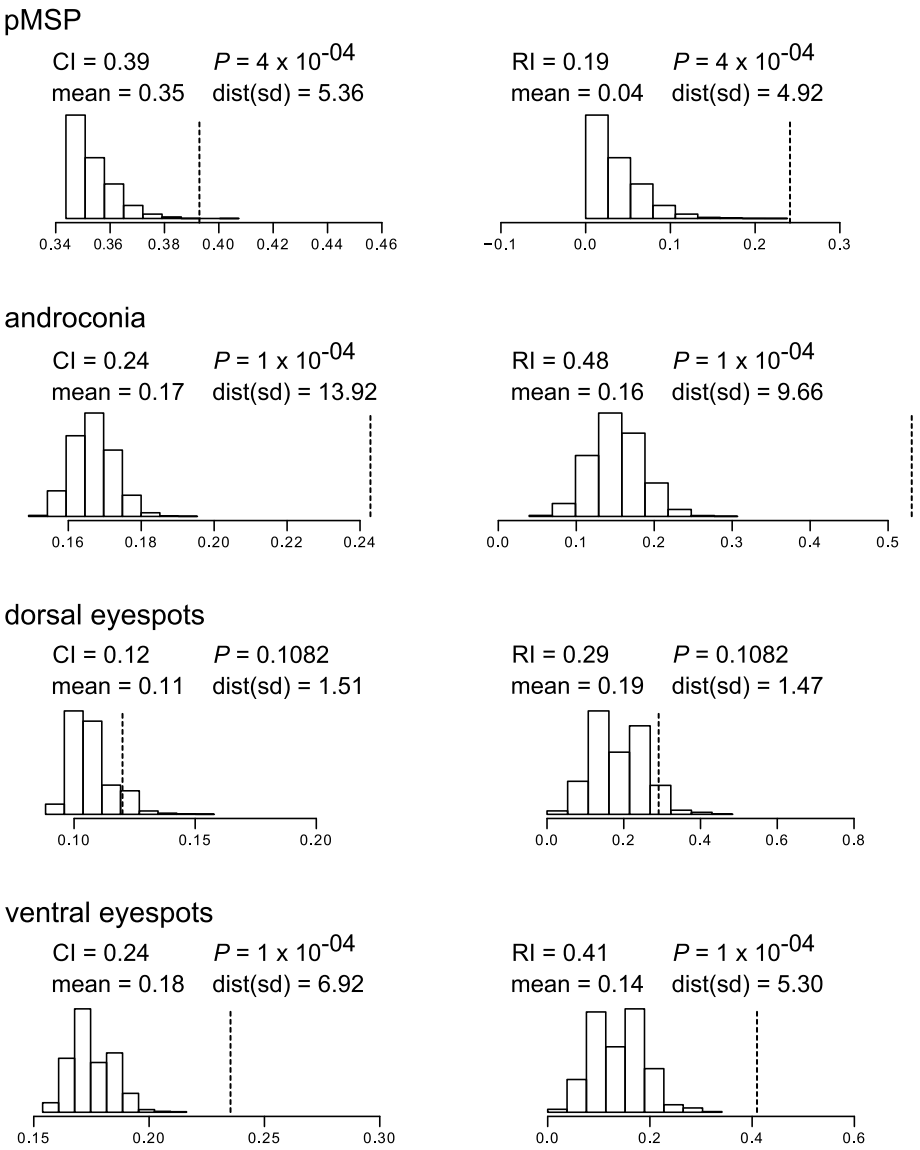


Figure 5.S11: Evidence for the presence of a phylogenetic signal in chemical and morphological traits.
The observed consistency index (CI) and retention index (RI) values (left and right graphs, respectively) are represented by vertical dotted lines, and their null distributions are represented by histograms. The *P* values, the mean values of the null distributions, and scaled distance between the observation and the null distribution [dist(sd)] are indicated above each graph. For the convenience of visual comparison between traits, abscissas are aligned and scaled with the standard deviation of each null distribution.

5.5.7 Diversification of pMSPs and androconia is uncoupled

Androconia have been used as a proxy to assess the existence and the importance of sex pheromone communication in male Lepidoptera (Phelan & Baker 1987), and are a key taxonomic trait in *Bicyclus* (Condamin 1973). Our analyses show that the number of differences in both androconial traits and pMSP components between pairs of species is affected by habitat in the same way: there are more differences between pairs of forest species than between pairs of savannah species (Figure 5.3). Because habitat affects both traits in the same way, we further investigated whether specific androconia were associated with the presence of specific pMSP components. To conduct this analysis, we identified twelve 'androconial units' by classifying as a single unit pairs of brushes which merge together despite originating in more than one wing space, and faint patches lying directly under brushes (see Figure 5.S12 for positions of these units). From the abundance data it was clear that most compounds were either found exclusively in a single androconial unit per species, or if found in several only as a trace in all positions except on dominant position. We therefore investigated which androconial unit contained the most of each selected pMSP for each species. The majority of pMSP components (65%) occurred in higher amounts in the 'cell brush' unit (unit 1; Figure 5.S12). The cell brush occurs in 31 of the 32 species we investigated (absent in *B. buea*), and this structure alone appears to contain a high proportion of the pMSP diversity we identified. The remaining 25% of the pMSP components occurred in parts of the wings which did not contain androconia. In most cases, the remaining androconial units (units 2–12; there are 1–5 units per species, see Figure 5.S12) generally lacked significant amounts of any pMSP components. Despite the fact that the number of androconial units seems to have increased during the diversification of the genus (Figure 5.S5), we did not find a one-to-one association between pMSP components and specific morphological structures. Most androconia may have lost a function in olfactory communication, or perhaps never had one. Our results indicate that the current diversity of androconia and pMSP components are independent and that these elaborate and fixed morphological structures may have a role in another, as yet unknown, function maintained by selection.

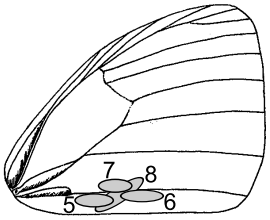
a Species	1 Cell brush (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	7 (%)	8 (%)	9 (%)	10 (%)	11 (%)	12 (%)	Rest of wings (%)
B. anisops	0	0			0	0							100
B. anynana	100	0			0	0							0
B. auricruda	100	0		0	0	0							0
B. buea	0	0	0			0				0			100
B. dentata	50				0				0				50
B. dorothea	100	0		0	0	0							0
B. ephorus	83		0					0		0			17
B. evadne	100				0								0
B. funebris	0	0				33							67
B. golo	80	0			0	0							20
B. graueri	100		0					0					0
B. ignobilis	100			0									0
B. jefferyi	67	0			0	0							33
B. madetes	100				0				0	0			0
B. mandanes	83	0	17	0	0	0							0
B. martius	0	25				50				25	0		0
B. medontias	80		0					0					20
B. mesogena	88	0	0	12		0							0
B. mollitia	100				0	0						0	0
B. safitza	50	0			0	0							50
B. sambulos	50		17	0	0					0			33
B. sandace	0	0	100	0		0							0
B. sangmelinae	50	17	0	0	0	0							33
B. sebetus	50		0					0					50
B. smithi	67				0	33						0	0
B. sylvicolus	0				0	0						100	0
B. taenias	0				0	0	0			100			0
B. technatis	100	0		0	0	0							0
B. trilophus	100		0	0	0					0			0
B. vulgaris	0	0				0							100
B. xeneoides	86	0	0			0			0	0			14
B. zinebi	100		0					0				0	0
	pMSP components primarily contained by each unit (%)												
	65	1	2.5	1	0	2.5	0	0	0	1	0	2	25

Figure 5.S12: Distribution of pMSP components among androconial units on *Bicyclus* wings.
(**a**) Columns represent each of the 12 androconial units (association of androconial hairpencil and patch, Nieberding *et al.* 2008) and the rest of the wings. Each pMSP component was usually present in a higher amount in a single androconial unit considered as actively releasing that component. Lines represent the percentage of the pMSP components assigned to each unit per species.

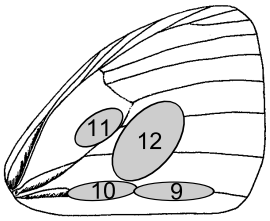
Species	Number of androconial units	Active androconial units (%)
B. anisops	4	0
B. anynana	4	25
B. auricruda	5	20
B. buea	4	0
B. dentata	3	33
B. dorothea	5	20
B. ephorus	4	25
B. evadne	2	50
B. funebris	3	33
B. golo	4	25
B. graueri	3	33
B. ignobilis	2	50
B. jefferyi	4	25
B. madetes	4	25
B. mandanes	6	33
B. martius	5	60
B. medontias	3	33
B. mesogena	5	40
B. mollitia	4	25
B. safitza	4	25
B. sambulos	5	40
B. sandace	5	20
B. sangmelinae	6	33
B. sebetus	3	33
B. smithi	4	50
B. sylvicolus	4	25
B. taenias	5	20
B. technatis	5	20
B. trilophus	5	20
B. vulgaris	3	0
B. xeneoides	6	17
B. zinebi	4	25
	Mean (s.d.)	Mean (s.d.)
	4.2 (1.0)	27.7 (13.6)

b

Forewing ventral surface



Forewing dorsal surface



Hindwing dorsal surface

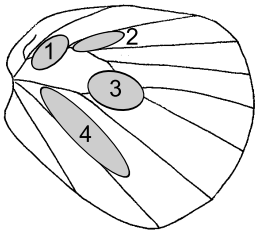


Figure 5.S12 (continued):

Blank cells correspond to androconial units absent in the species. Grey cells pinpoint androconial units containing 50% or more of the pMSP components found in the species. The last two columns show the total number of androconial units and the proportion active. The last line shows the proportion of all pMSP components present primarily in each unit. (**b**) Scheme showing the location of androconial units on wings.

5.5.8 Effect of the environment on the diversity of pMSP composition

We investigated the effect of habitat type (savannah, forest) on the diversity of pMSP composition using two approaches in addition to those described in the main text. First, we analysed the number of pMSP components per species as a function of habitat by using generalized linear models (GLM) with a Poisson distributed error. Since species diversity is higher in the forest habitat, we also included the number of sympatric species as a factor in the analysis. Model selection was based on corrected Akaike information criterion (Burnham & Anderson 2002, AICc) and the best model was:
Number of pMSP components $\sim$ *number of sympatric species* + *habitat* + *number of sympatric species : habitat*.

Since some of the effects were significant, we corrected for phylogenetic non-independence of species (Abouheif 1999). For this purpose we used the generalized estimating equations (Paradis & Claude 2002, GEE) in the R package APE (Paradis *et al.* 2004, R Development Core Team 2011, version 2.13.1 and 2.5-3 respectively). Results showed that forest species have a higher number of pMSP components than savannah species (effect size = 4.52; $P <$ 0.01; Figure 5.S13 and Table 5.S3). We also observed a significant interaction between habitat type and the number of sympatric species, which is difficult to explain: in forest habitat, the higher species diversity is associated with fewer pMSP components per species, while the opposite is true in savannah habitats. As these results were obtained using only three locations per habitat, this effect must be taken with caution and should be checked by sampling a larger number of locations per habitat.

Second, we examined the effect of habitat on pMSP composition by plotting the number of pMSP differences for pairs of species that either shared, or did not share, the same habitat. Species of different habitats did not differ more in pMSP composition than those sharing the same habitat, indicating a limited effect of habitat on pMSP differentiation at this geographic scale (Figure 5.S14). Most pMSP differentiation is present within the forest habitat, which was to be expected given that forest species have more pMSP components overall (Figure 5.S14 and Table 5.S3). Because habitat affects the number of pMSP differences between species, habitat was included as a factor in the main analysis of RCD on pMSP composition (Figure 5.3). The coding was '0' for savannah pairs of species, '1' for mixed pairs of species, and '2' for forest pairs of species.

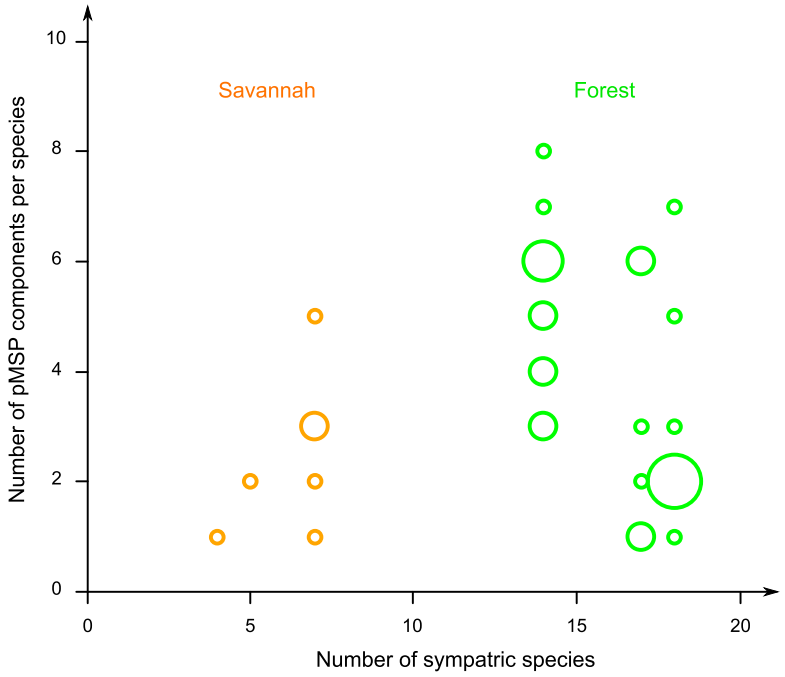


Figure 5.S13: Number of pMSP components per species as a function of habitat and the number of sympatric species surrounding the focal species.
The size of the circles is proportional to the number of species (1 to 4). Savannah species are in in orange and forest species in green.

Table 5.S3: Best fit model parameters and their significance for the generalized estimating equations.

Factors	Estimates	s.e.	t	*P*
Intercept	-0.68	1.16	-0.59	0.57
Number of sympatric species	0.30	0.16	1.87	0.09
Habitat (forest)	4.52	1.21	3.75	< 0.01
Number of sympatric species : habitat	-0.45	0.16	-2.77	0.02

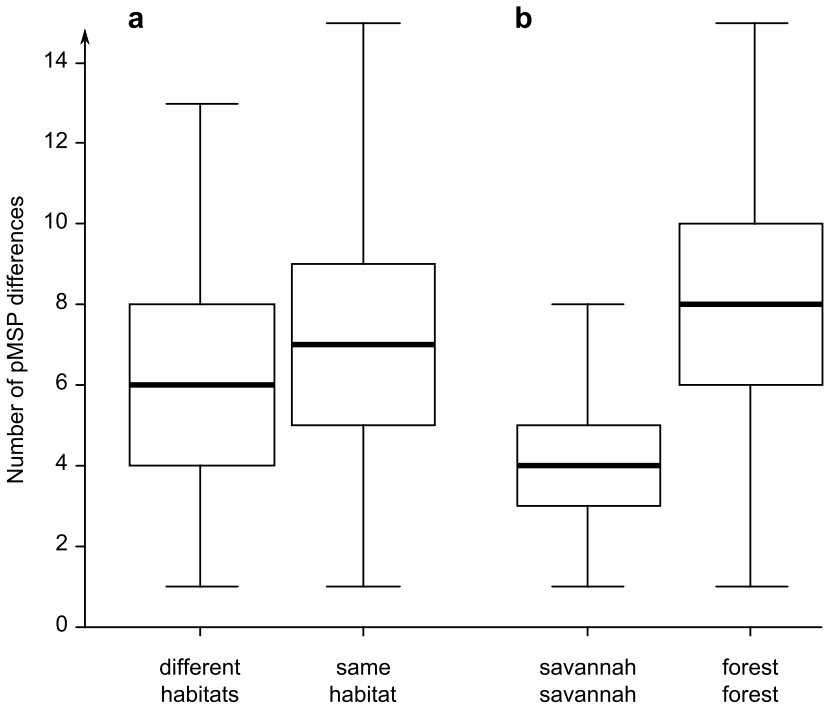


Figure 5.S14: Number of pMSP differences based on the habitat occupied by the pairs of species.
(**a**) Box-plots of the number of pMSP differences for pairs of species that either live in different habitats or share the same habitat. (**b**) Box-plots of the number of pMSP differences for pairs of species living both in savannah or both in forest habitat (subsets of the second category of panel **a**).

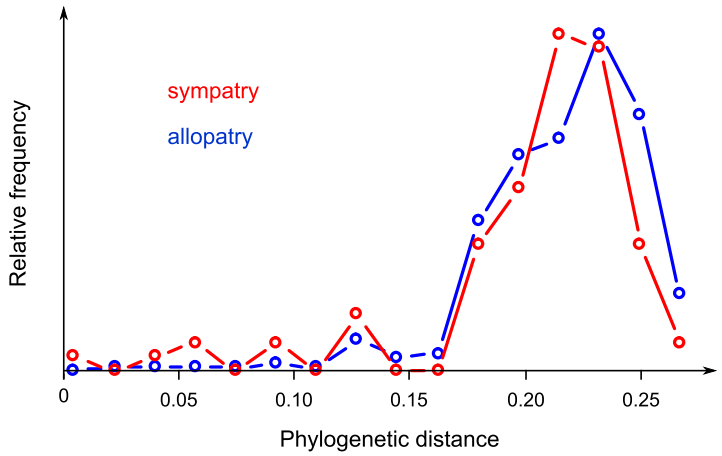


Figure 5.S15: Distribution of the phylogenetic distance between species depending on their sympatric or allopatric status in our study.
The distances are based on the Bayesian tree (Appendix 5.5.2). The frequencies (ordinate axis) are scaled between the two groups (90 sympatric and 406 allopatric pairs of species).

5.5.9 Sympatry and age of the pairs of species—the differential fusion hypothesis

We show that species sampled in allopatry are not more closely related than species sampled in sympatry (Figure 5.S15; one tailed permutation test; 9999 permutations, $P = 0.93$). This type of test should be interpreted with caution since species ranges can change over time (Losos & Glor 2003). Moreover, our assessment of sympatry only depends on the location where species were sampled, not on sympatry at the scale of the entire range of the species. In any case, this pattern is not in accordance with the differential fusion hypothesis (Templeton 1981) which predicts that allopatric species are more closely related than sympatric species, since sympatric species are the subset of allopatric species that were differentiated enough to coexist after secondary contact (Lukhtanov *et al.* 2005, Jiggins 2006a).

Of note, we obtained pMSP data for most sympatric species in each of four sampled locations during extensive field work, which limits the risk of underestimating the relative age of sympatric, compared to allopatric, pairs of species. Moreover, in our sample of 32 *Bicyclus* species, we found only two cases where the original taxonomic description (Condamin 1973) had missed cryptic species living in sympatry, namely for *B. mesogena* in Uganda and *B. mandanes* in Cameroon. These taxonomic changes reinforce our results as it reduces further the average phylogenetic distance between pairs of sympatric species.

Data accessibility

The new DNA sequences are accessible on Genbank, accessions KC786271 to KC786277. The raw GC-MS data, the different trait matrices and the different tree files are accessible on Dryad public repository[§].

Authors' contributions

P.M.B.B., O.B., P.M.B. and C.M.N. conceived and designed the study. The pheromone field sampling protocol was developed and tested by C.L., H.-L.W. and O.B. O.B. did the main data collection in the field (Liberia, Nigeria and Uganda) with the contribution of P.M.B.B. (Cameroon and Uganda) and C.M.N. (Cameroon). H.-L.W. performed chemical analyses, and H.-L.W. and C.L. identified compounds. P.M.B.B., O.B. and H.-L.W. did the post treatment of raw chemical data. O.B. conducted the scoring of androconia. P.M.B.B. did molecular analyses, carried out the phylogenetic and statistical data analyses. P.M.B.B. and C.M.N. wrote the manuscript with the contribution of O.B., H.-L.W., C.E.A., C.L. and P.M.B.

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[§]see `http://dx.doi.org/10.5061/dryad.768sd`

Chapter 6

Early divergence of male chemical blends in the African butterfly *Bicyclus anynana*, a candidate signal to trigger reproductive isolation*

*This chapter is an article in preparation authored by Paul Bacquet, Maaike de Jong, Oskar Brattström, Hong-Lei Wang, Freerk Molleman, Stéphanie Heuskin, George Lognay, Christer Löfstedt, Paul Brakefield, Alain Vanderpoorten‡ and Caroline Nieberding‡ (‡co-last authors).

Abstract: Strong divergence of secondary sexual traits among closely related species is often observed and suggests that these traits play a major role in the initiation of reproductive isolation and speciation. We compared the male-specific chemical profiles and the genetic structure of several populations of the African butterfly *Bicyclus anynana* (Butler, 1879). If chemical differentiation is a driver of reproductive isolation, 'pheromonal dialects' should be found among populations that are not yet genetically isolated. We estimated the genetic divergence among field-caught populations of *B. anynana* by sequencing the mitochondrial COI and nuclear CAD loci. Based on the properties of the previously identified male sex pheromone in *B. anynana*, we selected male-specific, repeated and abundant compounds within the chemical profiles in these populations, which formed their putative male sex pheromone (pMSP). We observed quantitative differences in some shared pMSP components as well as qualitative differences in the composition of the pMSP among populations. Specifically, similarly large differences in the pMSP components were found both between populations of the same species and between different *Bicyclus* species. In addition, *B. anynana* individuals from Kenya displayed differences in the pMSP composition within a single population that were not associated with genetic differences. Our results reveal that pheromonal dialects likely evolved within and among populations of *B. anynana*, and may therefore act as precursors of an ongoing speciation process.

6.1 Introduction

As Darwin (1871) already noticed and recent studies have recurrently demonstrated, sexual traits are often the most divergent characters among closely related species (Mendelson & Shaw 2005, Arnegard *et al.* 2010a, Safran *et al.* 2012). Differences in sexual traits have therefore been perceived as the signature of ongoing reproductive isolation and speciation (Allender *et al.* 2003, Boul *et al.* 2007, Ritchie 2007). Interspecific differences in traits might however accumulate subsequently to speciation and be the consequence rather than the cause of this process (Butlin 1987). Hence, a potential link between trait divergence and speciation can only be made if we observe trait differences that have evolved before or during the speciation process, i.e. at the level of populations or early stages of differentiation (Tregenza *et al.* 2000, Masta & Maddison 2002, Ritchie 2007, Amézquita *et al.* 2009, Grace & Shaw 2012, Oneal & Knowles 2013, Schwander *et al.* 2013).

Among the sexual traits potentially involved in differentiation, pheromones, which convey information among conspecific individuals in many species of mammals, reptiles, amphibians, fishes and insects (see Kikuyama *et al.* 2002, Johansson & Jones 2007, Smadja & Butlin 2009, Wyatt 2014 for review), may play a key role in the process of differentiation. Indeed, while species-specificity of sex pheromones maintains reproductive isolation between species sharing the same habitat (Löfstedt *et al.* 1991, Roelofs *et al.* 2002), evidence for intraspecific quantitative and qualitative variation in sex pheromones has been recurrently reported in Lepidoptera (e.g. *Agrotis segetum*: Toth *et al.* 1992, LaForest *et al.* 1997; *Ctenopseustis obliquana*: Clearwater *et al.* 1991; *Ostrinia nubilalis*: Klun *et al.* 1975, Roelofs *et al.* 1985). Some moth species showing population differences in the ratios of female sex pheromone components displayed no genetic divergence (Sperling *et al.* 1996, Groot *et al.* 2008, Nagoshi & Meagher 2008, Palacio Cortés *et al.* 2010). On the other hand, some taxa that had evolved differentiated sexual signals are already well isolated genetically (White & Lambert 1995, Sperling *et al.* 1996, Svensson *et al.* 2013). This supports the need of a genetic control of the taxonomic context—intra or interspecific—to identify if the chemical differences are relevant to a potential speciation event or to post-speciation interspecific divergence.

The Sub-Saharan butterfly genus *Bicyclus* (Lepidoptera, Satyrinae), which includes over 80 species, appears as an ideal model to explore the role of pheromone divergence in the process of speciation. In fact, up to 20 species can be found in sympatry in a single forest patch. The importance of sexual traits in the differentiation among species is suggested by the fact that the main taxonomic difference among species is the position and shape of the androconia, which are male wing patches and brushes formed by modified scales and assumed to produce the male sex pheromone components (MSP

components hereafter; Condamin 1973). Three MSP components have been identified in the model species *Bicyclus anynana*. Variation in their abundance among males is under sexual selection by female choice and indicates male age and their level of inbreeding (Nieberding *et al.* 2008, 2012, San Martin *et al.* 2011, van Bergen *et al.* 2013). Moreover, we recently identified the major male-specific compounds (putative MSP) in 32 *Bicyclus* species and showed that pairs of species differ on average by seven compounds (Bacquet *et al.* in press, see Chapter 5). Such a difference is of greater magnitude than what is known for female sex pheromone in moths (Linn & Roelofs 1989). We moreover unravelled a reproductive character displacement pattern at the scale of the genus which supports the role of this major male compounds in species recognition (Bacquet *et al.* in press) and potentially in *Bicyclus* speciation. If the divergence of male chemical profiles is involved in speciation, we expect to observe MSP differences between, or even within, populations of the same species, i.e. before completion of the speciation process. Therefore, we tested the hypothesis that, divergence in MSP accumulates in a context of low genetic differentiation.

6.2 Material and methods

6.2.1 Population sampling

We sampled four geographically distinct populations of *B. anynana* (Figure 6.1 and Table 6.1). Among the 160 sampled individuals, a subset of 35 males and 33 females was used for the chemical analyses (Table 6.1). Most of the males and females of each species in each location were sampled during a single trapping session of one week (Table 6.1 and 6.S1). We also sampled *B. anynana* individuals from two lab stocks, the first one originating from Malawi (Nkhata Bay, from 80 females in 1988, Brakefield *et al.* 2009) and used in most studies on *B. anynana*, and the second one sampled in 2006 from South-Africa (False Bay, from over 70 females, de Jong *et al.* 2010).

6.2.2 DNA sequencing

DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen) from a leg or a thorax piece kept in 100% ethanol at -20 °C or in glassine envelope at -80 °C. In total we sequenced 160 and 77 individuals for the mitochondrial gene cytochrome oxidase 1 (COI) and the nuclear gene carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD) following the protocols of Monteiro & Pierce (2001) and Wahlberg & Wheat (2008)[†]

[†]see `http://nymphalidae.utu.fi/Nymphalidae/Molecular.htm`

Table 6.1: Geographical distribution and size of the samples for genetic and chemical analyses of *B. anynana*.

Country	Location	Number of sequences			Number of chemical analyses		Date of chemical sampling
		COI	CAD	EF1α	♂	♀	
Uganda	Mburo	38	12	7	3	2	9/2009
Uganda	Ishasha	11	11				
Kenya	Watamu	27	16		9	8	3/2007
Malawi lab stock	Nkhata Bay	9			3	2	6/2010
South-Africa	False Bay	37	28	2	15		5/2007
						15	7/2006
South-Africa lab stock	False Bay	8			3	2	5/2010
South-Africa	Limpopo	30	10	3	5	6	7/2006

Detailed information for each individual are given in Table 6.S1.

(Table 6.S1; some COI sequences already available from de de Jong *et al.* 2011). The COI gene showed a strong similarity among all individuals not expected for a rapidly evolving marker compared to the nuclear CAD which displayed a larger diversity (Figures 6.2 and 6.S2). A few sequences for a third independent marker (elongation factor 1 α, nuclear) confirmed that the uniformity of the COI sequences was not reflecting the populations neutral divergence (Table 6.S4). As a confirmation, neutrality tests H of Fay & Wu (2000) and D of Tajima (1989) were both significant for the COI but not for the CAD gene (Table 6.S5). Altogether, these information suggest that COI underwent a selective sweep and is therefore inappropriate to describe the neutral genetic divergence of the populations in this species, leading us to discard this locus from subsequent analyses (Appendix 6.5.3).

6.2.3 Analysis of population structure and genetic diversity

We deduced the haplotypes forming each individual CAD diploid genotypes using the Excoffier–Laval–Balding algorithm (ELB) in Arlequin software (version 3.5.1.2) with default parameter values (Excoffier *et al.* 2003, 2005). Five independent runs of the ELB algorithm were compared, and we discarded individuals showing different coding phases across runs (seven individuals, Table 6.S1).

To represent the genetic distance among haplotypes, we constructed Median

Joining networks (Bandelt *et al.* 1999) with the software Network 4.6.1.1[‡]. To ensure the consistency of the network construction, several reconstructions were performed with variable epsilon values, and the less parsimonious links were deleted to simplify the network (Polzin & Daneshmand 2003). We also tested the consistency of network links by analysing partial datasets after removing the haplotypes occupying a central position in each full network. For sake of comparison we additionally reconstructed the Maximum Likelyhood phylogenetic trees (Appendix 6.5.2).

We investigated the distribution of the genetic diversity among populations with an analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) based on haplotype uncorrected pairwise differences. We performed 1000 permutations of the individuals among populations to test the significance of the observed pattern of genetic variation. We also calculated Φst between pairs of populations, a type of Fst based on haplotype dissimilarity (Excoffier *et al.* 1992). We run these analyses in Arlequin (version 3.5, Excoffier *et al.* 2005).

6.2.4 Extraction, analysis and repeatability of chemical profiles

Androconia were dissected from the wings of one side of freshly killed butterflies or from butterflies frozen alive and kept at -80 °C. At -80 °C the chemical compounds do not evaporate and these two treatments give the same extraction profiles. Each androconium was extracted in separate 2 ml screw cap vials (VWR International) with 100 µl of redistilled n-heptane (VWR International) containing 1 ng/µl of (Z)-8-tridecenyl acetate (Z8-13:OAc) as an internal standard. The remaining tissue of each dissected wing was also extracted in a vial with 300 µl redistilled heptane containing 0.33 ng/µl of the internal standard. The wing extracts were analysed on Agilent 5975 mass-selective detector coupled to an Agilent 6890 gas chromatograph (GC-MS), equipped with a HP-5MS capillary column (30 m × 0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific, USA). The oven temperature was programmed from 80 °C for 3 min, then to 210 °C at 10 °C/min, hold for 12 min and finally to 270°C at 10°C/min, hold for 5 min. Inlet and transfer line temperatures were 250 °C and 280 °C, respectively, and helium was used as the carrier gas. Compounds that eluted no later than tricosane were considered as the more volatile part of the chemical diversity displayed by these butterflies, and thus included in our analysis. Identification of wing compounds was done by comparing the GC retention times and mass spectra with commercially acquired or lab-synthesized authentic compounds on both non-polar (HP-5MS) and polar (INNOWax, 30 m × 0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific, USA) columns. Tentative structures were assigned for compounds that were not fully identi-

[‡]see `www.fluxus-engineering.com`

fied. The chemical composition and related information are summarized in the Appendix 6.5.4.

We determined the repeatability of chemical profiles (i.e. all compounds found in the chemical extracts, as opposed to the pMSP composition, see below) among field-sampled individuals within and between the four populations by using Spearman correlations on the amounts of compounds recognized as homologous (i.e. compounds displaying identical mass spectra and retention times across individuals).

6.2.5 Selection of putative male sex pheromone components

The chemical profile of *Bicyclus* butterflies consists of many compounds (Appendix 6.5.4). For example, in the model species *B. anynana*, over a hundred chemicals with low volatility have been identified on the different body parts, of which twenty significantly correlated with traits important in mate choice in this species (sex, age; Heuskin *et al.* 2014). Since behavioural or GC-EAD assays would be unrealistic to perform for each field-caught population, we used a standardized routine that was developed by Bacquet *et al.* (in press) to select a list of putative male sex pheromone (pMSP) among all the compounds observed per species. The selection criteria were based on behavioural, gas-chromatography coupled to mass-spectrometry (GC-MS) as well as gas-chromatography coupled to electro-antennographic detection (GC-EAD) data accumulated from the stock populations of *B. anynana* originating from Malawi and South-Africa (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013, and unpublished data). In summary, we first applied a threshold of 10% of the internal standard such that any compound at a lower amount than 20 ng per individual was discarded from the analysis to standardize the quality of peak identification among samples. After this filtering step, the absolute amount of each compound and its repeatability level among individuals were computed. A compound was considered repeatable if present in two males out of three. As the three behaviourally and electro-physiologically active MSP components identified in the Malawi stock of *B. anynana* are among the top third most abundant of the repeatable, and male-specific compounds (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013), we thus selected in every population the pMSP components following the same criteria. The female samples were used to assess the male specificity of the chemical compounds. A compound was considered as male-specific if it was five time more abundant, or more, than in female extracts sampled in the same population. Of note, our selection method of abundant and repeatable male-specific compounds that form the pMSP of *Bicyclus* species is in accordance with observations that MSP components that experience directional sexual selection are usually more abundant than other compounds (see Steiger *et al.* 2011, and examples in Lepidoptera: Iyengar *et al.*

2001, Andersson *et al.* 2007, Hymenoptera: Ruther *et al.* 2009, lizards: Martín & López 2010, or elephants: Greenwood *et al.* 2005).

6.2.6 Analysis of the chemical diversity

To describe the chemical diversity, we applied three types of dissimilarity indices on the chemical profiles of individuals. First, the Jaccard dissimilarity described qualitative differences in composition (presence and absence of compounds). Second, the Euclidean distance discriminated chemical profiles based on the absolute amounts of compounds and, thirdly, on the relative amounts of compounds (proportion of the sum of all compounds per individual). Using these dissimilarity indices, we performed permutational multivariate analyses of variance (PerMANOVA, adonis function in R package vegan; Oksanen *et al.* 2011) for estimating how much chemical variation was explained by sex and geographic origin of the individuals. PerMANOVA was preferred to the conventional MANOVA because its non-parametric permutational approach for testing significance is robust to the presence of large proportions of null data and is not restricted to the use of Euclidean distances (McArdle & Anderson 2001).

6.2.7 Comparison of genetic and chemical differentiation between pairs of populations.

To test the hypothesis that variation in MSP accumulates early between populations relatively to their neutral genetic divergence, we compared Φst between all pairs of populations based on either CAD distances or chemical distances in the similar framework with Arlequin (version 3.5, Excoffier *et al.* 1992, 2005). For chemical profiles, we used distance matrices based on presence-absence of compounds, absolute amounts of compounds and relative amounts of compounds. If, as we hypothesized, divergence in MSP is a potential cause of reproductive isolation and speciation in *Bicyclus*, we expect to observe a bias towards higher levels of pMSP differentiation as compared to neutral genetic variation.

6.3 Results

6.3.1 Patterns of genetic structure

B. anynana displayed significant genetic variation between populations based on the CAD nuclear marker (37% of the total genetic variance, $P < 0.001$, see AMOVA in Appendix 6.5.5 Table 6.S7). The Median Joining network of the CAD gene supported the isolation of a Ugandan clade, in which all but three divergent Ugandan haplotypes clustered together (black arrows in Figure

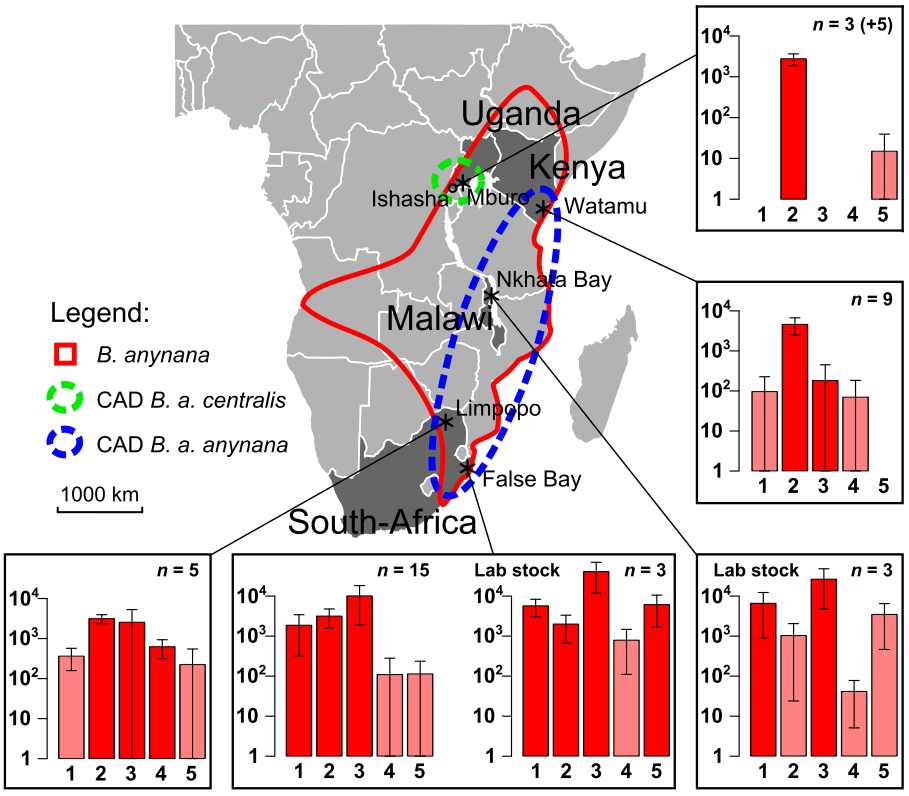


Figure 6.1: Geographic localization and pMSP composition of the populations of *B. anynana*.
Sampling sites are represented by asterisks excepted for Ishasha where only genetic data were sampled. Hypothetical distribution range derived from Condamin (1973) is represented in plain red. The two CAD haplotypes corresponding to *B. anynana* subspecies are in green and blue dashed line. The bar plots represent the log-transformed average amount (ng) of each pMSP component in each population (± S.D.; the error bar comprises zero if some individuals lack the compound). The sample size is given for each graph (within brackets for the backup preliminary sample from Uganda). The number below each bar codes the pMSP component: MSP1: Z9-tetradecen-1-ol; MSP2: hexadecanal; MSP3: 6,10,14-trimethylpentadecan-2-ol (the three active MSP identified from the lab stock of *B. anynana*, Nieberding *et al.* 2008); pMSP4: unidentified anynana #19; pMSP5: octadecan-1-ol (physiologically active in GC-EAD tests of individuals from False Bay, Nieberding *et al.*, unpublished data); (see Appendix 6.5.4 for details of their identification). The abundance of each pMSP component selected once in a species is represented for all the populations but the bars are light coloured in the populations where the compound was not selected as a pMSP component.

6.2). Similar results were obtained from phylogenetic reconstructions including the three Ugandan haplotypes that assembled with South-African and Kenyan haplotypes like in the Median Joining network (Appendix 6.5.2). These three haplotypes very likely belong to hybrids which had also the typical Ugandan haplotypes (see Appendix 6.5.6).

6.3.2 Patterns of chemical diversity

The total number of chemical compounds per male ranged from 8 to 16 (12.0 $\pm$ 3.3; average $\pm$ SD) and from 1 to 6 in females (2.6 $\pm$ 1.2; Appendix 6.5.7). Although we sampled a limited number of individuals for the chemical analyses, individuals from the same population showed repeatable chemical profiles (Figure 6.3). These profiles were significantly more similar within populations than between (P = 0.001 in males and P = 0.04 in females, test based on 10000 permutations, Figure 6.3).

Sex was the main factor explaining the differentiation of full chemical patterns among individuals (PerMANOVA analysis; n = 73 individuals; significant R^2 of 47%, 16% and 67% of the variance in presence, absolute and relative abundance of the compounds explained by sex, respectively; Table 6.2). When performing the PerMANOVA analysis on the two sexes separately, the variation in the presence and abundance of compounds was mostly explained by geographic location (PerMANOVA; n = 38 males and n = 35 females; significant R^2 of 50%, 59%, and 73% for males; and of 38%, 38%, and 75% for females; Table 6.S2).

We next aimed at spotting the most likely chemical compounds forming the MSP in these populations. After selection of the pMSP among the compounds forming the chemical profiles of males (see Methods), we found that the qualitative and quantitative variations in composition of the pMSP in males were mostly explained by geography as well (PerMANOVA; n = 38; significant R^2 of 51%, 59%, and 75% for the difference in presence, absolute and relative abundance of the compounds, respectively; Table 6.2). Specifically, the three MSP initially identified in the lab population of *B. anynana* originating from Malawi were present in all populations of *B. anynana* but Uganda where MSP1 and MSP3 were absent from male bouquet (Figure 6.1). Five additional males sampled at the same Ugandan location in 2007 lacked MSP1 and MSP3 as well which confirms that it is likely not a random effect due to the small sample size. Additional compounds (pMSP4 and pMSP5) were also selected in the South-African populations (Limpopo and False bay lab stock, respectively, Figure 6.1). The Kenyan population showed some peculiarities regarding the chemical profiles of males. First, some pairs of Kenyan males showed a much poorer correlation (between 0.2 and 0.4, mean = 0.61 $\pm$ 0.16 S.D.) than that found on average between pairs of male chemical profiles (Figure 6.3). We

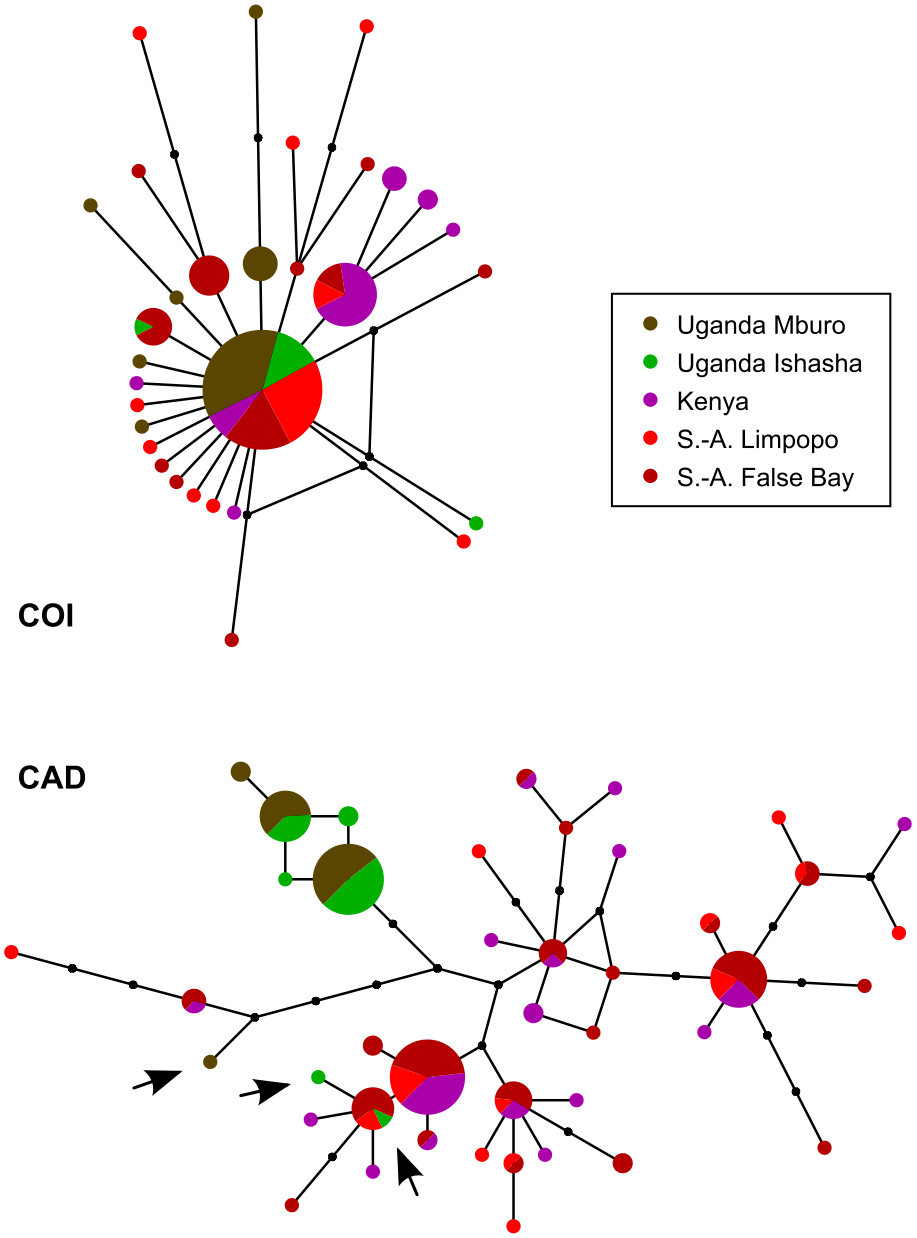


Figure 6.2: Haplotype networks of COI and CAD in *B. anynana*.
The surface of a circle is proportional to the number of individuals bearing one haplotype. Black circles represent intermediate haplotypes missing from the sampling. For sake of representation of the COI network, the distance between haplotypes is twice as large as in the CAD network. We only represented the simpler networks built with a null epsilon value as other reconstruction parameters showed consistent similar results. The three arrows pinpoint the probable introgressed haplotypes forming CAD hybrids in the population from Uganda.

also spotted that some Kenyan individuals had the same pMSP profile as the Ugandan population and thus missed MSP1 and MSP3, while other males did not, suggesting that in Kenya, a mixture of different chemical profiles could be present. Finally, quantitative, but no qualitative, differences were detected between either the pMSP or the chemical profiles of the lab adapted Malawian and the South-African lab and field-caught populations, despite over 20 years of lab acclimation for the Malawian stock population (Figure 6.1).

In summary, qualitative differences in the presence or the absence of population-specific pMSP components were observed between populations in *B. anynana*, but also within the Kenyan population.

Table 6.2: Proportion of the chemical variation explained by sex and geographic origin in *B. anynana*.

Sex	Compounds	Factors	Presence -absence R^2	Absolute abundance R^2	Relative abundance R^2
both	full profile	location	15% ***	30% ***	14% ***
		sex	47% ***	16% ***	67% ***
		interaction	8% ***	20% **	11% ***
males	full profile	location	50% ***	59% ***	73% ***
	pMSP	location	51% ***	59% ***	75% ***
females	full profile	location	38% ***	38% *	75% ***

Significance of the estimates: '*' if $P \leq 0.05$; '**' if $P \leq 0.01$; '***' if $P \leq 0.001$).

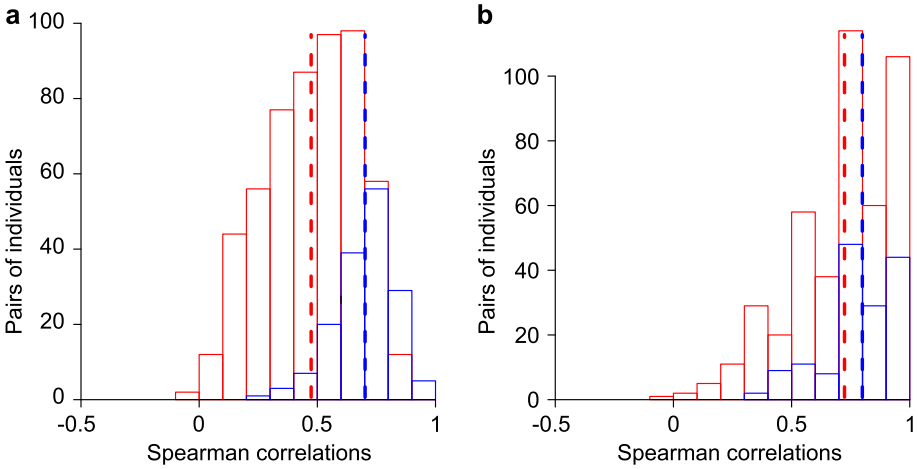


Figure 6.3: Distribution of Spearman correlations between pairs of *B. anynana* chemical profiles.
For each sex (**a**) males, (**b**) females, pairs of individuals of the same (in blue) or different (in red) populations were sampled. Dotted lines represent the means of each distribution.

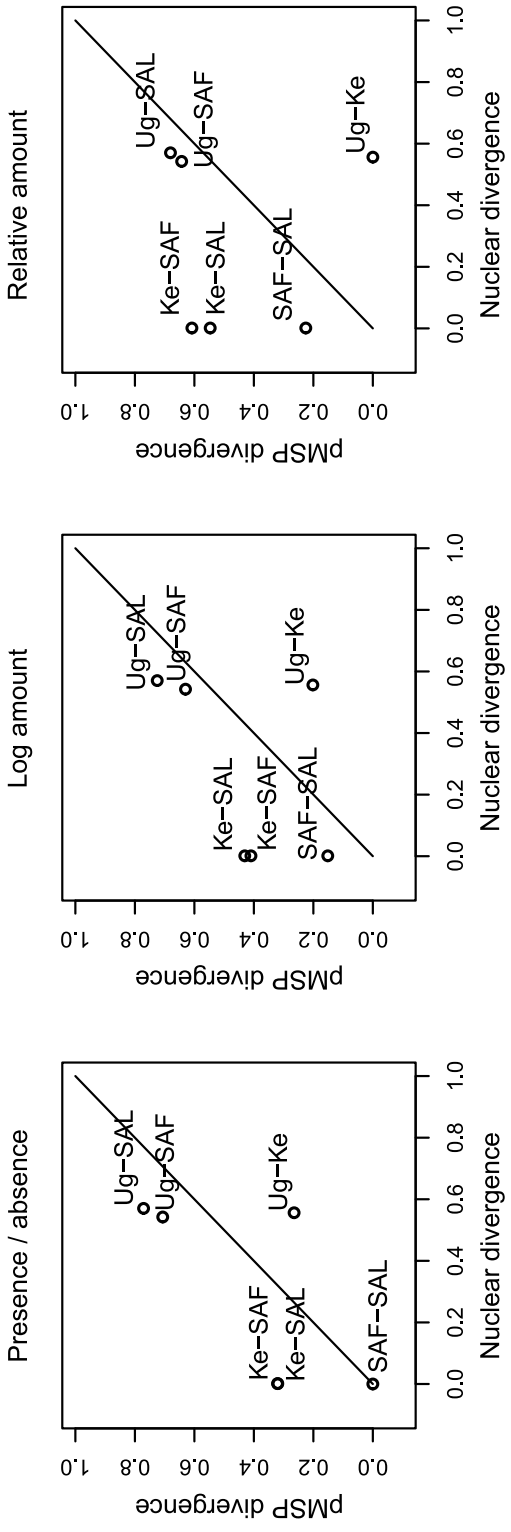


Figure 6.4: Comparison of the chemical and genetic differentiation between pairs of populations in *B. anynana*. Graphs are built using Φst values for estimating the differentiation of both the CAD gene and of the pMSP composition to allow direct comparison. Codes of populations are as follows: Ug: Uganda, SAF and SAL: South-Africa False Bay and Limpopo, and Ke: Kenya. A line was placed where genetic and chemical differentiations are identical.

6.3.3 Comparison of the patterns of genetic and chemical differentiations

The level of nuclear genetic divergence between pairs of *B. anynana* populations was lower than the corresponding level of pMSP differentiation, including presence-absence of pMSP components, absolute or relative variation in abundance (Figure 6.4). Although we present no statistical method to test it, this suggests that chemical divergence was concomitant, or preceded, genetic differentiation in *B. anynana*. One notable exception to this pattern was the comparison between the populations from Uganda and Kenya, wherein chemical differentiation was lower than neutral genetic divergence. This is because the Kenyan population displayed contrasted chemical profiles with some individuals bearing the Ugandan profile and others the Malawi-South-African one (see Figure 6.1, and results above). This increased the average differentiation within the population and therefore relatively decreased the chemical differentiation between males from Kenya and Uganda.

6.4 Discussion

We observed a strong differentiation in both the full chemical profiles and in the pMSP composition among *B. anynana* populations. The chemical differentiation involved qualitative differences in the composition, as well as quantitative differences in absolute and relative abundances. A large part of the variance in chemical profiles was due to the geographic isolation among *B. anynana* populations and particularly between the two subspecies of *B. anynana*, *B. a. centralis* in Uganda (Condamin 1968) and *B. a. anynana* in Malawi and South-Africa (Figure 6.1; Table 6.2). Importantly, 21% of the pairs of *Bicyclus* species previously investigated displayed one to four pMSP differences (Bacquet *et al.* in press, see Chapter 5). Thus, the qualitative differences observed between populations in this study were of the same magnitude as those observed among species and that participate to reproductive isolation across the genus (Bacquet *et al.* in press). Similar level of chemical differentiation was also found within a genetically undifferentiated population of *B. anynana* in Kenya (Figure 6.1). Overall, neutral genetic divergence between pairs of *B. anynana* populations was lower than variation in pMSP, lending support to our primary hypothesis that chemical divergence was concomitant, or preceded, genetic differentiation. This hypothesis is further supported by evidences of ongoing gene flow between the most divergent populations of the species: the sharing of a similar mitochondrial haplotype, and the three likely hybrid individuals sampled in Uganda. This raises the intriguing question that variation in pMSP is responsible for the evolution of premating isolation among *B. anynana* populations, and hence, a driver for speciation.

A follow up question would also be how frequent is this rapid divergence of chemical profiles in the *Bicyclus* genus? Preliminary data on genetic and chemical distances were gathered for three additional *Bicyclus* species (Appendices 6.5.1). In one of the three, *B. vulgaris*, we could detect similar qualitative differences of pMSP as in *B. anynana* (Appendices 6.5.7). Although the sample sizes are still low, this suggest that *B. anynana* does not display a marginal pattern of divergence.

Additional information would be necessary to determine whether the observed chemical differences are genetically fixed or plastic. Indeed, we performed chemical analyses on wild individuals sampled at different locations and time periods of the year. While MSP1 and MSP2 in *B. anynana* are fatty-acid derivatives and synthesized *de novo* (Liénard *et al.* 2014, Wang *et al.* 2014), terpenoid compounds such as MSP3 are likely derived from host plant compounds (Schulz *et al.* 2011) and potentially affected by host plant use. In addition, climatic or ecological conditions, like season, could be responsible of part of the observed variation (Landolt & Phillips 1997, Buckley *et al.* 2003, Groot *et al.* 2009, Geiselhardt *et al.* 2012, Larsdotter-Mellström *et al.* 2012). The *Bicyclus* genus is known for its seasonal polyphenism with dry and wet season forms varying in life history traits and displaying differences in wing patterns (mostly studied in *B. anynana*; Brakefield & Reitsma 1991, Windig *et al.* 1994, Brakefield *et al.* 2009, Prudic *et al.* 2011). However, several observations argue that part of the observed variation of MSP in *B. anynana* may be genetically based. First, preliminary results in *B. anynana* lab population originating from Malawi showed that the MSP composition was not affected by the temperature during development (the triggering factor of the two seasonal forms), and neither by host plant (maize or *Oplismenus sp.*, the natural host plant of *B. anynana*; Nieberding *et al.* 2008, and unpublished data). Second, we did not find MSP1 and MSP3 in the population of *B. anynana* from Uganda in five additional males sampled in 2007, which suggests that plasticity was likely not responsible for their particular MSP composition.

Our results should encourage future studies to rear in captivity wild-caught *Bicyclus* individuals from each population for a few generations to: i) confirm the observed qualitative and quantitative variations of pMSP components as well as their genetic or plastic basis, by standardization of developmental conditions; ii) investigate the physiological and behavioural activities of pMSP components as well as their fitness effect by GC-EAD and mate choice experiments, to test if the chemical differences have an effect on mate preferences between populations.

6.5 Supporting Information of the manuscript

6.5.1 Detailed list of sampled individuals

For comparison sake and to investigate the generality of the pattern observed in *B. anynana*, we also analysed preliminary sampled distinct populations of three other *Bicyclus* species *B. safitza*, *B. smithi*, and *B. vulgaris* (n = 41, 48, and 45, respectively, Table 6.S2). For these three, only three males and two females per population was used for the chemical analyses (Table 6.S2). These four species can be considered independent at the scale of the genus since they are relatively distant in the phylogenetic tree (10% K2P distance on average for the COI gene).

Two *B. anynana*, two *B. smithi*, two *B. vulgaris*, and one *B. safitza* were excluded from the analyses because of variable results across different runs of the ELB algorithm. These individuals originated either from the additional populations not sampled for chemical profiles (Cameroon and Ishasha, Uganda) or, in the case of *B. anynana*, from the False Bay population in South-Africa, one of the most numerously sampled of our study (n = 37). We are thus convinced that it does not impact our results.

Table 6.S1: Detailed information for each individual (separate excel file).
This table lists the precise geographic origin (Country, location, GPS coordinates), the date of collection, the collector, the sequences obtained (gene and reference of the primers) with related GenBank accession numbers, the haplotype code corresponding to the trees (Figure 6.S2), and correspondence with chemical sampling ('old GC-MS' refers to Ugandan additional *B. anynana* males mentioned in the main text). The seven individuals that showed variable results across different runs of the ELB phasing algorithm on CAD sequences are mentioned.

6.5.2 Phylogenetic reconstructions within the four *Bicyclus* species

Methods: Maximum Likelihood (ML) trees were reconstructed using the program Garli (Zwickl 2006, Bazinet & Cummings 2011). The model of sequence evolution GTR+G+I was selected to encompass the features of the best models of all four species and the two genes to allow comparison of phylogenetic reconstructions on a common basis. The model ranking was based on corrected Akaike Information Criterion (AICc) computed with JModeltest (Posada 2008). Final consensus trees for each species and each gene were obtained from 2000 bootstrap replicates starting from the best tree of 10 independent ML searches. We rooted the phylogenetic trees with closely related species: *B. campa* and *B. mollitia* for *B. anynana*; *B. funebris* for *B. safitza*; *B. buea*, *B. golo*, and *B. sophrosyne* for *B. smithi*; and *B. jefferyi* and *B. moyses* for *B. vulgaris* (Condamin 1973, Monteiro & Pierce 2001).

Table 6.S2: Geographical distribution and size of the samples for genetic and chemical analyses of *B. safitza*, *B. smithi*, and *B. vulgaris*.

Species	Country	Location	Number of sequences		Number of chemical analyses		Date of chemical sampling
			COI	CAD	♂	♀	
B. safitza	Nigeria	Yankari	7	7	1	2	10/2008
					2		11/2009
	Nigeria	Afi Mountains	3				
	Uganda	Mburo	23	16	3	2	9/2009
	Uganda	Ishasha	8	8			
B. smithi	Cameroon	West	25	16	3	3	4/2009
	Uganda	Kibale	20	15	3	2	10/2009
	Uganda	Ishasha	3	3			
B. vulgaris	Nigeria	Yankari	4	4	2	2	10/2008
					1		11/2009
	Nigeria	Afi Mountains	9	1			
	Cameroon	West	3				
	Cameroon	Center	5	5			
	Uganda	Mburo	14	11	3	2	9/2009
	Uganda	Ishasha	5	5			

Details for each individual are given in Table 6.S1.

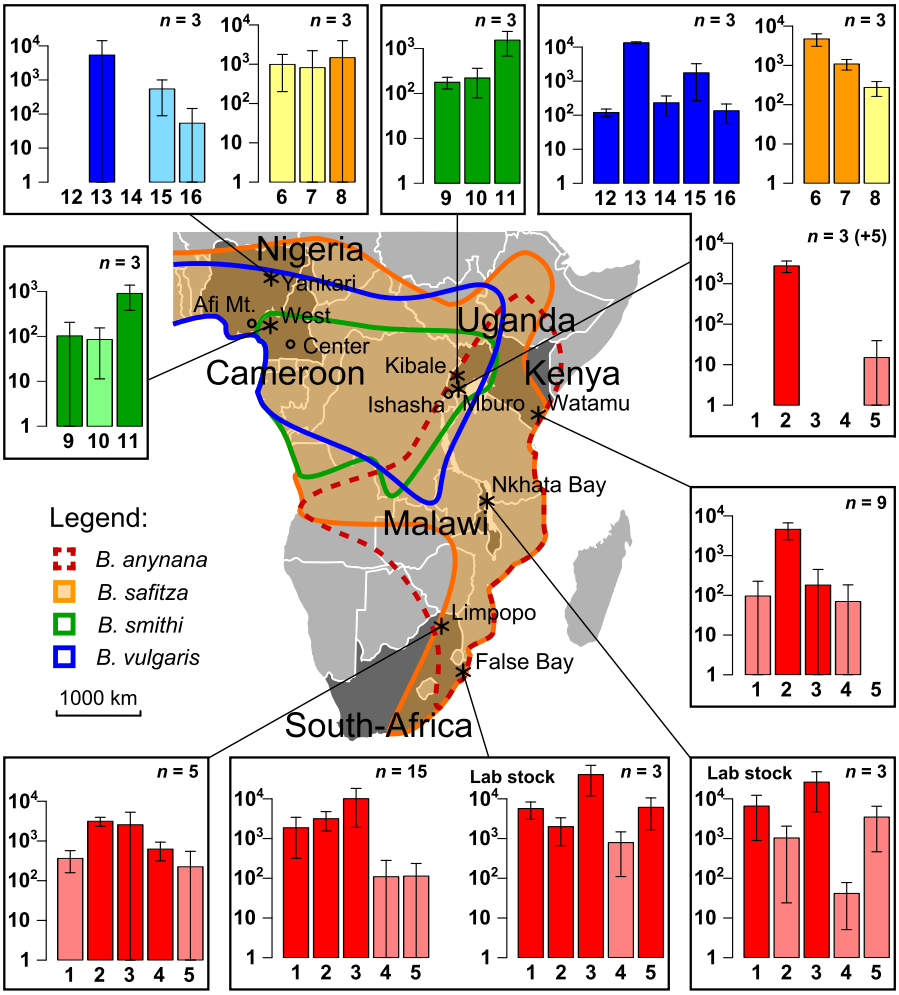


Figure 6.S1: Geographic localization and pMSP composition of the populations of *B. anynana*, *B. safitza*, *B. smithi* and *B. vulgaris*.
Sampling sites are represented by asterisks when both DNA and chemical samples were collected, and circles where we only collected DNA samples (Cameroon West: Fossong Ellelem and Bamena; center: Yaoundé, Ebogo and Dja, see Table 6.S1). Hypothetical distribution ranges are derived from Condamin (1973). The bar plots represent the log-transformed average amount (ng) of each pMSP component in each population (± S.D.; the error bar comprises zero if some individuals lack the compound). The sample size is given for each graph. The number below each bar codes the pMSP component: MSP1 to pMSP16. The abundance of each pMSP component selected once in a species is represented for all the populations but the bars are light coloured in the populations where the compound was not selected as a pMSP component.

Table 6.S3: Likelihood scores (log*L*) of ML trees with and without positive or negative constraints.

Species	COI gene Constraint	Constrained groups	log*L*	*P*	CAD gene Constraint	Constrained groups	log*L*	*P*
B. anynana	none	none	-1873.6		none	none	-1565.2	
	positive	(Ug) (Ke+SA)	-1881.1	< 0.01	negative	(Ug*) (Ke+SA)	-1574.7	0.03
B. safitza	none	none	-1748.5		none	none	-1378.3	
	positive	(Ni) (Ug)	-1751.7	0.24	positive	(Ni) (Ug)	-1382.9	0.30
B. smithi	none	none	-2139.5		none	none	-1489.6	
	negative	(Ca) (Ug)	-2160.9	< 0.01	positive	(Ca) (Ug)	-1536.4	< 0.01
B. vulgaris	none	none	-1644.1		none	none	-1402.2	
	negative	(Ni) (Ug)	-1669.3	< 0.01	positive	(Ni) (Ug)	-1407.7	0.20

Constrained groups of populations are delimited by parentheses.

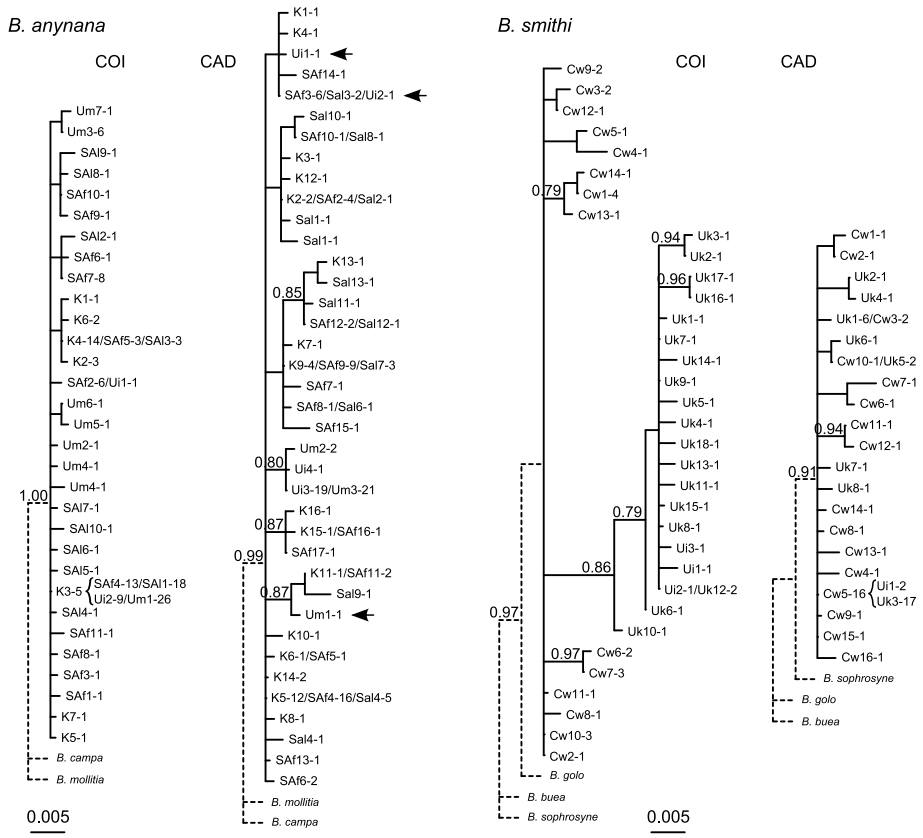
The *P* values result from the S.H. test to compare the trees with and without constraint.

*For the CAD in *B. anynana*, three haplotypes are omitted in the constraint of the Ugandan individuals. These three CAD haplotypes are present in Ugandan individuals together with typical Ugandan haplotypes but more similar to Kenyan and South-African ones (pointed by black arrows in Figures 6.2 and 6.S2). These three haplotypes are likely introgressed from the southern populations and the three individuals are likely hybrids (see results in main mext and Appendix 6.5.6).

We performed the test of Shimodaira and Hasegawa (1999; S.H. test hereafter) to compare likelihood scores of trees reconstructed with different constraints in order to test more specifically the support for population (defined according to the sampling location) separation in different clades of the trees. We used positive and negative constraints to test the support of population separation in different clades. Positive constraints forced all individuals of the same population to be kept in a single clade in the trees during the reconstruction. Conversely, negative constraints forced the individuals of each population to be distributed across different clades. We used positive constraints to test the support of observed separation of some populations in distinct clades, and negative constraints to test the support of observed admixture of populations. To compare the different hypotheses, each corresponding best tree were imported into R program (Paradis *et al.* 2004, R Development Core Team 2011), the branch lengths were optimized with the functions pml and optim.pml for the tree likelihood to be subsequently compared with S.H. tests (package Phangorn, Schliep 2011). The likelihoods of the trees for each hypothesis and the *P* values of the tests are reported in the Table 6.S3.

Results: B. anynana displayed significant genetic variation between populations, yet smaller when using the mitochondrial COI marker than the CAD nuclear marker. The ML phylogenetic reconstructions showed that the genetic divergence for COI was not associated with the geographic origin of the populations (Figure 6.S2) and constraining the COI tree to display geographic clades significantly decreased its likelihood (S.H. test: $P < 0.01$; Table 6.S3). In contrast, the ML phylogenetic tree using the CAD gene separated most of the Ugandan haplotypes from other localities (BT of 80% for the Ugandan clade). Preventing Ugandan haplotypes to form a clade in the CAD tree significantly decreased its likelihood (S.H. test: $P = 0.03$; Table 6.S3).

The ML phylogenetic reconstructions using COI supported the genetic differentiation of geographic clades in *B. smithi* (bootstrap support, BT hereafter, of 86% for separating Uganda from Cameroon) and *B. vulgaris* (BT of 71% for grouping Nigerian haplotypes, Figure 6.S2). The geographically intermediate *B. vulgaris* from Cameroon grouped with either Nigerian or Ugandan haplotypes showing the coexistence of the two mitochondrial lineages in Cameroon. For the two species, the clade separation in the COI gene was supported by S.H. tests (Table 6.S3). In contrast, the CAD gene showed no genetic differentiation between populations of the two species. *B. safitza* did not display geographic clade either for COI or for CAD, despite the distance separating Nigeria and Uganda (approximately 2600 km, Figures 6.S1 and 6.S2). Constraining the tree for the presence of the clades did not improve the likelihood and S.H. test revealed no significant differences (Table 6.S3).



6.5.3 Incongruence between COI and the nuclear markers in *B. anynana*

To assess whether the COI or the CAD marker was a better signal of the evolutionary history of *B. anynana*, we obtained additional sequences for the nuclear marker EF1α for seven Ugandan individuals, two South-African individuals from False-Bay and three from Limpopo (Tables 6.1 and 6.S1). We measured the average number of pairwise differences between all individuals for each of the three genes. The average divergence between populations is larger than the average divergence within populations for the two nuclear markers, but not for the COI (Table 6.S4). This confirmed the deep nuclear divergence found for Ugandan individuals compared to more southern populations, which is also in accordance with the description of a Ugandan subspecies, using morphological traits, *B. anynana centralis* (Condamin 1968). Since COI is supposed to evolve faster than the nuclear markers (EF1α is used to resolve deep nodes in phylogenetic studies in butterflies), this results means that COI is unreliable to

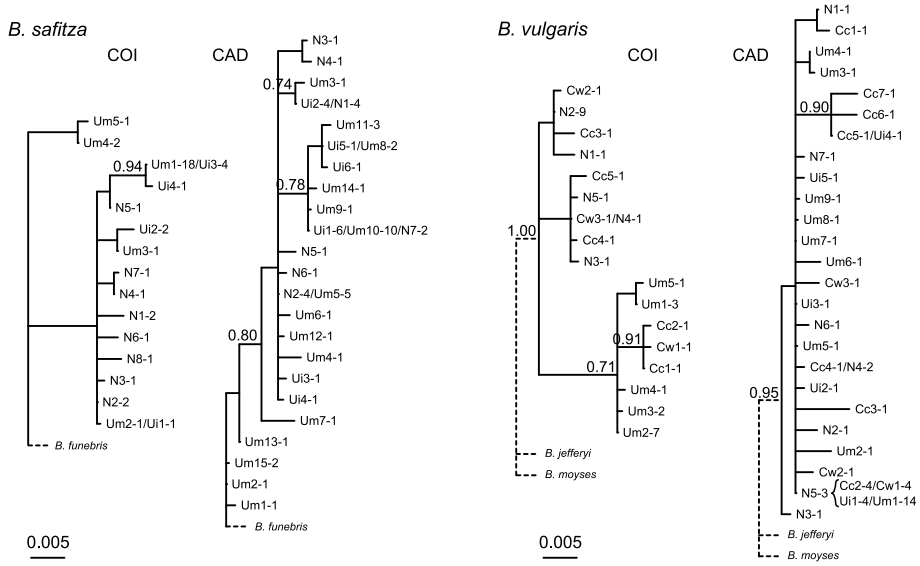


Figure 6.S2: Phylogenetic reconstructions of the species trees using ML on COI and CAD gene sequences.
Internal nodes with bootstrap values above 0.50 are represented, and values above 0.70 are specified. Each tip represents a haplotype named according to the geographic origin of sampling (N for Nigeria; Cw and Cc for central and West Cameroon; Uk, Um and Ui for Kibale, Mburo and Ishasha in Uganda; K for Kenya; SAf and SAl for False-Bay and Limpopo in South-Africa; see Figure 6.S1 and Table 6.S1). Numbers after the dashes indicate the numbers of repeated haplotypes in each population. To save space, the dashed branches linking the species to their outgroups are not scaled. The three arrows pinpoint the CAD haplotypes likely introgressed in the Ugandan population of *B. anynana*.

describe the neutral genetic divergence of the populations in this species. In the three other species investigated, COI display, as expected, a larger divergence between populations (Appendices 6.5.2 and 6.5.5).

This relative mitochondrial uniformity in *B. anynana* suggests a recent spread of one haplotype across populations (mitochondrial introgression). Such a pattern of mitochondrial discordance is not uncommon and justifies the use of several independent markers (Funk & Omland 2003, Bazin *et al.* 2006). The erasing of mitochondrial diversity can be due to drift (stronger in the mitochondrial genome with lower effective population size due to haploidy and maternal inheritance), selection of an advantageous mutation (selective sweep facilitated by the absence of recombination in this genome), or selection by symbiotic interaction with bacteria like *Wolbachia* (reviewed in Ballard & Whitlock 2004). According to de de Jong *et al.* (2011), the neutrality of the polymorphism in COI sequences was not met for several *B. anynana*

populations, which would reject the first hypothesis of fixation by drift. We applied neutrality tests H of Fay & Wu (2000) and D of Tajima (1989) with the DnaSP software (version 5.10.01; Librado & Rozas 2009), and observed that both significant for the COI but not for the CAD gene (Tables 6.S5). This further supported the hypothesis of a recent sweep on the mitochondrial polymorphism.

Table 6.S4: Number of pairwise differences between individuals for the three genes.

Pairwise differences	COI	CAD	EF1α
within Uganda	0.86 ± 0.48	0.19 ± 0.13	0.00 ± 0.00
within S.-A.	1.40 ± 0.82	2.10 ± 0.83	0.30 ± 0.28
between Uganda and S.-A.	3.23 ± 1.48	6.49 ± 2.16	2.23 ± 0.90
ratio between / within	1.43	2.83	7.43

The analysis was performed with MEGA5 and the standard error computed from 1000 bootstrap (Tamura *et al.* 2011).

Table 6.S5: Neutrality tests.

Gene	Tajima (1989)		Fay & Wu (2000)	
	D	*P*	H	*P*
COI	-2.39	< 0.01	-16.81	< 0.01
CAD	-1.46	> 0.10	0.61	0.40

P values of the H test were computed from 1000 coalescent simulations based on the observed diversity value (Theta in DnaSP).

6.5.4 List of chemical compounds detected in the four *Bicyclus* species

Table 6.S6: List of compounds detected in the four species (separate excel file).
This table lists all compounds present above the threshold of 10% of the internal standard and their abundance in each individual of the four species. The pMSP components are highlighted in the first column. The subsequent columns describe the type of the compounds (chemical family), their assigned structure and the details of their identification (type of column for the GC, representative mass fragments). All GC-MS files are available upon request.

Figure 6.S3: Mass spectra of the pMSP components (separate pdf file).
The second mass spectra below each pMSP component (except pMSP9 and pMSP16) belong to reference compounds for comparison sake.

Table 6.S7: Summary of the genetic variation between pairs of populations for the COI and CAD genes.

Species	Pairs of populations		COI AMOVA		Φst	CAD AMOVA		Φst
B. anynana	Uganda	Kenya	among:	20% ***	0.43 ***	among:	37% ***	0.56 ***
	Uganda	S.-A. False Bay	within:	80%	0.09 ***	within:	4% *	0.54 ***
	Uganda	S.-A. Limpopo			0.04 ***	within ind.:	59% **	0.57 ***
	Kenya	S.-A. False Bay			0.30 ***			0.00
	Kenya	S.-A. Limpopo			0.28 ***			0.00
	S.-A. False Bay	S.-A. Limpopo			0.04 **			0.00
B. safitza	Nigeria	Uganda	among:	46% ***	0.46 ***	among:	21% *	0.22 ***
			within:	54%		within:	30% ***	
						within ind.:	49% ***	
B. smithi	Cameroon	Uganda	among:	66% ***	0.66 ***	among:	9% ***	0.10 **
			within:	34%		within:	5%	
						within ind.:	85%	
B. vulgaris	Nigeria	Uganda	among:	94% ***	0.94 ***	among:	5%	0.06 *
			within:	6%		within:	24% ***	
						within ind.:	72% ***	

AMOVA and pairwise Φst are based on uncorrected pairwise genetic differences. The displayed percentages correspond to variation among populations, within populations, and in addition for CAD, within individuals.

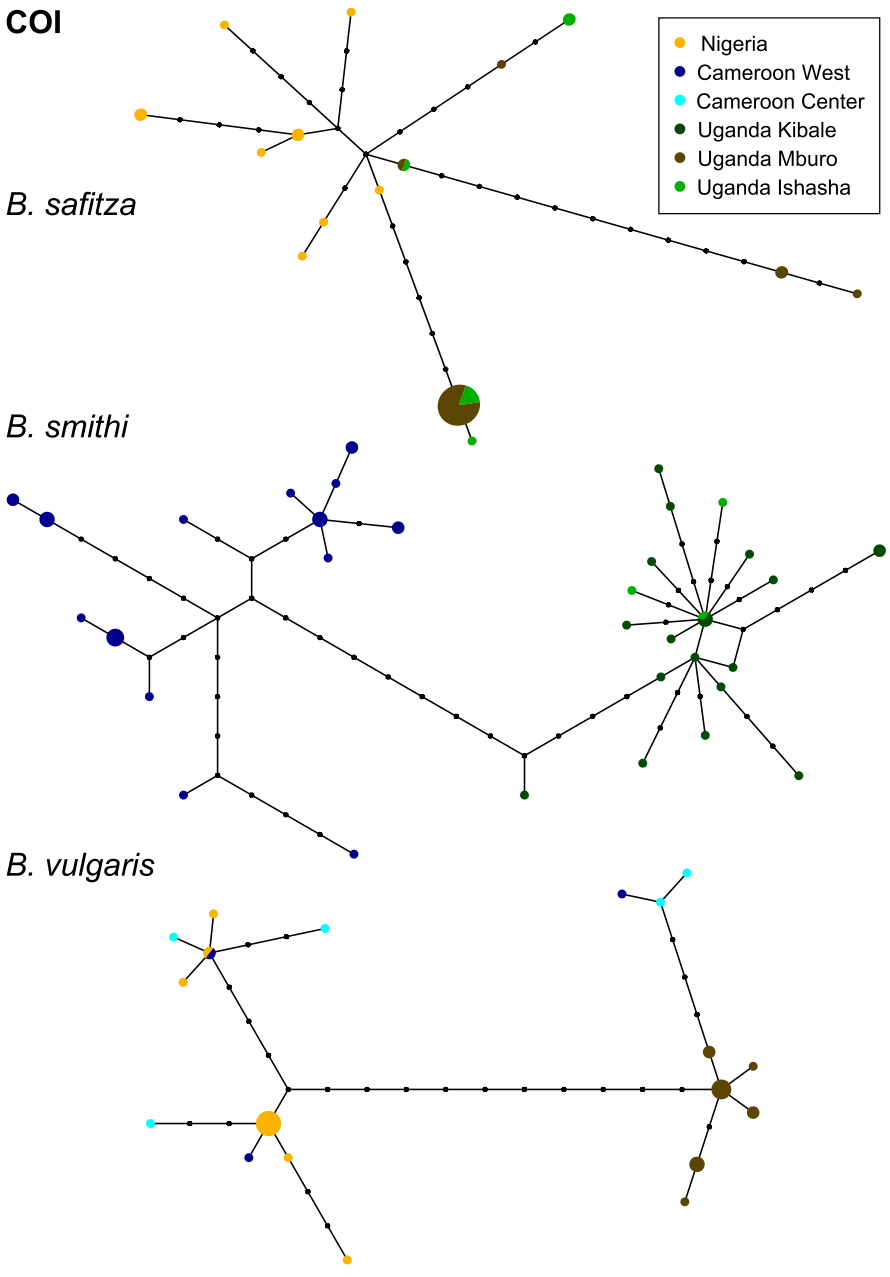
Significance of the estimates: '*' if $P \leq 0.05$; '**' if $P \leq 0.01$; '***' if $P \leq 0.001$.

6.5.5 Genetic differentiation within the four *Bicyclus* species

The Median Joining networks confirmed the genetic structure of the three species as found in the trees: we found a neat COI geographic differentiation in *B. smithi* and *B. vulgaris* while there was no pattern of genetic structure for *B. safitza* despite the large geographic area sampled (Figure 6.S2). *B. safitza* is abundant and well adapted to anthropogenic ecosystems (observations in Makerere University campus in Kampala, Uganda, and see also Munyuli 2012), such that it may experience little geographic or ecological barrier to gene flow despite its large geographic distribution.

The AMOVA and pairwise Φst between populations supported in general the COI genetic differentiation observed in trees and networks between populations in *B. smithi* and *B. vulgaris*. In *B. smithi*, AMOVA and individual Φst additionally revealed a significant divergence for the CAD (Table 6.S7). Despite no separation into clades, in *B. safitza* the AMOVA and pairwise Φst detected a significant effect of geography in the distribution of genetic diversity (46% and 21% of the variance explained for COI and CAD, respectively, Table 6.S7).

Of note, the divergence between the mitochondrial clades found within *B. smithi* and *B. vulgaris* (average uncorrected pairwise difference of 1.96% $\pm$ 0.34 S.D. and 1.53% $\pm$ 0.17, respectively) were under the level known for other Lepidopteran subspecies (3.4% in *Heliconius erato*, Brower 1994, or 2% in *Spodoptera frugiperda* host plant strains, Kergoat *et al.* 2012) , suggesting that we are dealing with intraspecific genetic variability.


COI
B. safitza
B. smithi
B. vulgaris
Nigeria
Cameroon West
Cameroon Center
Uganda Kibale
Uganda Mburo
Uganda Ishasha

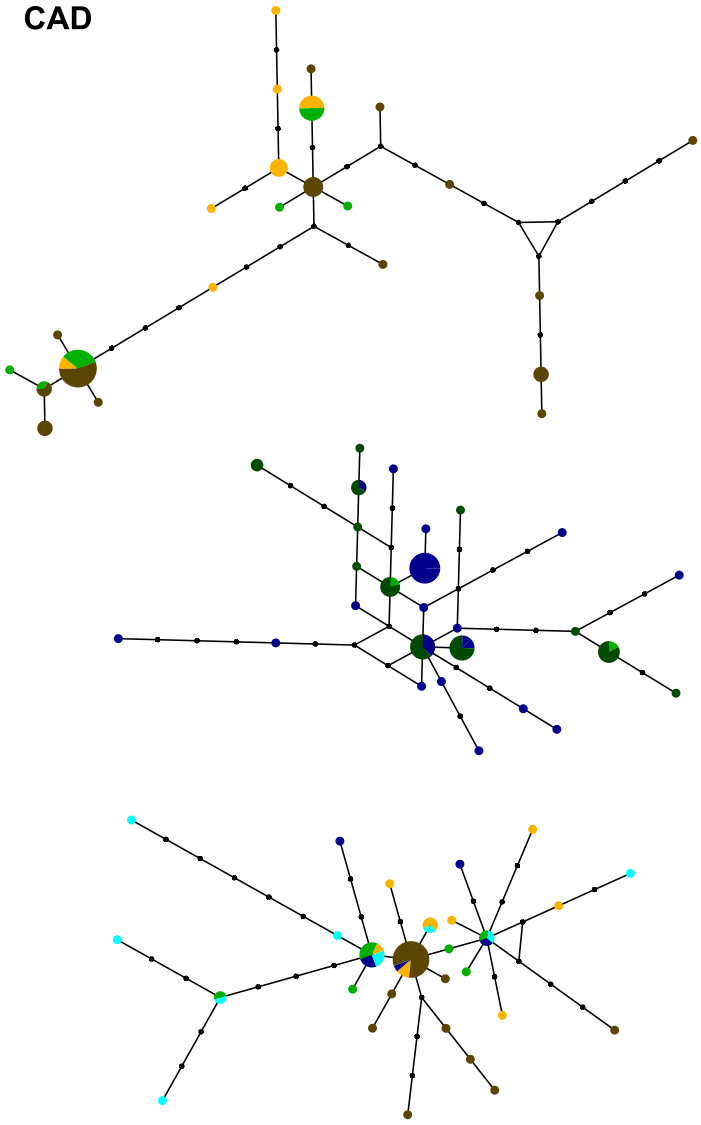


Figure 6.S4: Haplotype networks of COI and CAD in *B. safitza*, *B. smithi* and *B. vulgaris*.
The surface of a circle is proportional to the number of individuals bearing this haplotype. Black circles represent intermediate haplotypes missing from the sampling. We only represented the simpler networks built with a null epsilon value (see Methods) as other reconstruction parameters showed consistent similar results.

Table 6.S8: Number of individuals displaying the different genotypes in Uganda and in the other populations (S.-A./Kenya) for five nucleotide positions of the CAD gene.

Clade		Position 1		Position 2		Position 3		Position 4		Position 5	
Uganda		CC	20	TT	20	CC	20	AA	20	AA	20
n = 23	ind. Mu005	CC	1	TT	1	AC	1	CA	1	TA	1
	ind. Iu043	AC	1	AT	1	AC	1	CA	1	AA	1
	ind. Iu049	AC	1	AT	1	AC	1	CA	1	TA	1
Local proportion of the allele		C	0,96	T	0.96	C	0.93	A	0.93	A	0.96
S.-A./ Kenya		CC	0	TT	9	CC	0	AA	18	AA	0
n = 52		CA	4	TA	28	CA	0	AC	20	AT	0
		AA	48	AA	15	AA	52	CC	13	TT	52
								TC	1		
Local proportion of the allele		A	0,96	A	0.56	A	1.00	C	0.45	T	1.00

The three divergent haplotypes of the Ugandan individuals 'Mu005', 'Iu043', and 'Iu049' are highlighted in Figures 6.2 and 6.S2).

6.5.6 Origin of Ugandan heterozygotes in the CAD gene of *B. anynana*

The three CAD haplotypes sampled in Uganda but present out of the Ugandan clade belonged in fact to three heterozygous individuals, also displaying the typical Ugandan haplotypes. These three Ugandan individuals had heterozygous positions for two and three of the three positions that bore in all other sampled *B. anynana* individuals fixed differences between the Ugandan and the Kenyan and South-African populations (Table 6.S8). The three heterozygous individuals also presented a heterozygous state for two other positions fixed in Ugandan individuals but still variable in more Southern populations (Table 6.S8). Given the frequency of these mutations, the probability of being heterozygous (by mutation) for at least three of these five positions was very low in the Ugandan population ($P = 0.008$, 0.0004, and 8.56×10^{-6} for three, four and five heterozygous positions as observed for these three individuals, Table 6.S9). It was even more surprising to observe no individual heterozygous for only one or two positions ($\chi^2 = 5128.61$, $P = 0.0001$ obtained from 10000 Monte Carlo simulations; Table 6.S10). Thus the probability that these three individuals bear alleles both typical from Uganda and from other populations, that differ at several positions, due to mutation only is very low. It is far more likely that they inherited these heterozygous genotypes from recent hybrid matings between Ugandan and more southern individuals. Based on the strong divergence of the clades at the nuclear level, the differentiation of the populations must be ancient compared to pairs of populations investigated in the other species (Appendix 6.5.5), yet reproductive isolation is not yet complete given the presence of the three hybrids.

Table 6.S9: Probability of observing different combinations of genotypes at the five positions of CAD fixed in Uganda.

Number of heterozygous positions	Probability of observation in Uganda	Expected	Observed
0/5	0.58	14	20
1/5	0.32	8	0
2/5	0.07	2	0
3/5	0.0076	0	1
4/5	0.0004	0	1
5/5	0.000009	0	1

For example the probability of being fully homozygous for the more frequent state at these five positions is the product of the squared probability of the states at each position,
that is: $P(C_{pos.1})^2 \times P(T_{pos.2})^2 \times P(C_{pos.3})^2 \times P(A_{pos.4})^2 \times P(A_{pos.5})^2$
or: $0.96^2 \times 0.96^2 \times 0.93^2 \times 0.93^2 \times 0.96^2 = 0.58$

Table 6.S10: χ^2 tests designs and results.

Contingency table subset*	χ^2	*P* value estimation method**	Degrees of freedom	*P* value
full table	5128.61	Monte Carlo	NA	9.9×10^{-5}
0/5–1/5	11.04	conventional	1	0.0009
1/5–5/5	15973.58	Monte Carlo	NA	9.9×10^{-5}

* number of heterozygous positions considered, see Table 6.S9.
** when the *P* value of the χ^2 test could not be obtained by conventional method, we used 10000 Monte Carlo simulations.

Table 6.S11: Summary of the number of compounds* per population and sex.

Number of compounds		*B. anynana*											
		males						females					
		Um	Ke	Ma	SAl	SAf	SAflab	Um	Ke	Ma	SAl	SAf	SAflab
Per individual	maximum	11	12	14	18	15	19	4	5	6	4	4	2
	mean	9.0	9.6	12.3	16.0	12.4	14.7	3.5	2.0	5.5	2.5	2.5	2.0
	minimum	6	7	9	13	9	9	3	1	5	2	1	2
Mean ± S.D. of the sex		12.1 ± 3.2						2.6 ± 1.2					
Per location		14	22	15	29	25	20	4	5	6	4	6	2
Mean ± S.D. of the sex		20.8 ± 5.8						4.5 ± 1.5					

Number of compounds		*B. safitza*				*B. smithi*				*B. vulgaris*			
		males		females		males		females		males		females	
		N	Um	N	Um	Cw	Uk	Cw	Uk	N	Um	N	Um
Per individual	maximum	12	19	4	8	21	21	11	16	10	23	2	9
	mean	9.3	15.0	3.5	6.0	17.3	20.3	7.7	13.0	7.3	21.0	1.5	7.5
	minimum	7	12	3	4	12	20	1	10	5	19	1	6
Mean ± S.D. of the sex		12.2 ± 4.2		4.8 ± 2.2		18.8 ± 3.4		9.8 ± 5.4		14.2 ± 7.8		4.5 ± 3.7	
Per location		19	21	5	9	26	31	13	17	15	30	2	9
Mean ± S.D. of the sex		20.0 ± 1.4		7.0 ± 2.8		28.5 ± 3.5		15.0 ± 2.8		22.5 ± 10.6		5.5 ± 4.9	

*Compounds were counted after the removal of small peaks < 10% of the IS.

Populations are coded as follows: Um and Uk for Mburo and Kibale in Uganda; K for Kenya; M for Malawi; SAf, SAflab and SAl for False-Bay (wild caught and lab reared) and Limpopo in South-Africa; N for Nigeria, Cw for western Cameroon.

6.5.7 Chemical differentiation within the four *Bicyclus* species

The full chemical profile of *B. safitza* and *B. vulgaris* displayed a similar number of compounds to *B. anynana* (i.e. 8–16 in males and 1–6 in females), while we found a larger number of compounds in *B. smithi* (18.8 $\pm$ 3.4 and 9.8 $\pm$ 5.4 for males and females, respectively; Table 6.S11).

In these three species, the repeatability of the male chemical profiles within populations ranged between 0.49 $\pm$ 0.24 and 0.70 $\pm$ 0.15 (mean $\pm$ S.D., Spearman correlations, n = 3 males per population) except for *B. safitza* and *B. vulgaris* from the Nigerian populations (0.19 $\pm$ 0.30 and 0.37 $\pm$ 0.23, respectively).

One to five pMSP components were selected in the different *B. safitza*, *B. smithi* and *B. vulgaris* populations (Figure 6.S1). Similarly to *B. anynana*, *B. vulgaris* displayed differences in pMSP composition between populations: pMSP12 and pMSP14 were absent from *B. vulgaris* in Nigeria (Figure 6.S1; Appendix 6.5.4). In *B. safitza* and *B. smithi*, the observed differences in pMSP composition between populations were not due to the absence of some compounds but to variations in their amounts that determined whether they were selected as pMSP components or not. Because of the small sample size, PerMANOVA analyses were not informative for *B. safitza*, *B. smithi*, and *B. vulgaris* (Table 6.S12). Yet, in *B. vulgaris*, 84% (PerMANOVA, n = 6, P = 0.10) of the qualitative variation in pMSP composition among individuals was explained by geography, and this was based on the absence of the two compounds in the Nigerian population. *B. safitza* seemed to have strong differences in pMSP absolute amounts with 48% explained by geography (PerMANOVA, n = 6, P = 0.10).

In conclusion of the preliminary analysis of these three species, the patterns of qualitative chemical differentiation observed between populations of *B. anynana* was not anecdotal since present in two out of four species. It has to be kept in mind that given the small sample size in *B. vulgaris*, we cannot exclude that part of the qualitative variation in pMSP composition between the two populations was due to chance.

Table 6.S12: Proportion of the variation of male and female chemical profiles explained* by the geographic origin in *B. safitza*, *B. smithi*, and *B. vulgaris*.

Species	Sex	Compounds	Presence -absence R^2	Absolute abundance R^2	Relative abundance R^2
B. safitza	males	full profile	42%	43%	8%
		pMSP	20%	48%	6%
	females	full profile	50%	85%	90%
B. smithi	males	full profile	51%	50%	60%
		pMSP	20%	23%	25%
	females	full profile	44%	35%	24%
B. vulgaris	males	full profile	46%	36%	31%
		pMSP	84%	36%	35%
	females	full profile	79%	81%	38%

*None of these results were significant.

Authors' contributions

PMBB and CMN designed the study. FM provided preliminary data. MDJ, PMBB, OB, and CMN collected the butterflies. SH and GL performed preliminary chemical analyses. CL and HLW developed the pheromone sampling protocol and HLW performed final chemical analyses. PMBB did the molecular work, the chemical data processing and the statistical analyses. PMB and CMN provided funding for the field work in Africa. AVP advised on the molecular genetics analyses and provided early funding for molecular lab work. PMBB, AVP, and CMN wrote the paper with the help of MDJ, CL, FM and OB.

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Chapter 7

Behavioural significance of the pMSP components

In Chapter 5 and 6, clear patterns about putative male sex pheromones (pMSP) divergence were unraveled. The underlying hypothesis that accompanied these results is that the selected pMSP components are used as chemical signals for mate choice, and more specifically to avoid interspecific mating. However, excepted for *Bicyclus anynana*, and for the particular population from Malawi, no solid evidence about which chemical is part of the male sex pheromone (MSP) exists for other species and populations. During the repeated attempt to publish the Chapter 5, and now more recently the Chapter 6 of this thesis, this weakness has naturally recurrently been raised by the reviewers, who requested additional evidence of the behavioural activity of these pMSP components in more populations of the *Bicyclus* genus. This was also initially the second part of this PhD program. We thus attempted to produce additional evidence of the functional activity of some pMSP components in two experiments. In the first experiment, we took advantage of a field trip by Sille Holm to receive field-caught individuals of the species *B. smithi*, which we submitted to gas-chromatography coupled to electro-antennographic detection (GC-EAD) at the University of Lund (Sweden, in collaboration with Prof. C. Löfstedt) to detect the existence of a physiological response of female antennae to some of male chemical wing compounds. The second experiment was done in the laboratory on *B. anynana* and aimed at testing the effect on *B. anynana* females of the presence of a pMSP component of *B. safitza* on *B. anynana* male wings.

7.1 Physiological response of females to male chemical compounds in *B. smithi*

In Lepidoptera, as most chemical ecology work, GC-EAD studies are usually performed by testing the physiological activity of female sex pheromones (FSP) on male antennae (Ando *et al.* 2004). In moths, males are well known to have very sensitive antennae that allow individuals to detect FSP emitted at low concentration by females emitting a few nanograms of active chemical signal at tens to hundreds of meters away (Phelan 1997). In contrast, while studies reported electro-physiological responses to plant compounds (Fadamiro *et al.* 2010, Sourakov *et al.* 2012) it remains very difficult to obtain GC-EAD results using female antennae in response to MSP in moths. There are almost no studies at all testing either FSP on male antennae, or MSP on female antennae in butterflies, which are thought to rely much more on visual than chemical signals. Exceptions concern GC-EAD positive response with MSP and female antennae in *B. anynana* (Nieberding *et al.* 2008), and in the castniid moth *Paysandisia archon* (Sarto i Monteys *et al.* 2012). For *B. anynana*, these responses were notoriously difficult to obtain and concern a small part of the tested individuals. However it constitutes a necessary first step in the identification of chemical compounds relevant in female mate choice. Moreover, GC-EAD has the advantage of testing all the compounds of a chemical profile without knowing their exact identification. Other techniques are simple EAD where compounds are individually puffed to the antennae (no parallel GC run), and single cell recording where individual sensilla are tested for their sensitivity to individual compounds. These two alternatives necessitate pure compounds and therefore their correct identification and synthesis before their physiological activity is tested.

In order both to learn the technique and to try to obtain the GC-EAD results of Nieberding *et al.* (2008), I performed the first series of tests on *B. anynana* individuals from the lab stock. The second series was performed on *B. smithi* wild individuals.

7.1.1 Materials and Methods

Butterflies

B. anynana lab stock in Lund University originated from the lab of A. Monteiro in Yale University, USA. They were reared on maize in a climate chamber at 27 °C and 70% humidity. Seven females were used for this species. *B. smithi* were collected from the field in Kibale forest (Uganda) in late May 2012 by Sille Holm (Institute of Ecology and Earth Sciences, Tartu University, Estonia). Only nine females and eight males of this species were captured. We kept the

females in cages in the same climate chamber and fed them with bananas for a minimum of four days before the experiment. Males were fed for two days before the extraction of the wing chemicals.

Chemical samples

MSP of *B. anynana* and major compounds (pMSP) of the male chemical profiles of the two species are known chemicals. Therefore we could prepare solutions of standard compounds at higher concentration than one can obtain by extracting live butterflies. For *B. anynana*, we used standards of the three MSP components (MSP1: Z9-tetradecenol, MSP2: hexadecanal, MSP3: trimethyl-pentadecan-2-ol (hereafter TMP2ol), trimethyl-pentadecan-2-one (MSP3 related, hereafter TMP2one), and vanillin (common compound in *Bicyclus*, see Chapter 5). We additionally tested three 'non MSP components' as controls since the large amount of compounds presented to the antennae seemed to elicit a physiological response regardless of the nature of the compound. We chose hexadecane (hereafter 16:Hy), Z8-tridecenyl acetate (hereafter IS since used as internal standard in the previous Chapters 5 and 6), because they were never observed in *Bicyclus* (at least in our 32 species investigated in Chapter 5), and β-farnesene (70% pure, hereafter βF) to have a terpene type compound (the two former would in nature be fatty acid derivatives). In *B. smithi* the selected pMSP components are the trimethylpentadecatrien-2-yl acetate (TMP2Oac), the octadecenyl acetate (18:Oac), and the Z9-tricosene (Z9-23:Hy). We used solution with tricosane (23:Hy), Z9-23:Hy, pentacosane (25:Hy), and 18:Oac. All the tested solutions are described in Table 7.1.

We additionally prepared male whole wing extracts to also test the physiological activity in females of other, minor, compounds. We cut the wings of one side of the individuals and put them in 1.5ml glass vials with redistilled GC grade hexane for one day. We used four half males extracted in 400 µl hexane per sample that we further filtered and concentrated (volume divided by two by evaporation under vacuum). Using different individuals for one sample allows testing female sensitivity to average chemical profiles. For convenience the samples are described in terms of individual per 100 µl (four half males in a final volume of 200 µl gives an average of 1 individual 'diluted' in 100 µl).

Table 7.1: List of GC-EAD runs.

Species tested	Chemical sample composition	Chemical sample concentration*	Antennae runs	Whole head runs	Number of females
B. anynana	standards: MSP1, MSP2, MSP3, TMP2one, vanillin	10 ng/µl	1	4	3
B. anynana	standards: MSP1, MSP2, MSP3, TMP2one, 16:Hy, IS, βF	125 ng/µl	2	11	5
B. anynana	standard: MSP1	20 ng/µl		1	1
B. anynana	standard: MSP2	100 ng/µl		1	1
B. anynana	standard: MSP3	200 ng/µl		1	1
B. anynana	standard: TMP2one	200 ng/µl		1	1
B. anynana	standards: MSP1, MSP2, MSP3, TMP2one, 16:Hy, IS, βF	60 ng/µl	2	4	3
B. anynana	individual extract	1 ind./100 µl	1		1
B. anynana	individual extract (reconcentration of above)	2 ind./100 µl	1		1

B. anynana	standards: MSP1, MSP2, MSP3, TMP2one	250 ng/µl	1		1
B. smithi	individual extract, concentrated twice	2 ind./100 µl	5	2	4
B. smithi	individual extract, concentrated twice	2 ind./100 µl	9	2	6
B. smithi	individual extract	1 ind./100 µl	1		1
B. smithi	individual extract (reconcentration of above)	2 ind./100 µl	7	1	5
B. smithi	23:Hy, Z9-23:Hy, 25:Hy, 18:Oac	100 ng/µl	5		3
B. smithi	23:Hy, Z9-23:Hy, 25:Hy, 18:Oac	25 ng/µl	8	1	5

*The concentration for individual extracts is given in number of individual-equivalent per 100 µl of solvent (depending on the compound, the concentration varies between 5 and 60 ng/µl in extracts with 2 individuals per 100 µl of *B. smithi*, see Figure 7.2c and d).

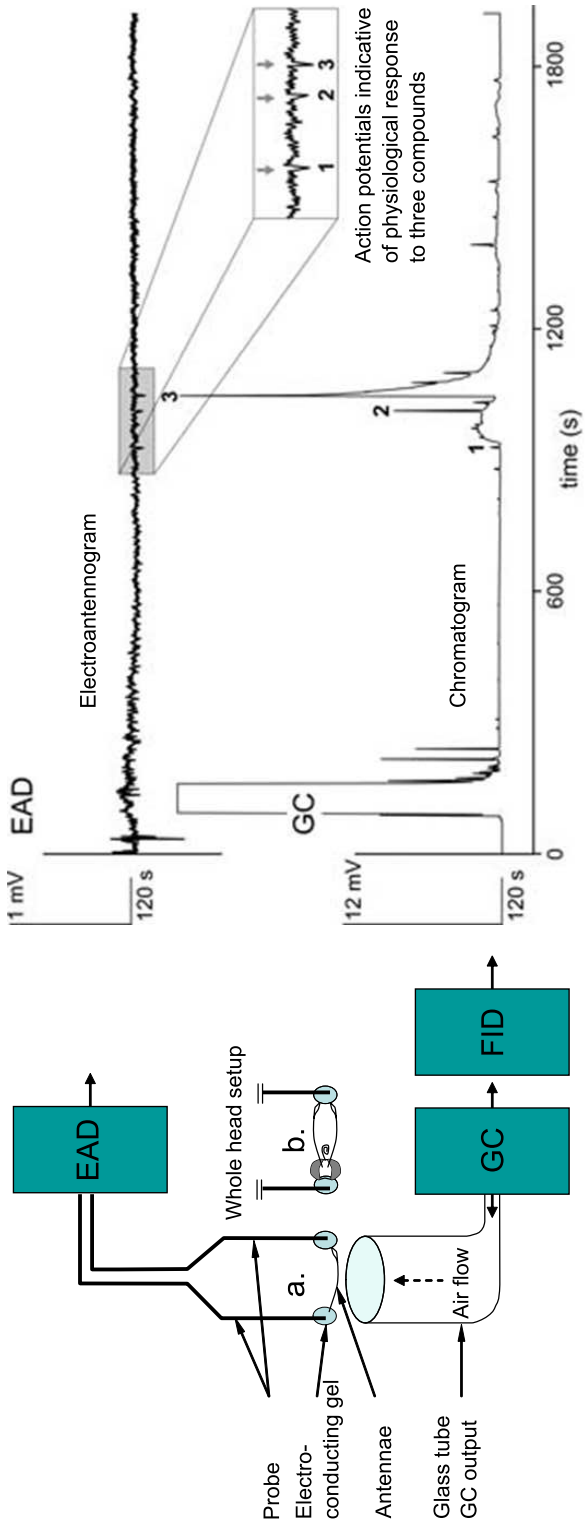


Figure 7.1: GC-EAD setup.
The two types of setups are represented, (**a**) single antennae, (**b**) whole head. On the right is an example of positive physiological response (three pulses on the electroantennogram) in parallel to the chromatogram of a male profile in *B. anynana* (right part of the figure from Nieberding *et al.* 2008).

GC-EAD analyses

Chemical samples were injected in a splitless mode (2 µl) in an Agilent 7890 GC equipped with a flame ionization detector (FID) and an EAD. The FID was equipped with a HP-INNOWax column (30 m × 0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific, USA) and run with hydrogen as a carrier gas (constant flow of 1 ml/min). The temperature of the injector was 230 °C. The oven temperature program started at 80 °C and directly raised at 10 °C/min to 210 °C, held for 10 min and raised again at 10 °C/min to 230 °C and held for 3 min (8 min for individual extracts, to empty the column of late compounds). Single antennae or the whole head with both antennae were mounted between the PRG-2 EAG (10× gain) probe (Syntech, Kirchzarten, Germany) with an electro-conductive gel (Blågel, Cefar, Malmö, Sweden). In both cases the tips of the antennae were removed to offer electrophysiological contact with the probe. Single antennae were easier to mount because of a lesser risk of movement compared to the whole head. However the whole head offers the advantage of a higher sensitivity (Hong-Lei Wang, personal communication). Several GC-EAD tests could be performed on the same antennae or head since they stayed alive for several hours (their 'physiological death' is detectable by the sudden appearance of a strong instability of the EAD signal). The individuals were freezed right after the cutting of their antennae. The scheme of the setup is presented on Figure 7.1. FID and EAD signals were processed with the program GC-EAD Pro Version 4.1 (Syntech, Kirchzarten, Germany).

7.1.2 Results

The summary of all the runs is presented in Table 7.1. We obtained no positive electrophysiological response for any of the compounds, either with *B. anynana* or with *B. smithi*. Apparently physical responses were recorded when the compounds were injected in higher concentration (around 100 ng/µl, Figure 7.2a and b). However this seemed not to be electrophysiological response since they were detected for any compound, including unlikely MSP components and the solvent itself (Figure 7.2b). Moreover they were not presenting the typical pattern of increase followed by an abrupt decrease of the voltage usually observed for action potentials (see Figure 7.1)

7.1.3 Discussion

The sensitivity of the antennae of females compared to males is expected to be lower since males are the sex under sexual selection, in order to detect the female sex pheromone from long distance in moths, and to produce a sufficiently abundant male sex pheromone to attract the female in butterflies. Indeed, in moths, female sex pheromone is detected by males at tens to hundreds of meters

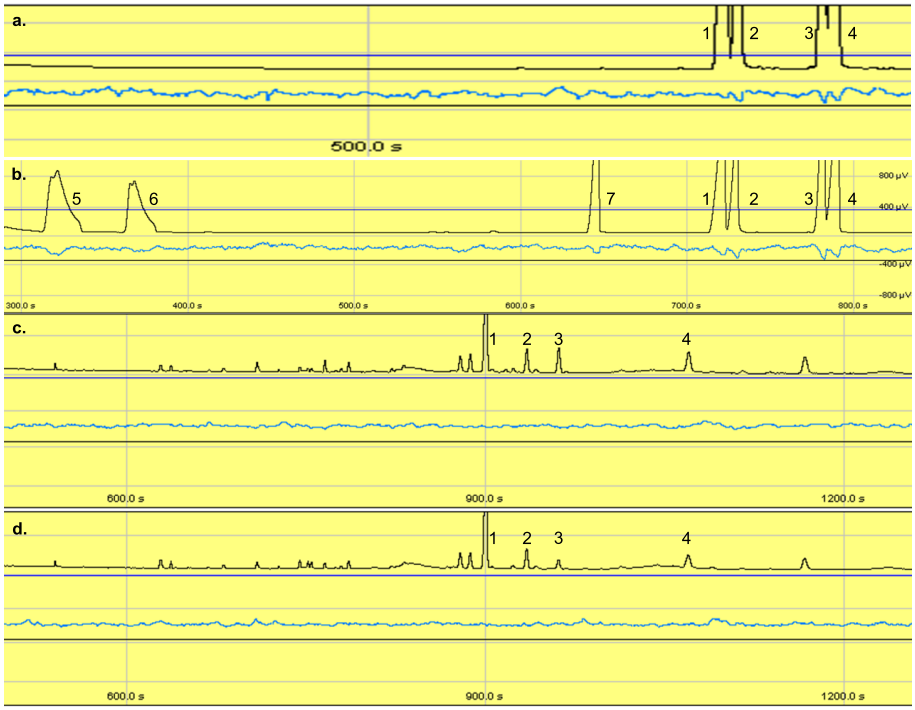


Figure 7.2: Examples of GC-EAD recordings
For each case **a** to **d**, the black curve represents the chromatogram and the blue curve the electro antennogram (both expressed in µV as a function of retention time). Panels **a** and **b** correspond to *B. anynana* recordings with solutions of standard compounds (1: TMP2one, 2: MSP2, 3: MSP3, 4: MSP1, 5: 16:Hy, 6: Z8-13:OAc, 7: βF). Panel **a** corresponds to the standard mix at 10 ng/µl, and panel **b** the standard mix at 125 ng/µl. They illustrate the physical response to any of the compounds once in large concentration. Panels **c** and **d** represent recordings of *B. smithi* with two individual extracts (both at a concentration of 2 individuals per 100 µl; 1: Z9-23:Hy, 2: tricosadiene, 3: TMP2Oac, 4: 18:Oac). Given the known amount of these four compounds in *B. smithi* males, Z9-23:Hy (1) should be present at 60 ng/µl and the three others around 5 ng/µl.

away, and males are selected to be highly sensitive to a very small amount of the relevant compounds (a few nanograms emitted by a female, in general, Phelan 1997). This is likely the main reason why it is so difficult to get physiological responses of female antennae for male MSP components in butterflies. In *B. anynana*, the results obtained by Nieberding *et al.* (2008) were acquired from a very small fraction of the tested individuals (seven out of 50). In the study presented here, no such sample size was available either for *B. anynana* or for *B. smithi*.

An important technical point should be accounted for. The effective amount that reaches the antennae with the GC-EAD setup is 60 times smaller than what is injected initially in the GC. Therefore if we inject 2 µl of a solution at 100 ng/µl, only 3 ng of compound reaches the antennae. Injecting higher concentration saturates the column and reduces its separation efficiency. If we take the example of *B. smithi* pMSP Z9-23:Hy, 3 ng represent 1/1000 of what is detected in a male wing extract. This could be a likely explanation of the lack of positive result. The two alternative protocols mentioned in introduction (EAD and single cell recording) could be good solutions to counteract the lack of sensitivity of GC-EADs. Both are routinely used in moths and allow to directly puff the compounds to the antennae and therefore to test realistic amounts of the compounds. For example, EAD was successfully used in *Pieris* butterflies with MSP and female antennae (Andersson *et al.* 2007).

Another technical issue could be the presence of inhibitory compounds in the environment where we performed the experiment. For example, (Su *et al.* 2011) described an attenuation effect of DEET (N,N-diethyl-m-toluamide) on the response of *Drosophila* olfactory receptor neurons. A compound with similar mass spectrum to DEET (major mass fragments of 91, 119 and 190) was detected in some of our 32 species samples. However, since positive GC-EAD response was obtained for other species (*B. anynana* and moths), we can think that no such compound is effectively preventing the physiological response in the experimental environment.

To conclude, even if this one shot experiment was not successful, it raises some issues that could be controlled in the future. The main improvement will be to present a higher amount of the pMSP components to the female antennae by removing the GC part of the setup.

7.2 Antagonist effect of a pMSP component of *B. safitza* on the mate preference of *B. anynana* females

In *B. anynana*, three chemical compounds form the volatile male sex pheromone (MSP) and increase male mating success during male courtship (Nieberding *et al.* 2008). From an interspecific point of view, a male from another species with other compounds but lacking these three MSP components will likely have a lower probability to mate with *B. anynana* females than *B. anynana* males, as absence or reduction of *B. anynana* MSP decreases *B. anynana* male mating success (Costanzo & Monteiro 2007, Nieberding *et al.* 2008).

A second, possibly complementary, mechanism that could help individuals avoiding incompatible heterospecific matings would be the recognition by the female of heterospecific compounds that would lead her to reject courting heterospecific males. This mechanism is described and well known in moths in which males attraction and upwind flight is stopped by the presence in the female sex pheromone of a heterospecific component or ratio of components (Löfstedt *et al.* 1990, Fadamiro & Baker 1997, Vickers & Baker 1997, Larsson *et al.* 2002, Gemeno *et al.* 2006, Takanashi *et al.* 2006, Van Vang *et al.* 2006, Linn *et al.* 2007, Lelito *et al.* 2008).

Since we observed that pMSP composition was likely used as a species recognition signal based on the presence of species-specific chemical components (Chapter 5), we tested the hypothesis of antagonist effect on female mate choice of some heterospecific compounds in *Bicyclus*. Across most of its range, *B. anynana* cohabitates with the morphologically similar *B. safitza*, the most widespread species of the genus (Condamin 1973). Although the two species are not closely related in the phylogeny, heterospecific matings have been observed, although rarely, in the field and in the lab (P. Brakefield and M. de Jong, personal communications). Previous data on *B. safitza* from Malawi attest for the presence of trimethylpentadecan-2-ol (termed MSP3 in *B. anynana*) and Z11-octadecenal (hereafter Z11-18:Ald) as major compounds (unpublished data, Table 7.2). We have no information on the presence of other compounds in *B. safitza* in Malawi but these two compounds were the ones selected as pMSP in the Ugandan population (Chapter 5 and 6). The MSP1 of *B. anynana* (Z9-tetradecenol) is also present in *B. safitza* males in Uganda, but not MSP2 (hexadecanal). Therefore, we predicted that *B. anynana* females may be able to avoid mating with *B. safitza* males thanks to the presence of Z11-18:Ald or to the absence of MSP2. Here, we thus experimentally tested the antagonistic effect of Z11-18:Ald on mate acceptance of *B. anynana* females by manipulating the chemical profile of *B. anynana* males. We expected that males perfumed with the heterospecific compound would have a decreased mating

success compared to control males perfumed with the natural *B. anynana* MSP blend.

Table 7.2: Absolute amounts per individual of each male-specific, most abundant, compound in the blend of *B. anynana* and *B. safitza* in the wild.

Country	Uganda		Malawi	
Species	*B. anynana*	*B. safitza*	*B. anynana*	*B. safitza*
MSP1	absent	0.2 ± 0.1	3.2 ± 1.6	NA
MSP2	1.4 ± 0.3	absent	0.5 ± 0.3	NA
MSP3	absent	4.7 ± 0.9	12.8 ± 6.0	0.4^*
Z11-18:Ald	absent	1.0 ± 0.2	absent	0.9^*
Number of males	3	3	3	8
Date of capture	Oct. 2009	Oct. 2009	Mar. 2007	Mar. 2007

Amounts are in µg (mean $\pm$ standard error).

NA if no information on the presence of the compound.

* no standard error available because all males were pooled in one extract to identify the isomeric structure of the chemicals).

Of note, both *B. anynana* and *B. safitza* present large genetic differences between population of Uganda and Malawi (Chapter 6, Monteiro & Pierce 2001).

7.2.1 Materials and Methods

Experimental setup

B. anynana from the Malawian lab stock (Brakefield *et al.* 2009) were reared on maize in a climate chamber with a temperature of 27 °C and of 70% of relative humidity close to the wet seasonal conditions in Malawi. After emergence, males and females were kept in separate cages and fed with banana and water. We used 6–9 days old virgin males and 2–5 days old virgin females to maximize their willingness to mate. Males and females were given a number written with permanent ink on the ventral side of one hindwing to be tracked during the experiment. We additionally dusted the genitalia of the males with coloured 'rodent-tracking' fluorescent dust using a different colour for each competing, differently 'perfumed', group; the dust is transferred to the female partner's genitalia during mating (Joron & Brakefield 2003), so mating events could be checked after the experiment. Colours were switched across groups between trials. These two manipulations were performed the day before each experiment to minimize the processing of the individuals the day they were tested.

We supplemented half of *B. anynana* males with either the putative sex pheromone component typical of Malawian *B. safitza*, Z11-18:Ald (perfumed males) or with only the solvent in which the component had been diluted,

hexane (control males). It took about 20 minutes to perfume 40 males for each replicate. In three first replicates, we perfumed one group after the other and the last group to be perfumed tended to mate more but also later (Table 7.3 and Figure 7.3). To avoid this potential effect of the time elapsed between the application of the perfume and the experimental testing of the butterflies, both groups were perfumed simultaneously in the following replicates (males perfumed one by one but alternating the treatments). Pure Z11-18:Ald was provided by the lab of Erik Hedenström (Sundsval University, Sweden). We added 14.32 µg of Z11-18:Ald per male by dropping 5 µl of a solution at 1.432 µg/µl on each side of the males (only 2.86 µg in the three first replicates). This corresponds to 15 times the amount that is measured on *B. safitza* male wings in Malawi (Table 7.2, the equivalent of 10 males was used by Nieberding *et al.* 2008). Adding more than the natural amount found on wings compensates at least partly the evaporation of the compound since 50% of the extract is lost immediately after application (Nieberding *et al.* 2008). Since changes of ratios between components were shown to become biologically significant after 2.5 to 3 hours (Nieberding *et al.* 2008), we let the experiment last for 2 hours. Exactly 20 females and 20 males of each perfumed group were released in a 4.20 $\times$ 2.15 m (9 m^2) and 1.90 m high climate room with maize plants. This density is definitely superior to what is observed in nature (Brakefield & Reitsma 1991) but the density in nature is not uniform and perching males can be found locally in high densities in the field (Brakefield & Reitsma 1991) and in big enclosure (green house) in the lab (Joron & Brakefield 2003). The room was lit by a set of SHP sodium, HTX MetalHalide and high UVA and UVB lamps, and external sunlight complemented the wavelength spectrum. This set up allowed us to mimic the range and intensity of wavelengths observed in natural African daylight. During the two hours, the identity of mating individuals and the starting and ending time of mating was recorded. After the two hours, females were inspected under ultraviolet illumination for fluorescent dust transferred at mating to cross-check the group-identity of their mating partners. Occasional double matings were scored as 1 for both perfumed and control group in the contingency table.

We tested for heterogeneity among samples using Fisher exact test because we observed in some treatments less than five matings per replicate. We then employed Goodness of fit test to compare the proportion of mating in the two groups (pooled replicates) to the null expectation of 50/50. We finally compared the time of mating between males from the two treatments controlling for the replicate with GLM.

Power analysis

To assess the likelihood to detect an antagonist effect of the additional compound on female mate choice with the sample size and number of replicates of our experiment, we performed a power analysis. The goal was rather informative since the available time to perform the experiment before resubmission did not allow to increase the number of replicates for this experiment. We simulated the following scenario: for the four pooled replicates, within the two hours of experiment, each of the 20 females will mate (once) with a probability p(m) and if the mating occurs, the female will choose a control male with the probability p(c). This scenario was simulated 1000 times with random Binomial sampling for different conditions: p(m) ranging from 0.1 to 0.9 and p(c) ranging from 0.5 to 0.9. For all these 45 conditions, we performed binomial tests (robust to small sample sizes and convergent with Goodness of fit for larger ones) and recorded the proportion of *P* values inferior or equal to 0.05 among the 1000 simulations. All the statistical analyses were carried out using the R language and environment for statistical computing and graphics (R Development Core Team 2011).

7.2.2 Results

We excluded from the analyses the three first replicates because of the potential bias linked to the time separating the application of each treatment and the experiment. Females did not mate significantly more with control males (n = 28) than with perfumed males (n = 21; χ^2 = 1 with 1 degree of freedom, P = 0.32; Fisher exact test for heterogeneity: P = 0.28), although control males tended to achieve more matings than perfumed males (57% of all matings; Table 7.3). Of note, none of the replicates displayed a significant difference in the number of mating between treatment when tested separately (data not shown). Regarding the time at which a mating occurred, perfumed males mated significantly earlier than controls excepted for the third replicate (GLM with combined treatment and replicate effects: perfume effect = -41.1, P = 0.02; interaction perfume $\times$ third replicate = 74.8, $P < 0.01$; see Table 7.3 and Figure 7.3). The large proportion of individuals mated within two hours (31%) indicated that conditions were favourable and males well capable of courting females despite the treatments.

The results of the power analysis are presented in Table 7.4. In previous similar experiments performed in the lab, when a preference was observed it usually ranged from 0.6 to 0.7, and the usual proportion of mating ranges from 0.4 to 0.5. In these conditions, the probability to detect a preference with four replicates of this experiment was 41%.

Table 7.3: Number of matings and mating latency for control and perfumed males for the different replicates

Experiment conditions			Number of mating events		Mean ± s.d. time at mating (min)	
Date	Experi-mentator	First group treated	ctrl	perfumed	ctrl	perfumed
21/6/2013	PB	ctrl	6	9	28 ± 9	41 ± 34
22/6/2013	PB	ctrl	8	9	35 ± 32	54 ± 31
23/6/2013	PB	perfumed	9	8	60 ± 43	19 ± 13
24/6/2013	PB	none	5	9	69 ± 47	28 ± 13
25/6/2013	PB	none	7	5	46 ± 27	13 ± 12
27/6/2013	CN	none	10	4	41 ± 38	75 ± 46
28/6/2013	CN	none	6	3	48 ± 25	37 ± 16

CN: Caroline Nieberding, PB: Paul Bacquet

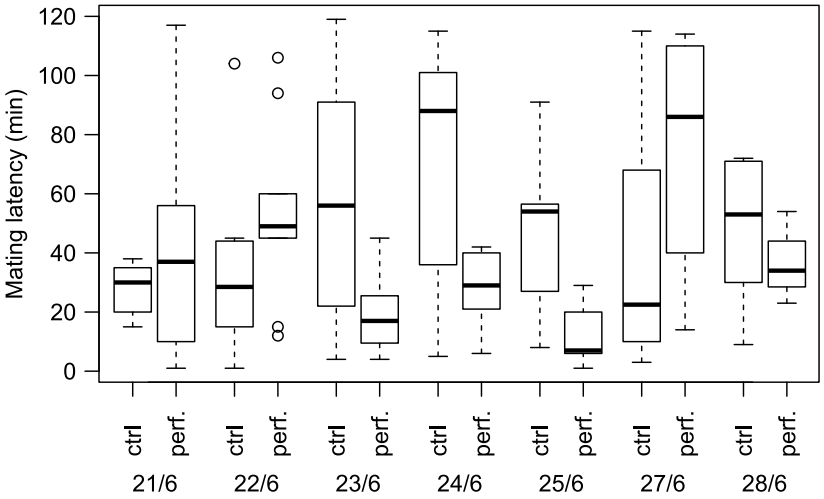


Figure 7.3: Time of latency for the mating of the perfumed and control males for each replicate.

Table 7.4: Probability of detecting a preference depending on the strength of the preference and the proportion of females mating within two hours.

Proportion of mating	Preference 0.5	0.6	0.7	0.8	0.9
0.9	3%	36%	92%	100%	100%
0.8	4%	31%	90%	100%	100%
0.7	3%	26%	83%	100%	100%
0.6	3%	26%	75%	99%	100%
0.5	3%	21%	69%	96%	100%
0.4	3%	17%	57%	93%	100%
0.3	3%	11%	44%	83%	99%
0.2	3%	8%	30%	61%	91%
0.1	2%	4%	11%	23%	53%

The black square highlights usually observed range of conditions and the grey square highlights the conditions observed in this experiment.

7.2.3 Discussion

While the number of mating was slightly larger for the control males, the males perfumed with *B. safitza* pMSP component mated significantly earlier. This result is quite unexpected and can either mean that females prefer males with this heterospecific compound, or that this compound increased the mating motivation of the males. We cannot distinguish between the two scenarios as we did not record male courtship activity in the experiment. Most interestingely, in both cases, it would suggest that *B. anynana* females, and/or males, have olfactory receptors for detecting the presence of Z11-18:Ald, a specific pMSP component of *B. safitza*.

We can speculate, in the case that the perfume increased female preference, that it is a case of preference for a novel signal as it seems to happen often under to sexual selection (Elias *et al.* 2006, Rodríguez *et al.* 2013). In the case that the perfume increased male courtship activity, it suggests that *B. anynana* males are able to adjust courtship investment, which is known in *B. anynana* to relate to mating success (Fischer 2006), in response to the biotic environment that surrounds them during courtship.

In both scenarios, it is to be noted that the effect of the perfume seems to have occurred at the beginning of the experiment since otherwise, perfumed males would also have mated with a larger number of females in addition to have mated earlier. It may be a sign that the compound evaporated more than expected during the course of the experiment. This would need to be checked

by GC analysis of perfumed males with this particular compound regularly during two hours after application. Yet, given that the number of carbons of Z11-18:Ald is even larger than the compounds used previously (MSP1, MSP2 and MSP3) in such 'perfumed' mate choice experiments, it is unlikely that it evaporated more quickly than in two hours.

The power analysis revealed that the chance to detect a potential preference given the predicted conditions was weak (41%) and that more replicates would be needed to unravel it if it is present. If our quantitative assumptions are realistic, 10 replicates would allow a probability of detection of 70%. It is to be noted that we underestimated the proportion of mated female which reached 70% across the seven replicates but also predicted a higher preference than the 0.57 observed across the four last replicates. These values reduce the predicted chance to detect a preference to 26% and 63% in the cases of four and ten replicates.

To finish with, it has to be kept in mind that this lab stock was collected from the field 25 years ago (in 1988, Brakefield *et al.* 2009), and that the risk of hererospecific mating does not exist any longer. Therefore if female preference (or reluctance) was costly, it has possibly been lost.

Chapter 8

General discussion

The subject of this thesis was to evaluate the role of sexual olfactory communication in the evolution of reproductive isolation using a butterfly genus (*Bicyclus*) as a model system.

We aimed at characterising the diversity of male chemical profiles across field-caught species and populations in the butterfly genus *Bicyclus*, and to identify plausible factors affecting the evolution of these chemical profiles and the role they can play in reproductive isolation and speciation.

The underlying hypothesis was that, in contrast to other types of sexual signals (visual, acoustic), olfactory communication allows a wide variety of signals to coexist without interference/competition, such that species that are differentiated for their 'olfactory niche' are not in competition to find, communicate with, and access, mating partners.

In this discussion, I will summarize the results obtained in the different parts of the thesis, discuss the general relevance of these results across taxonomic groups, discuss the implication of the methodological approach used to produce the results, and propose perspectives for future studies.

8.1 Summary of the results: role of chemical traits in reproductive isolation

8.1.1 Divergence of chemical traits between species

This work unravelled that among the 32 species we studied, the large diversity of pMSP is organized according to biologically relevant patterns (see Chapter 5). Indeed, the pMSP composition displayed a phylogenetic signal (phylogenetically more related species share more compounds than more distantly related species), a pattern of reproductive character displacement (RCD; sympatric species display more differences of composition than allopatric pairs of species after correction for phylogenetic distance), and an environmental effect of forest versus savannah on the number of compound per species (that is forest species had larger numbers of compounds compared to savannah species). Regarding the subject of this PhD, the most interesting pattern observed is the second: pMSP are more different between species sampled in sympatry than between species sampled in allopatry. The underlying scenario is that closely related species, or lineages within species, develop more distinct mate recognition signals and preferences to avoid unfit mating between lineages when they co-occur (sympatry) than if they do not (allopatry). We also observed that the effect of RCD was stronger for closely related species and was blurred when more distantly related species were compared, which is expected since these species are more exposed to unfit mating than species differentiated for a longer period of time.

As any pattern in biology, the RCD pattern we found for pMSP composition may be explained by different scenarios other than avoidance of maladaptive matings. Pairs of related species with similar reproductive characters could be absent in sympatry compared to allopatry because of competitive exclusion (species sorting) or because they merge genetically (differential fusion), both phenomena being due to an insufficient level of differentiation acquired between populations while they were previously evolving in allopatry. Yet, these two alternative phenomena imply that species pairs observed in sympatry are less related genetically than in allopatry. As we found that sympatric pairs of species were not less related than allopatric pairs in our dataset, we could thus rule out species sorting or differential fusion as likely alternative explanations of the observed RCD pattern.

Similarly, another scenario that we took into account is that of ecological character displacement (ECD). When ecology plays a role in the evolution of reproductive characters, ecological divergence between lineages in sympatry can cause the displacement of these reproductive traits. We have concluded that ECD is less likely than RCD for driving the evolution of pMSP composition for two reasons. First, because pMSP was not more different when species lived

in different habitats than when living in the same habitat, and second, because differences in pMSP composition were unlikely due to host plant adaptation. In addition, the larger number of pMSP compounds per species in forest compared to savannah habitats is not responsible of the RCD pattern since it was accounted for in the analyses.

Since avoidance of maladaptive mating seems the most likely to explain pMSP differences between *Bicyclus* species, we conclude that pMSP components are probably used as a species recognition signal. We next further refined this hypothesis by testing the evolution of pMSP composition between populations of several *Bicyclus* species. Indeed, RCD can be observed on sexual signals between populations within species if reproductive isolation is not complete between diverging lineages. This pattern refers to reinforcement, which can be distinguished from RCD that takes place between already isolated species in which latter case the RCD pattern is supposed to occur to reduce the cost associated with heterospecific, sterile, matings (Butlin 1987). It is only in the case of reinforcement that the divergence of the trait is considered to play a role in the speciation process by reducing gene flow between lineages (Butlin 1987).

8.1.2 Divergence of chemical traits within species

For the evolution of pMSP to be considered as part of the speciation process, one has to show that the chemical differentiation takes place before the genetic isolation of two lineages, and that these early differences cause premating reproductive isolation. In the Chapter 6, we have studied the differences of pMSP composition between individuals within species, and we have controlled for the genetic differentiation of these populations. In the model species *B. anynana* for which we had the best sampling, we observed that one population, from Uganda, is lacking two of the three MSP components previously identified in the population originally sampled in Malawi (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013). The Uganda population also displayed a neat genetic divergence compared to the Kenyan, Malawian, and South-African populations based on two nuclear genetic markers. This is in accordance with the previous description of the Ugandan *B. anynana* lineage as a distinct subspecies, *B. a. centralis* (Condamin 1968). However, three Ugandan individuals were found that had very probably hybrid genotypes between Uganda and more southern populations, which indicated that gene flow still occurs between the two *B. anynana* subspecies and that these two lineages are not yet isolated as good species. Moreover, all *B. anynana* populations shared the same mitochondrial haplotype, testimonial of recent genetic introgression from one lineage into the other. We also observed differentiation in the pMSP composition of different individuals of a single population (Kenya) while no genetic difference was

present between individuals of this population. All these results point that pMSP differentiation has likely predated genetic differentiation within *B. anynana* across its natural distribution range in Africa.

We also sampled 3 other *Bicyclus* species, although only three males from two populations for each species, to get a first preliminary insight about the generality of the pMSP differentiation pattern found in *B. anynana* across the genus. In *B. smithi* and *B. safitza*, we observed no pMSP differentiation between populations despite some divergence in the mitochondrial gene in *B. smithi*. In *B. vulgaris*, the divergence in the pMSP composition was as large as between *Bicyclus* species and was accompanied with mitochondrial divergence. It suggests that pMSP divergence within species as observed in *B. anynana* is not anecdotal.

Overall, our results suggest that the same differences in pMSP composition that are found between 32 species and used for species recognition, are also found within populations still exchanging genes, before genetic differentiation occurs, and could be the cause or at least a major factor of speciation within this genus. The very important last step that would close the loop of this demonstration would be to show experimentally that intraspecific polymorphism also exists in female preference and can lead to assortative mating within species, the basis of the speciation process.

8.2 Large diversity of male chemical profiles

The major male compounds we selected as forming the potential male sex pheromone displayed a large diversity of chemical structures across species. Most of the pairs of species did not share any pMSP component. This was expected as other types of sexual displays used like visual (e.g. colours) or acoustic (e.g. songs in birds, Seddon *et al.* 2008) sexual signals are often more divergent between closely related species than non-sexual signals (Mendelson & Shaw 2005, Arnegard *et al.* 2010a, Safran *et al.* 2012). Nevertheless, few studies have investigated the diversity of chemical profiles across a large assemblage of closely related species and none had yet attempted to target the male sex pheromone components. Conceptually close studies include Schulz *et al.* (2004) who focused on the biosynthetic origin of 13 androconial compounds (among at least 15) from 46 species and subspecies of Ithomiine butterflies. The rate of evolution of these compounds across the group of tested species seems high but the authors acknowledged that a more solid phylogeny of the group would be needed for producing strong conclusions. A recent study by Hernández-Roldán *et al.* (2014) also described the 125 distinct chemical compounds found in the scent organs of wings and legs of nine species of the butterfly genus *Pyrgus* (Hesperiidae). They observed some species specificity considering the whole

chemical spectrum, and in some species, differences between populations were of the same magnitude as found between species. The closest study to ours was performed by Symonds *et al.* (2009) on 19 species of Tefritid flies of the genus *Bactrocera*. They gathered from the literature the putative sex pheromone components (male rectal gland secretions) and related them to a phylogeny and the sympatry or allopatry of the studied pairs of species. They reported a strong species specificity, a rapid evolution of the composition since more changes are observed in terminal branches of the tree, and some phylogenetic signal. No clear effect of sympatry was observed on chemical differences. In a technically more advanced study, Zimmermann *et al.* (2009) identified for 15 sympatric species of orchid bees of the genus *Euglossa*, the chemical compounds that males collect and store in their tibial pockets and display for courtship. They found a strong homoplasy of major compounds across the phylogeny, suggesting a rapid turnover from a limited number of compounds between closely related species. They also observed stronger dissimilarities between species than expected under random evolution of the blends, which suggest a role of these blends in species recognition. A major discrepancy between this study and ours is that Zimmermann *et al.* (2009) only analysed compounds that are collected by individuals and excluded *de novo* synthesized lipids, although they can have a behavioural significance in other Hymenoptera species (Eltz *et al.* 2007). A last study about the evolution of olfactory signals in a phylogenetic framework is the one by de Oliveira *et al.* (2011) who described the evolution of the 36 cuticular hydrocarbons (CHC) observed in both sexes of seven species from the clade of the cactophilic fly *Drosophila buzzati*. They reported a strong specificity of the compounds in relation to sex, species and populations and also found a phylogenetic signal. So far, a few studies have thus assessed that chemical compounds assumed to play the role of male secondary sexual traits display a large diversity in structure, a strong species-specificity, and a rapid rate of evolution between closely related species. However, a tendency of stronger diversity and stronger species specificity seems to emerge for male sex pheromone compared to other pheromone functions. For example, Symonds and collaborators studied the evolution of aggregation pheromones in bark beetles and *Drosophila* flies and showed that these chemicals were more conserved between species and composed of relatively less compounds than sex pheromones (Symonds & Elgar 2004b,a, Symonds & Wertheim 2005).

Our results satisfy the prediction that chemical traits are particularly suited to generate a strong diversity and a rapid differentiation between males of closely related species compared to other types of secondary sexual signals such as visual or acoustic ones. Since the compounds investigated in *Bicyclus* are most probably synthesized *de novo* by the butterflies (Schulz *et al.* 2011, Liénard *et al.* 2014, Wang *et al.* 2014), and that distant species of *Bicyclus* sometimes display the same pMSP components (see Chapter 5, Figure 5.2), the

observed diversity and the inferred rapid changes in pMSP composition likely reflects the absence of constraints at the genetic level for the biosynthesis of the compounds. This is in accordance with recently published studies about the genetics of the production of chemical signals that have unravelled that first, the genes involved in the biosynthesis of sex pheromones form large families within the genome of insects including butterflies (Keays *et al.* 2011, Liénard *et al.* 2014). Moreover, changes in sex pheromone composition were shown to be induced by single mutations, or by a rapid change in gene expression. For example, Shirangi *et al.* (2009) observed a rapid evolution of the expression of a desaturase gene involved in the addition of double bonds to CHC (putative female sex pheromone components) across the *Drosophila* genus. Some of these changes in expression were due to deletions in the sequence of the cis-regulatory region of the gene. Lassance *et al.* (2010) showed that modifications in the coding sequence of a fatty acid reductase gene (an enzyme to transform fatty acids into alcohols) in the moth *Ostrinia nubilalis* account for the difference in ratio of the two female sex pheromone components contributing to reproductive isolation between the two strains of the species. As a third case study, Albre *et al.* (2012) discovered in two related genera of moths (*Ctenopseustis* and *Planotortrix*), that differences between sister species in the composition of the female sex pheromone was related to differential expression of a shared desaturase gene. Since similar types of compounds (fatty acid derivatives) and similar types of enzymes (reductases, desaturases) are active in moths as well as in *Bicyclus* (Liénard *et al.* 2014), we can expect that the same type of genetic determinism in male sex pheromone in the *Bicyclus* genus is responsible for the rapid evolution, as found in moths.

For rapid evolution of olfactory communication to be possible, both signal and perception through olfactory receptors (OR, see Figures 1.2 and 1.3) must be able to change in concert. It is indeed another peculiarity of chemical communication compared to other types of (visual, acoustic) communications, that specific olfactory receptors are necessary to detect particular signals. In this context, Gould *et al.* (2010) discovered that the genetic bases of reproductive isolation by male detection of female sex pheromones between the two moth species *Heliothis subflexa* and *H. virescens* was located in a single quantitative trait locus containing four OR genes. In an elegant study, Sakurai *et al.* (2011) have shown in the moth *Bombyx mori* that the replacement of a single olfactory receptor gene by that of another species could trigger the complete courtship behaviour of the transgenic male toward the heterospecific females producing the sex pheromone component perceived by the transgenic OR. Recently, Leary *et al.* (2012) identified a single nucleotide change responsible for the shift of sensitivity of an OR from a compound to another, and showed that this genetic change mimics the observed difference in sex pheromone preference between the moths *Ostrinia nubilalis* and *O. furnicalis*. Together these examples on the

molecular evolution of olfactory receptors show that: i) a modification in the olfactory receptor sensitivity can have a simple genetic determinism (such as the change of the coding sequence of an OR gene), ii) more importantly, that a change in the olfactory preference for the odour produced by potential mating partners can occur on this simple basis without modification of the central neural system and of the behaviour, and iii) that the entire courtship behaviour of males can be directed to females of another species on the simple basis of changing the chemical perception of one OR in the male antenna.

These recent results suggest that the simple genetic determinism of both chemical compounds biosynthesis and associated olfactory reception underlying preference leads to limited constraint and allows rapid evolution of olfactory communication using sex pheromone blends. This could explain the rapid evolution and diversity of pMSP in the *Bicyclus* genus.

The lability of the male chemical composition and the endless number of 'olfactory niches' that could be exploited in response to selection make chemical communication a well suited channel for the development of species-specific and private signalling. As a consequence, this lability could rapidly cause divergence within and between populations and drive the initial stages of reproductive isolation.

Since the olfactory mode of communication is the most ancient, but also the most widespread mode of communication across taxa, this point is relevant regarding the entire animal kingdom. We thus advocate to increase research on chemical communication in the field of sexual selection and speciation. Indeed many cases of species recognition rely on olfactory signals in insects and some are now found in vertebrates too: newts and potentially reptiles, fishes, bats, rodents (reviewed in Wyatt 2014). A case where the evolution of olfactory communication may have helped speciation is the *Drosophila* group. This system is a textbook example of large-scale reproductive character displacement (RCD) with the description by Coyne & Orr (1989) of larger prezygotic isolation compared to postzygotic isolation in species pairs occurring in sympatry. This example suggests that the evolution of premating signals is determinant in reproductive isolation but does not provide information about which signals are involved. More recently, several cases of reproductive olfactory character displacement were unravelled in different pairs of *Drosophila* species suggesting that olfactory communication may be involved in the original large scale pattern of RCD (Higgie *et al.* 2000, Ortiz-Barrientos *et al.* 2004, Higgie & Blows 2007, Grillet *et al.* 2012, Dyer *et al.* 2014, see also Singh & Singh 2013). In addition, some species of Hawaiian *Drosophila* flies are known to use olfactory signals during courtship display, and species specificity in CHC was described (Alves *et al.* 2010), suggesting a potential role for the evolution of olfactory sexual signals in this striking radiation.

8.3 Ultimate causes of the evolution of chemical sexual signals

While secondary sexual traits very probably arose because of sexual selection, their evolution may not only be due to this force (Allen *et al.* 2011). In this part of the discussion I will address the possible evolutionary forces that may act on the pMSP composition in the *Bicyclus* genus.

In the basic model of sexual selection described by Lande (1981), preferences match traits on a line of equilibrium. However, once the equilibrium is attained, the evolution of both trait and preference is subject to any other force, starting by drift as a null model of evolution. Since we observe a phylogenetic signal in pMSP composition both at the level of the genus and across populations within species (Chapter 5 and 6), it is not possible to rule out drift as a factor of pMSP evolution yet drift is theoretically not possible to demonstrate either since it is the null hypothesis. Additional evolutionary forces include natural or sexual selection if their direction is randomly fluctuating through time (Losos 2008). Indeed, if the selected optimum in pMSP composition is changing rapidly, the rate of evolution of pMSP composition is expected to be high. pMSP evolution is fast in the *Bicyclus* genus supporting the scenario of the fluctuating selection, but this hypothesis remains strongly speculative.

An obvious force that acted on pMSP evolution is selection for species recognition and avoidance of unfit hybrid mating that most likely caused the RCD pattern. As mentioned in the general introduction and in the discussion of the Chapter 5), not everybody agrees on the type of selection, either natural or sexual, being involved in this process of reinforcement or RCD, the most usually invoked one being the natural selection (Butlin 1987).

Natural selection could also have acted on the evolution of pMSP composition through illicit receivers, predators or parasitoids, which would detect males (and females subsequently to mating) thanks to the presence of MSP. For example, in *Pieris brassicae* butterflies, a parasitoid wasp hitchhikes on females following the smell of male antiaphrodisiacs that are transferred to the female during mating, to eventually parasitize the eggs laid by the female on the host plant (Huigens *et al.* 2009). High specialization of these illicit receivers could be an alternative explanation to the observed RCD. Indeed, one of two closely related lineages of host sympatric species using the same MSP composition would gain a strong advantage by escaping a coevolution cycle with a parasitoid that would remain specialized in the other, evolving more slowly, lineage of the butterfly pair of species. As for RCD, this phenomenon would primarily take place in sympatry. An indication that this scenario is not happening, at least as a rule in *Bicyclus*, is that differences between species seem to develop by the appearance of new components rather than by loss of shared components

(see Chapter 5). Escaping the detection of an illicit receiver would likely be more efficient by the loss of pMSP components. Solving this question would necessitate knowledge about the guild of associated parasites and predators of *Bicyclus* species which so far is not available. It would also be valuable to look for transfer of male compounds to the female during mating, which is not likely the case of pMSP that was sampled from the wings in *Bicyclus*.

Other scenarios including ecological pressure that could affect the evolution of pMSP are rather part of sexual selection. The first one is sensory bias (or sensory exploitation, Dawkins & Guilford 1996) where males would exploit female natural preference for some chemical compounds, for example those used for host plant detection. Although we again have no way to test this scenario with actual knowledge on *Bicyclus* as data on host plant preference is very incomplete, this scenario could be involved in the evolution of pMSP composition given the high proportion of plant derived pMSP components (potentially 44% depending on the correctness of the predicted biosynthetic origin; Chapter 5). However sensory bias is not likely to have repeatedly caused reproductive isolation since plant derived pMSP component do not display a stronger species specificity than other, more numerous *de novo* synthesized compounds (see Chapter 5).

The second ecology-based scenario of sexual selection is sensory drive. It occurs when divergent environmental conditions affect the optimal efficiency of signal transmission provoking divergence of the signal because of sexual selection for enhanced conspicuousness (Endler 1992). Since we did observe a larger number of compounds in forest (more humid) habitat, it seemed plausible that species compensate in this ecosystem for lower signal transmission (Symonds & Elgar 2008) by having a larger number of compounds forming the pMSP. However there was no difference in volatility of the pools of compounds used in forest versus savannah when testing for differences in chain length, or presence of specific chemical groups (Chapter 5).

Pure sexual selection would affect the evolution of pMSP composition if the chemical compounds were involved in male-male competition used in premating combats, as antiaphrodisiac transferred to the female during mating to decrease sperm competition, or as arrestant to induce females to accept mating. No characteristic behaviour usually associated with the use of sexually selected traits for the first two functions have been described in *Bicyclus* (see the discussion of the Chapter 5). In *B. anynana*, females remain attractive to males after mating (a new mating can be performed by a female within less than two hours in the lab, with realistic population density conditions), thus antiaphrodisiacs are not likely to be used. Therefore in this species only the arrestant function seems relevant since MSP allows males to mate with females (Nieberding *et al.* 2008).

To finish with, since pMSP are in most cases composed of several chemical

compounds, it is possible that the different components have a different function and hence, a different evolutionary dynamic (Bro-Jorgensen 2010). In *B. anynana*, hexadecanal (MSP2) is important for female acceptance, increases with age and decreases with inbreeding (Nieberding *et al.* 2008, 2012, van Bergen *et al.* 2013). Females prefer males with a higher amount of MSP2 which seems to be a condition-dependent sexual signal and shows no broad sense heritability (Nieberding *et al.* 2012). On the contrary, MSP1 and MSP3 (tetradecenol and trimethyl-pentadecan-2-ol) are heritable and are not, or less linked, to age and inbreeding (Nieberding *et al.* 2012, van Bergen *et al.* 2013). If MSP2 is a honest signal of male quality, we expect female preference to be conserved as well as the signal itself as a consequence. This is what we observed with our intraspecific study where MSP2 is the compound showing the least quantitative variation across populations and individuals compared to the other selected MSP components. Across species, MSP2 also displays a relatively low rate of evolution with shared presence between related species (Figure 8.1). It contrasts with MSP1 and MSP3 which are variable across populations in *B. anynana* and are less often shared between related species in the tree of the genus (Figure 8.1).

To conclude, MSP are multiple signals that evolve under various potential selection pressures. This makes probably impossible the task of identifying these pressures with a broad scale study as presented in Chapter 5.

8.4 Discussion of the methods

The results obtained in this thesis are mainly based on a protocol of chemical analysis and a method of selection of the putative male sex pheromone components. Even if we have tried to choose the most relevant method, anyone could argue against some of its features, and many did. It therefore seemed relevant to discuss this matter.

8.4.1 Chemical analysis protocol

Our chemical analysis protocol consisted of the extraction of *Bicyclus* wings with heptane as a solvent, and running the extracts through a non-polar column for gas chromatography with a particular program of temperature for the separation of the compounds (see methods in Chapters 5 and 6). This protocol is particularly suited for the analysis of fatty acid derivatives of 10 to 40 carbons, including those usually produced by female moths to attract males, or the three components of the male sex pheromone of *B. anynana*. The retention time window from which the compounds were scored, corresponds to the volatile portion of these expected types of compounds. However, some volatile compounds

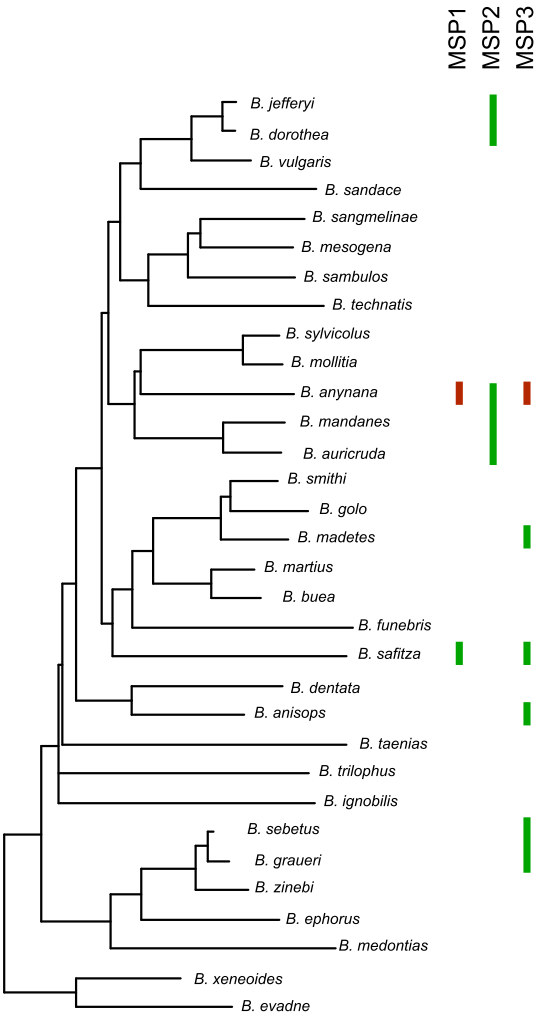


Figure 8.1: Distribution of the three MSP components identified in the lab stock of *B. anynana* across the genus.
The red bars indicate that we observed population variation within species for the presence of the MSP component, and green bars indicate no population variation or the sampling of only one population for the species. MSP1: Z9-tetradecenol, MSP2: hexadecanal, MSP3: trimethyl-pentadecan-2-ol.

were later detected out of this window (in *B. martius*; Wang *et al.* 2014) and other compounds may not be extracted or detected with this protocol. Therefore our results concern only a part of the whole volatile chemical profile of the species.

As recently highlighted by Heuskin *et al.* (2014), a certain part of the less or non-volatile cuticular lipids are linked to age, sex, and body parts and could also be involved in a functional chemical signaling in *B. anynana*.

8.4.2 Selection of putative male sex pheromone components

Similarly, the method of selection of the compounds as putative MSP components adds a second bias to the production of the final dataset. Although we supported it with theory and based it on solid experimental data, it involves some extrapolation that could have been performed according to different choices. We postulated that the MSP of all species, as observed in *B. anynana*, was composed of the top third most abundant, repeated, male-specific compounds. First our method completely excludes the minor male compounds. Although none of them seemed active in the study of *B. anynana*, we recognize that they could have a behavioural significance in other species. However, whether they are or not used for species recognition or another function does not change the fact that major compounds behave like species recognition signals. Secondly, the proportion of compounds falling in this selection is thus dependent on the total number of compounds male specific and repeatable within a species, which varies across species (Figure 8.2). This corresponds to the assumption that species with higher number of repeated male compounds have a larger number of MSP components as well. A potential confirmation that the choice of this proportional threshold is realistic, is that the two compounds selected in *B. safitza* previously elicited GC-EAD response in a few samples performed in Löstedt's lab in Lund University (Nieberding *et al.*, unpublished data). Unfortunately, no additional results on physiological activity of pMSP could be obtained to support or correct our selection (see Chapter 7).

It is important to note that this standardized method of selection had the advantage to avoid circular reasoning, since we selected compounds as being under sexual selection (male sex specificity, repeatability and abundance in males) and tested them for species-specificity and role in reproductive isolation. Despite potential biases linked to our assumptions, this method unravelled sensible biological patterns of phylogenetic relatedness and reproductive character displacement that act as confirmation that the major compounds that we selected have a biological function. We have identified no reason why our assumptions would artificially produce these results. Other studies usually analyse the whole chemical profile (Tregenza *et al.* 2000, Schulz *et al.* 2004, Zimmermann *et al.* 2009, Runemark *et al.* 2011) which could blur a potential

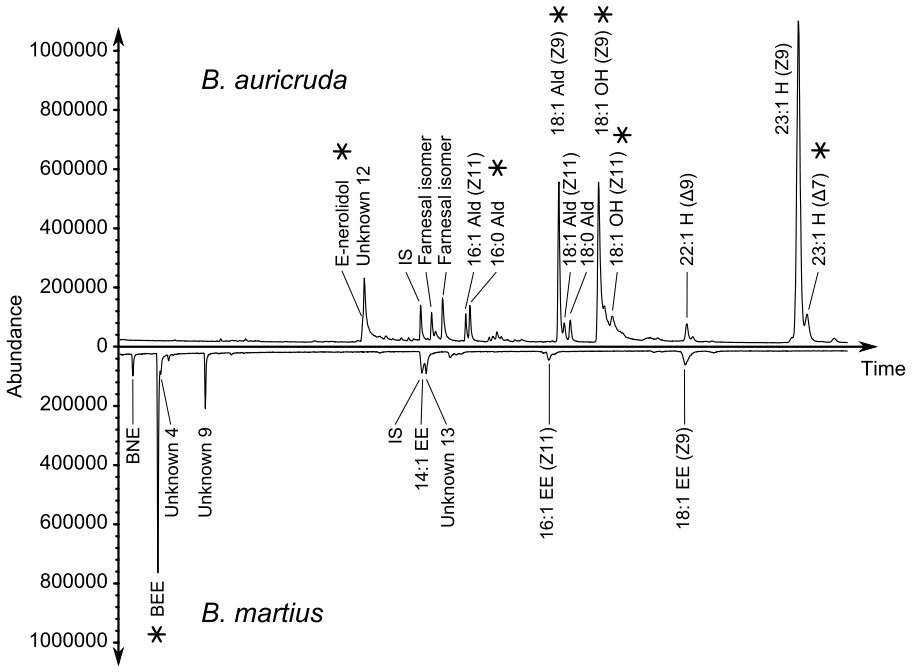


Figure 8.2: Discrepancy in the number of compounds present in the profile of different *Bicyclus* species.
B. auricruda displays 24 repeated male specific compounds and *B. martius* only 7. The presented samples correspond to the cell brush (structure 1a) in *B. auricruda* (from Uganda), and the androconial patch 6 on the dorsal surface of the forewing in *B. martius* (from Nigeria, see Figure 5.S3 for the androconia nomenclature). Asterisks highlight the pMSP components of the two species (two pMSP components were not present in these wing parts: octadecanol in *B. auricruda* and isobutyl Z11-hexadecenoate in *B. martius*). Compound names abbreviations are the following: IS is the internal standard, 16:1 Ald is the hexadecenal, 16:0 Ald is the hexadecanal, 18:1 Ald is the octadecenal, 18:0 Ald is the octadecanal, 18:1 OH is the octadecenol, 22:1 H is the docosene, 23:1 H is the tricosene, BNE is the probable contaminant N-ethyl-benzenamine, BEE is the ethyl benzoate, 16:1 EE is the ethyl hexadecenoate, 18:1 EE is the ethyl octadecenoate.

pattern only concerning sexual traits, or choose some compounds of interest (Schwander *et al.* 2013). In our case, analysing the totality of the chemical compounds observed in the 32 species would necessitate a nearly complete reanalysis of the raw data. This is so because we did not establish, from the beginning, the correspondence between homologous compounds across species and because of the huge total number of compounds (about 1200 since 40 compounds were detected in average per species). This is the drawback of a large dataset analysis such as ours.

8.5 Perspectives

In the field of speciation and chemical ecology, contrary to *Drosophila* flies (Coyne & Orr 1989, Hallem & Carlson 2006, Higgie & Blows 2007, Yukilevich 2012) or moths (Roelofs *et al.* 2002, Groot *et al.* 2006, Dopman *et al.* 2010), *Bicyclus* is a study system still in its infancy. Therefore an endless list of research questions remains to be addressed both in the field of speciation and of chemical ecology, although it will benefit from the methodological advance developed in other systems. The currently most limiting methodological issues are the difficult rearing of species or populations other than the Malawian *B. anynana* stock population used over the last 25 years (Dopman *et al.* 2010), and the lack of an efficient routine Gas Chromatography coupled to Electoantennographic Detection (GC-EAD) protocol to test the physiological response of female butterfly antennae to various chemical compounds. Finding a solution for these two constraints would be of the highest help for this research line.

In this last part of the thesis, I will identify the most relevant research questions for unravelling speciation in *Bicyclus*. I will then propose some experiments useful to study the evolution of MSP signals and of female preference, since a key to rapid evolution of reproductive isolation through chemical communication is dependent on how easily perception and production of new chemicals is possible to achieve at the molecular level. Finally, I will end with perspectives on chemical communication research that are currently made possible thanks to the development of genetic tools. Though I classified these perspectives into three parts, I believe they are very much connected and interdependent.

8.5.1 The study of speciation using the *Bicyclus* model system

Research on speciation could always need additional study systems since it is a complex melt of intertwined processes based on diverse traits and ecological interactions that can hardly all be studied with a single species group to achieve generalization. Despite the study of speciation is already advanced in other

butterflies (*Heliconius* genus), insects (Drosophilid flies), or various tropical taxa (Cichlid fishes or Amazonian frogs), the ecology and taxonomic origin of *Bicyclus* makes it unlikely that research on this new system will be redundant with other systems. For example, if *Heliconius* genus is the most related system in this research field, it differs from *Bicyclus* by the conspicuous aposematic and mimic function of its wing patterns that reflect very different ecology and consequently differences in the selective forces shaping their evolution. The *Bicyclus* genus is primarily attractive for research on speciation because it is composed of a large number of visually inconspicuous species that seem to rely on chemical cues for species recognition (see general introduction). Therefore, although a lot has to be learned about *Bicyclus* speciation, the study of this system will definitely enrich the field through the novelty of the trait involved (chemical signal) and its ecological implications. A first step toward the understanding of (*Bicyclus*) speciation is to identify the reproductive barriers: pre-mating and through which trait, post-mating, ecological and geographic. This should next allow asking more theoretical questions about which evolutionary force (natural and sexual selection) and in which ecological context speciation occurs for this group of butterflies. Basic large scale studies to get a global idea of some trends of the *Bicyclus* radiation are still needed. To start with, a more complete phylogenetic reconstruction (with both species and populations, and based on more genes, Wahlberg & Wheat 2008) and better data on the distribution ranges would allow to infer the biogeographic history of the genus. Without this basis, we are currently unable to detect if some clades have been more speciose, which confronted to ecological variables and sexual trait evolution would tell a lot about the factors favouring speciation (Wagner *et al.* 2012). We could expect for example that species belonging to more speciose clades would display more MSP components (Byers 2006).

The goal of these large scale studies as the one presented in this thesis is also to identify groups of species more suited for further fine scale studies. In this regard, the study of genes and of different phenotypic characters (pMSP, or eyespots in *Bicyclus*) across contact zones between differentiating lineages can be very informative on the selective pressures in action (Jiggins & Mallet 2000, Gay *et al.* 2008). In the particular case of chemical traits, the data gathered during this PhD thesis allowed to discover ongoing divergence of chemical profiles within several species which thus would be good candidates for finer scale studies. For example, the divergent population of *B. anynana* from Uganda or the polymorphic population from Kenya, and *B. vulgaris* which presents a contact zone between two mitochondrial strains with putatively different MSP composition in Cameroon are the best examples of likely interesting contact zones. Until the resolution of the major methodological difficulty of rearing new species or populations in lab conditions, it should be possible to answer some basic questions about pre- and post-mating isolation *in situ*, thanks to longer

field work sessions (two months). This would help understanding which barrier arose first and for example if the RCD pattern we detected originates from reinforcement or from species interference between already isolated species. For example keeping males of one sister species with female of another in cages would inform on premating isolation and the possibility of heterospecific mating. Similarly, forced heterospecific mating could inform on the extent of postmating isolation thanks to measure of viability and fertility of the potential offspring. In a more elaborated setting, proposing a choice between males of two sister species, to one type females either with blocked or unblocked antennae would inform on the use of MSP in species recognition. The monitoring of female mate choice in the population of *B. anynana* from Kenya could inform on within species assortative mating. This experiment would be performed in captivity by capturing couples to sample DNA and chemical profiles of males. Non-exclusive expectations would be heritability of the preference (related females should prefer chemically similar males), assortative mating (females should prefer genetically related males), or inbreeding avoidance (preference of less related males). These longer sessions would definitely be beneficial to also gather data on the ecology of the species which are critically lacking: following individuals in their natural environment is the only way to know how frequent interspecific courtship is within *Bicyclus*, which host plants are used (are the species specialized and how overlapping is their host plant use?), or which parasitism do *Bicyclus* species suffer (do we observe strong specialization of parasites to separate *Bicyclus* species?). More robust ecology based characters other than ventral eyespots, maybe the shape of the wings, their venation pattern, or their overall coloration (excluding eyespots), are lacking in our comparative study (Chapter 5) to compare ecological and sexual trait divergence (see examples in Arnegard *et al.* 2010a, Martin & Mendelson 2014).

8.5.2 Congruent divergence of olfactory traits and preferences for these traits

A key hypothesis developed during this work and discussed above is that both chemical traits and preferences for these traits can evolve rapidly in response to selection, partly because they suffer few intrinsic (genetically based) and extrinsic (little competition in the olfactory niche thanks to private communication) constraints. A straightforward perspective would be to test it in the lab with experimental evolution.

The development of premating isolation by shift in female preference in response to maladaptive mating could be tested by altering female compatibility with a part of the males and adding chemical cues in the other. We expect females to develop a preference toward some new compounds. Compatibility could be reduced to zero by mechanically blocking copulatory organs of males.

To improve the probability of recruitment of new female olfactory receptors, the chemical cue added on compatible males should strategically be a blend of compounds, and these compounds should be minor compounds already present in *B. anynana* or in closely related species (so OR could already be present in the female without being associated with a particular preference). Preliminary test should ensure that none of the treatment (blocking, sham blocking of copulatory organs, and sham perfuming of the males) influences the preference of females or the motivation of the male for courtship.

Since MSP2 is a trait indicating genetic quality in *B. anynana*, it would be interesting to test how quickly females could shift their preference toward another trait in response to the appearance of cheaters among males. These cheaters could be introduced artificially by adding MSP2 on inbred males, for example. Practically, this would necessitate allowing mating in a restricted time period only to prevent effect of evaporation of the compound. Although MSP2 seems to be a 'solid' honest signal since it is relatively conserved in *B. anynana* and related species (Figure 8.1), this would challenge the scenario proposed in the general introduction about a turnover of male sexual signals because of the loss of honesty in a dynamic similar to sexual conflict. A recent investigation of the diversity of less volatile cuticular lipids in *B. anynana* revealed that a significant proportion of the compounds are linked to age, sex or body parts (Heuskin *et al.* 2014). Therefore it is to be expected that potentially recruited compounds honestly linked to condition will be part of this less volatile range of the chemical spectrum.

It may be reasonable to test the heritability of other male compounds (non-MSP) and of female preference. This would serve as a control for the two proposed experimental evolution protocols. Indeed, if no heritability is present for compounds or preference (for example because the lab stock has lost some diversity), time loss could be avoided by cancelling a long lasting experiment with poor probability of success. If heritability is present but the traits never evolve, this could be a cue that *B. anynana* relies less on olfaction than hypothesized.

8.5.3 Genetic bases of chemical communication

With the recent sequencing of both a transcriptome of *B. anynana* dedicated to the study of chemical communication (Nieberding *et al.*, in preparation), and the whole genome of the species (Brakefield, Monteiro, Beldade and collaborators, in preparation), we can expect that more genes involved in chemical communication will be identified in a close future in addition to the first ones recently found (Liénard *et al.* 2014). The candidate genes of biosynthesis pathways that are currently being tested are derived from existing knowledge from the female sex pheromone system in moths and therefore

concern the biosynthesis of fatty acid derivatives compounds. However in *Bicyclus*, almost a half of the major male compounds we selected as pMSP components are not fatty acid derivatives (Chapter 5), therefore additional work will be necessary to investigate the genetic bases of enzymes involved in potentially very different biosynthesis pathways (or functions of recruitment and re-emission of host plant compounds). A transcriptome approach is thus relevant to avoid bias in targeting known pathways.

The identification of OR genes may be more advanced than for the biosynthesis since a large list of OR sequences have been expressed in empty neuron mutant *Drosophila* and their sensitivity and specificity tested with libraries of diverse odorants (Hallem & Carlson 2006). Moreover, genes of both OR sensitive to compounds derived from plants and to compounds synthesized *de novo* (fatty acid derivatives in moth or CHC in *Drosophila melanogaster*) have already been identified (Tanaka *et al.* 2009, Wanner *et al.* 2010, Carraher *et al.* 2012, Zhang & Löfstedt 2013).

Once several genes underlying pheromone production and female perception will be identified, the comparison of pattern of expression and sequence polymorphism for these genes in populations or species could be performed. This would unravel the genetic mechanism underlying recent changes in chemical signals and preference or allow understanding if the same compound originates from the same pathway or is detected by the same OR in different lineages (as was started for gustatory receptors in *Heliconius* butterflies, Briscoe *et al.* 2013, and for desaturase genes in *Drosophila*, Shirangi *et al.* 2009). Ultimately, analysis of the sequence polymorphism of biosynthesis and OR genes could help to unravel the selective pressure at work on the evolution of chemical communication (e.g. purifying or directional selection, Keays *et al.* 2011).

All of these possible perspectives integrating research on speciation, sexual selection, genetics and chemical communication, give *Bicyclus* research a very exciting future.

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