

Flower colour variation across a hybrid zone in *Antirrhinum* as perceived by bumblebee pollinators

Emmanuelle Tastard · Christophe Andalo ·
Martin Giurfa · Monique Burrus · Christophe Thébaud

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Abstract To assess if pollinators' behaviour could explain the maintenance of hybrid zones between different flower colour morphs, we analyzed flower colour variation in an *Antirrhinum* hybrid zone using spectrometry and a model of bee perception. Some colours generated by hybridization were not observed in any *Antirrhinum* species and even appeared to be rare among angiosperms. Variation in flower colours within the hybrid zone was continuous; the most similar colours were predicted not to be discriminated from one another in natural foraging situations. However, when compared at a scale corresponding to bees' foraging range, some flower colours could be discriminated from all colours displayed by neighbouring plants. This could affect pollinator behaviour and explain lower visitation rates within the centre of the hybrid zone. Behavioural studies involving bumblebees and plant mixtures of parental and hybrid flower colours carefully characterized with appropriate visual models will be necessary to test this hypothesis.

Keywords Hybrid zone · Flower colour · Bee visual model · Pollination ecology

Introduction

Hybridization is a critical issue in evolution. It generates a wide variety of phenotypes which may promote adaptation (Stebbins 1959; Arnold 1992). In most cases, however, related populations, when they come into contact after having evolved in separate geographical areas, co-occur in narrow and stable hybrid zones (Mayr 1963; Endler 1977). Several factors can contribute to the stability of such hybrid zones, including selection against hybrids, positive assortative mating within populations, and species segregation by habitat (Barton 1979; Barton and Hewitt 1985). In flowering plants, where many lineages are dependent on biotic vectors for pollen export and reception, pollinator behaviour is thought to contribute to maintaining hybrid zones through effect on mating patterns and individual reproductive success (e.g. Campbell et al. 1997; Emms and Arnold 2000). However, the mechanisms underlying this contribution remain poorly known.

A number of authors (e.g. Meléndez-Ackerman et al. 1997; Wesselingh and Arnold 2000; Campbell et al. 2002; Ippolito et al. 2004) have examined the behaviour of pollinators when confronted to both parental and hybrid phenotypes and found little evidence for behavioural mechanisms that could maintain the genetic differences between populations. However, their conclusions may have little bearing on the role played by pollinators in nature since they rely upon experiments in which a limited number of hybrid phenotypes were used. This contrasts with hybrid populations which usually display very diverse phenotypes and often contain phenotypes that exceed the

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E. Tastard · C. Andalo · M. Burrus · C. Thébaud (✉)
Laboratoire Evolution et Diversité Biologique, UMR 5174,
CNRS-Université Paul Sabatier, 31062 Toulouse cedex 9,
France
e-mail: thebaud@cict.fr

M. Giurfa
Centre de Recherches sur la Cognition Animale, UMR 5169,
CNRS-Université Paul Sabatier, 31062 Toulouse cedex 9,
France

phenotypic range of the parental species (Stebbins 1959; Rieseberg et al. 1999). Notably, hybrid zones between closely related species with different flower colours usually display a wide diversity of flower colours, including extreme or novel phenotypes relative to parental species (Whibley et al. 2006). Since visual signals produced by flower colour play an essential role in aiding identification by pollinators (Chittka et al. 1999; Gumbert 2000; Chittka and Spaethe 2007; Dyer et al. 2007), phenotypic diversity in flower colour as expressed in hybrid zones could influence the behaviour of pollinators with regard to parental and hybrid phenotypes.

Colour variation in hybrids has often been assessed using measures of pigment concentration (Schemske and Bradshaw 1999; Streisfeld and Kohn 2005), optical densities (Campbell et al. 1997), or light reflectance (Meléndez-Ackerman 1997; Streisfeld and Kohn 2005). However, while these measurements provide useful proxies for phenotypic diversity in floral colour traits, none of them directly reflects the colour space as perceived by pollinators. Pollinator perception of colour notably depends on the spectral sensitivity of photoreceptors and on colour coding in the central nervous system. Colour perception in important pollinators such as honeybees and bumblebees has been thoroughly studied for several decades (Daumer 1956; von Helverson 1972; Menzel and Backhaus 1991; Chittka et al. 1992; Peitsch et al. 1992). This has led to several models of colour perception, which allow predicting how well a bee will be able to distinguish two flower colours (Backhaus and Menzel 1987; Chittka 1992). It is also well-known that bees can treat different visual stimuli as equivalent if the perceptual similarity of these stimuli is high enough to promote such generalization (Giurfa 1991; Gumbert 2000). Thus, if we are to understand how pollinators could influence the dynamics of hybrid zones through their patterns of flower visitation, it is crucial to describe colour phenotype variation in hybrid zones by analyzing it in the framework of bee colour perception.

In this study, we quantified colour phenotypes in a hybrid zone formed by two bee-pollinated snapdragon subspecies, the magenta flowered *Antirrhinum majus pseudomajus* and the yellow flowered *A. m. striatum*, and analyzed this information using the colour hexagon, a generalized model of bee colour vision (Chittka 1992). Since all individuals were georeferenced, we were able to examine how flower colour as perceived by pollinators varied spatially across the hybrid zone and to discuss its significance in relation to mechanisms that could explain the maintenance of hybrid zones. In this *Antirrhinum* hybrid zone, Whibley et al. (2006) found that a locus affecting patterns of floral pigmentation showed a very narrow cline in allele frequencies, whereas other linked loci showed no such clinal variation, thus suggesting that

the maintenance of the cline was due to direct selection on floral pigmentation. However, the mechanisms of selection remain unknown. Our observations that pollen reception is the major limiting factor in seed production in this system suggest that pollinators are likely candidates as agents of selection (E. Tastard and C. Andalo, unpublished data).

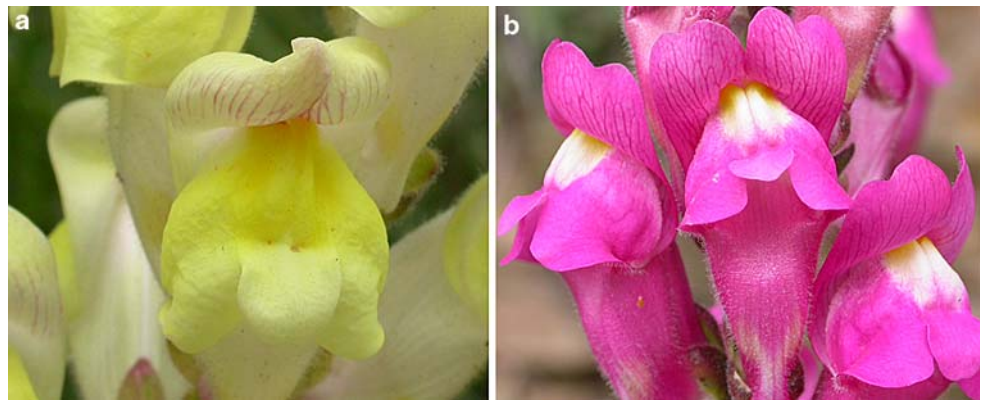
Specifically, we addressed the following questions: (1) Does hybridization produce uncommon, no-analogue, colour phenotypes? Since bee pollinators are usually flower constant and therefore choose previously visited phenotypes, uncommon phenotypes could have a lower probability of visitation. (2) Do the colours of hybrids appear as part of a continuous variation between the two parental *Antirrhinum* subspecies in the colour perceptual space? Such pattern of variation could, in principle, favour generalization to all phenotypes by pollinators and prevent populations from maintaining their differences owing to increased gene flow. However, insect pollinators only perceive colours at relatively small spatial distances (Giurfa et al. 1996; Dyer et al. 2008) and move mostly between neighbouring plants (Waser 1982; Collevatti et al. 2000; Saleh and Chittka 2007), which implies that they perceive colour variation only at a very local scale. Therefore, there may be discontinuous transitions at spatial scales that are biologically meaningful for pollinators even if flower colour variation seems continuous when considering all the plants of the hybrid zone together. Thus, (3) is flower colour variation locally discontinuous in some parts of the hybrid zone? If so, this could provide clues for understanding the role of pollinators in the maintenance of the hybrid zone.

Materials and methods

Study system

Antirrhinum majus is a short-lived perennial (1–3 year) which produces annual inflorescences of zygomorphic flowers from May to June in our study area (see below). Several subspecies have previously been described, among which the magenta flowered *A. m. pseudomajus* and the yellow flowered *A. m. striatum* (Fig. 1). These two subspecies occupy non overlapping geographical regions throughout most of their range in the Pyrénées. However, where they come into contact, natural hybridization occurs, giving rise to hybrid zones that are strikingly diverse in flower colour (Whibley et al. 2006). The role of pollinators in propagating *A. m. pseudomajus* and *A. m. striatum* is likely to be of central importance because both subspecies are self-incompatible. Thus seed production depends crucially on pollinator visitation. Main insect pollinators are large bees such as bumblebees (*Bombus* spp.) and carpenter bees (*Xylocopa* spp., Hymenoptera, Apidae; Whibley 2004).

Fig. 1 Parental phenotypes:
(a) *Antirrhinum majus striatum*,
(b) *A. m. pseudomajus*



Plant and flower sampling

Sampling of the hybrid zone

In June 2004, sampling was performed across a hybrid zone in the Toses valley south of Puigmal, northern Catalonia (Spain), on a 2-km long transect starting from an area with only yellow-flowered phenotypes to an area with only magenta-flowered phenotypes. In this zone, individuals are spatially arranged according to the typical clinal structure of hybrid zones relative to flower colour (Barton and Hewitt 1985; Whibley et al. 2006). All individuals sampled ($n = 558$) were located using a GPS system with 2-meter precision. To analyse flower colour variation relative to geographic location, flower colour was visually scored using a previously defined scoring system: a score was allocated for each colour component (magenta and yellow) on the basis of overall appearance of the corolla (see Fig. 2B in Whibley et al. 2006). Although it would have been desirable to obtain reflectance measurements of every plant in the hybrid zone, time and logistic constraints prevented us from doing so. Instead, we predicted an approximate location in the bee colour perceptual space for some of the plants sampled in the hybrid zone, on the basis of their colour visual scores. This was possible because all plants had identical flower morphology and *A. majus* petals have low reflectance levels in the UV. Thus, in 2004–2005, one or two flowers (mean = 1.51) per plant were collected on a total of 124 plants, so as to cover most of the colour range generated by hybridization, as perceived by humans, and specify the relation between human visual scores and flower colour in the bee colour space. Upon collection, flowers were visually scored, stored in an icebox and brought back to the laboratory for spectral colour analyses which were performed within 48 hours (see below).

Sampling of other *Antirrhinum* species

To compare flower colour range among hybrids with that of other *Antirrhinum* taxa with diverse floral phenotypes,

15 species or subspecies occurring naturally in South-western Europe (including one species of *Misopates*, the sister genus of *Antirrhinum*) were grown in a greenhouse under standard conditions. Spectral colour analyses were carried out on 1–2 (mean = 1.3) flowers of each of 1–4 (mean = 1.6) plants per taxon. In *Antirrhinum*, differences in flower colour among species and subspecies are known to be highly genetically determined (Stubbe 1966; Schwinn et al. 2006; Whibley et al. 2006). Thus, we assume that flower colours were unaffected by the greenhouse growing conditions.

Floral colours through the bees' eyes

To analyze floral colours as perceived by bees, we used the 'colour hexagon', which is a generalized colour opponent space (Chittka 1992). Colour reflectances can be converted into colour space coordinates by considering the opponent processes defining the space in order to assign them a given locus in the hexagon. Colour loci that lie close to each other within the hexagon are perceptually similar while distant colour loci are perceptually different to bees. Flower reflectance was measured using an S2000 reflectance spectrophotometer (Ocean Optics Inc., Dunedin) calibrated against white and black standards. We measured reflectance on the upper and lower lobes of the flower's corolla. The top of the upper lobe and the lateral part of the lower lobe of the corolla were dissected with a razor blade, and flattened on a black support. Percent reflectance was measured in the wavelength range between 300 and 700 nm, which encompasses the visible spectrum for bees (Menzel and Backhaus 1991). To represent colours in the perceptual space (hexagon), we assumed that colour receptors were adapted to a natural background provided by the average reflectance of several green leaves and used the spectral sensitivity functions of the three types of photoreceptors and the standard function D65 representing daylight illumination. Electrophysiological recordings of photoreceptor sensitivity performed on many hymenopteran species, including six species of *Bombus* and one

species of *Xylocopa* have shown that spectral sensitivity functions of these bees are broadly similar, with a short-wavelength, a medium wavelength and a long-wavelength receptor peaking in the ultraviolet, blue and green region of the spectrum, respectively (Peitsch et al. 1992). We therefore used the spectral sensitivity curves obtained for *Bombus terrestris* as a template both for *Bombus* spp. and *Xylocopa* spp. encountered in our study area (Peitsch et al. 1992).

Statistical analyses

To check the repeatability of our measurements, reflectance spectra were recorded four times on the upper lobe and four times on the lower lobe of two flowers from each of 16 plants collected in the field. The location in the colour perceptual space was calculated for each spectrum. Using one-way analysis of variance, we estimated the repeatability (calculated as the intraclass correlation coefficient, r_i ; Zar 1984, p. 323) of the location in the colour perceptual space at the flower level and at the individual level.

To ascertain that the location in the colour perceptual space could be predicted from the magenta and yellow scores assigned visually, we performed a MANOVA analysis using data from individual plants sampled for reflectance measures ($n = 124$). The coordinates in the colour perceptual space were considered as the dependent variables. The magenta and yellow scores were considered as the independent factors. Both scores influenced significantly the location in the colour perceptual space (Wilks' Lambda = 0.319; $F_{2,119} = 126.81$; $P < 0.001$ and Wilks' Lambda = 0.370; $F_{2,119} = 101.48$; $P < 0.001$ for the magenta and yellow scores, respectively) which suggests that flower colour locations in the colour perceptual space could be approximately predicted from the visual scores in our system (see supplementary material for further accuracy assessment of the prediction). Plants for which no reflectance measures were available were therefore assigned the mean colour location calculated for plants with the same magenta and yellow visual scores.

To determine if two flower colours can be discriminated by pollinators, we computed pairwise colour distances among the locations of individual plants within the bee colour perceptual space and compared them to a discrimination threshold. Since discrimination abilities are highly dependent on the experience of individual bees and experimental conditions (Dyer and Chittka 2004a, b; Gjurfa 2004), we chose a threshold of 0.1 hexagon units (Euclidean distance) which is the minimum colour distance that enables reliable flower constancy in several bee species (Chittka et al. 2001). Such a threshold is likely to provide a behaviourally meaningful estimate of discrimination in the field. To assess flower colour variation in the

hybrid zone, we plotted the locations of the plants of the hybrid zone together with those of the plants from other *Antirrhinum* taxa in the colour perceptual space. To determine if hybridization between *A. m. pseudomajus* and *A. m. striatum* generated extreme or novel flower colours, we tested whether some of the hybrids could be discriminated from all other sampled taxa. In practice, we tested whether the flower colour distance between each plant from the hybrid zone and each plant of the other taxa exceeded the colour difference threshold necessary to ensure forager constancy. To assess if colours found in the hybrid zone that were different from other *Antirrhinum* taxa's colours were perhaps uncommon in angiosperms in general, we compared their locations in the colour perceptual space with those of 1063 plants from 573 species analyzed by Chittka et al. (1994).

In order to explore the reasons why some flower colours found in the hybrid zone might be otherwise little or not represented in other angiosperms, we calculated spectral purity of colours for plants from the hybrid zone and other *Antirrhinum* taxa since this parameter can influence pollinator behaviour (Lunau 1990; Lunau et al. 1996). Spectral purity is maximal for monochromatic lights, whose loci lie on the spectral line delineating the colours that are visible for bees (Lunau 1990). We calculated it as the colour distance from the neutral point (the centre of the colour perceptual space defined by the green leaf background, see above) to the locus of the focal colour, related to the colour distance from the neutral point to the point of the spectral line with the corresponding wavelength, following Lunau (1990). We tested whether the hybrid phenotypes that were discriminable from all other *Antirrhinum* taxa had unusual spectral purity values by comparing their colour purity with that of the other *Antirrhinum* taxa and of the 573 angiosperm species analyzed by Chittka et al. (1994, Table 1) using Student's t -tests.

To determine whether flower colour variation in the hybrid zone could be considered as a continuum, we tested if the distribution of phenotypes in the colour perceptual space is such that any colour phenotype lying between parental colours cannot be discriminated from its nearest neighbours by bee pollinators because the colour distance between nearest neighbouring points in the colour perceptual space is always under the discrimination threshold. To achieve this, we performed a hierarchical clustering analysis on the data set obtained in the hybrid zone using the smallest colour distance between points in different clusters as the criterion to merge them (*agnes* function in the *cluster* package of R software, version 2.6.1). When several plants had the same coordinates in the colour perceptual space, duplicates were removed from the dataset for this analysis. In the resulting dendrogram, the length of the branches is the distance between the corresponding

clusters in the colour perceptual space, which represents the colour difference perceived by bees. This colour distance was compared to the discrimination threshold; flower colour variation can be considered as continuous if all branch lengths in the dendrogram are under this threshold.

To determine if there was continuity in flower colour variation at a scale corresponding to pollinators' perception, we performed the same clustering analysis in the neighbourhood of each plant, within an empirically determined radius. Since bumblebees usually visit sequentially neighbouring plants (Zimmerman 1981), we chose a value of 6 m for this radius because most plants in our dataset had at least one neighbour within that distance. To ensure that the choice of this 6-m radius did not affect our conclusions, we also performed separate analyses using radii of 3 m and 12 m. To visualise if variation in flower colour is locally continuous or not across the hybrid zone, we plotted the length of the longest branch of each dendrogram against the location of the focal plant along the transect defined by the linear regression of the UTM-coordinates of the plants. A y-coordinate above the discrimination threshold (0.1 hexagon units) would indicate that there is at least one plant within 6 m around the focal plant which can be discriminated from all others by bee pollinators, thus signalling a discontinuity in colour variation.

Results

We found a high repeatability of spectral data at the flower level for both petal parts (upper lobe: $r_I = 0.96$ for X and Y, lower lobe: $r_I = 0.99$ for X and Y). At the individual level, repeatability was high as well (upper lobe: $r_I = 0.76$ for X and $r_I = 0.61$ for Y, lower lobe: $r_I = 0.84$ for X and $r_I = 0.82$ for Y). In all analyses, general trends observed for upper and lower lobes were very similar. However, the data for upper lobes were noisier because magenta veins often appear in this part of the corolla. Thus, we only report results obtained using data from lower lobes. Note that veins are extremely thin and cannot be therefore detected by flying bees due to the poor spatial resolution of the compound eyes (Giurfa and Vorobyev 1997). Even recent measures reporting improved visual detection performances for bumblebees (Dyer et al. 2008) imply that veins would be only perceived at an extreme close-up, when the bee is practically on the flower. Thus, veins introduced noise in our measures only because the measuring device had to be placed directly on the petals but not because they are susceptible to affect bee choice at a distance.

In the bee colour perceptual space, plants from the hybrid zone occupied an area that was nearly as large as the area covered by plants from 15 *Antirrhinum* taxa, indicating that in the hybrid zone variation in flower colours as

perceived by bees is comparable to that found in the *Antirrhinum* lineage (Fig. 2). The colour of most hybrids was undistinguishable from the colour of at least one plant of the sampled *Antirrhinum* taxa. However, there were some notable exceptions, some phenotypes clearly falling outside the range of colours found in *Antirrhinum* taxa. When compared with the set of 1063 flower colours from 573 angiosperm species analyzed by Chittka et al. (1994), these outliers also appeared to fall into areas of the bee colour perceptual space where data points are relatively scarce (Fig. 3). Their loci were biased towards the right part of the hexagon, lying closer to the spectral line of this perceptual space. This closeness means that their spectral purity is high. Indeed, we found that they were significantly purer than the colours of *Antirrhinum* taxa for which we have data ($t = 5.84$, $df = 23.97$, $P < 0.001$, Fig. 4) and those of the 573 angiosperm species analyzed by Chittka et al. (1994; $t = 12.08$, $df = 10.59$, $P < 0.001$, Fig. 4).

Samples from the hybrid zone revealed nearly perfect continuity in flower colour between *A. m. pseudomajus* and *A. m. striatum*. In the clustering dendrogram (Fig. 5), all branch lengths were below 0.1 hexagon units, except one that was slightly above this value (0.105). This result indicates that the colour distance between clusters in the bee colour perceptual space was below or very close to the discrimination threshold at every clustering level. Therefore, no flower colours are likely to be discriminated from the most similar ones that can be found in the hybrid zone. At the scale of the hybrid zone, flower colour variation can thus be globally considered as a perceptual continuum from a bee's perspective.

To analyze the geographic structure of colour variation, we performed, in the neighbourhood of each plant, a clustering dendrogram of flower colour in the colour perceptual space and plotted the length of the longest branch against spatial distance across the hybrid zone (Fig. 6). In most cases, particularly in the centre of the hybrid zone, this length was above 0.1 hexagon units, meaning that at least one flower colour in the neighbourhood of the focal plant could be discriminated from all others. Flower colour therefore seems to be locally discontinuous in most places of the hybrid zone, and nearly everywhere in the central part of the hybrid zone. This pattern was consistent irrespective of the radius values used to select the set of neighbouring individuals around each focal plant (results not shown).

Discussion

Hybrid zones between animal-pollinated plant species have been studied extensively (e.g. Campbell et al. 1997; Weseloh and Arnold 2000). Most previous studies

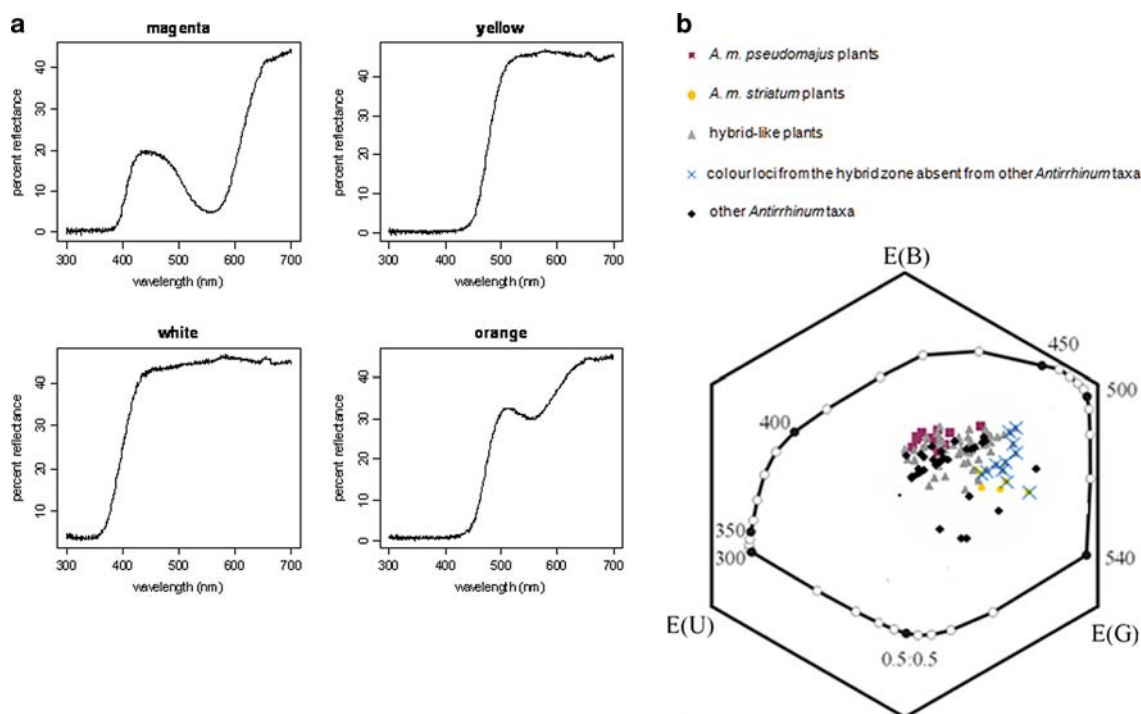


Fig. 2 (a) Examples of floral reflectance spectra for parental phenotypes ('magenta' and 'yellow') and two hybrid phenotypes greatly differing in their patterns of pigmentation ('white' and

'orange'). (b) Colour loci in the bee colour perceptual space. The spectral line (solid line) connects the loci of monochromatic lights (Chittka et al. 1994, Fig. 2)

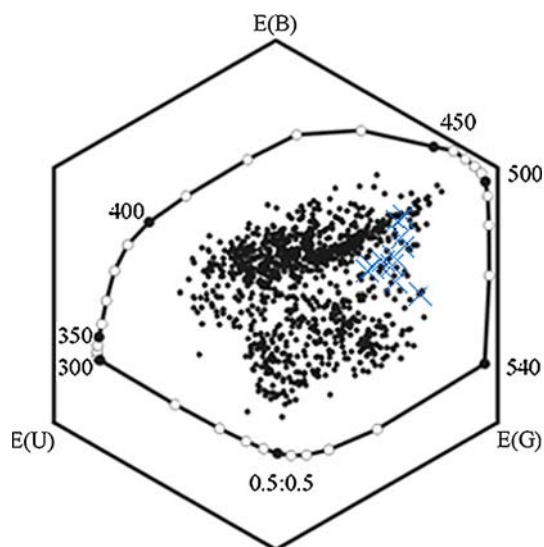


Fig. 3 Colour loci in the bee colour perceptual space. *Dots*: colour loci of 1063 flowers from 573 species (Chittka et al. 1994, Fig. 2). *Crosses*: colour loci from the hybrid zone which are absent from other *Antirrhinum* taxa. The spectral line (solid line) connects colour loci of monochromatic lights (Chittka et al. 1994, Fig. 2)

addressing the influence of pollinators on hybrid zones' dynamics were performed in arrays or common gardens with a limited number of hybrid phenotypes. Focusing on flower colour, our study analyzed (1) the full diversity of colour phenotypes observed in natural hybrid zones and (2)

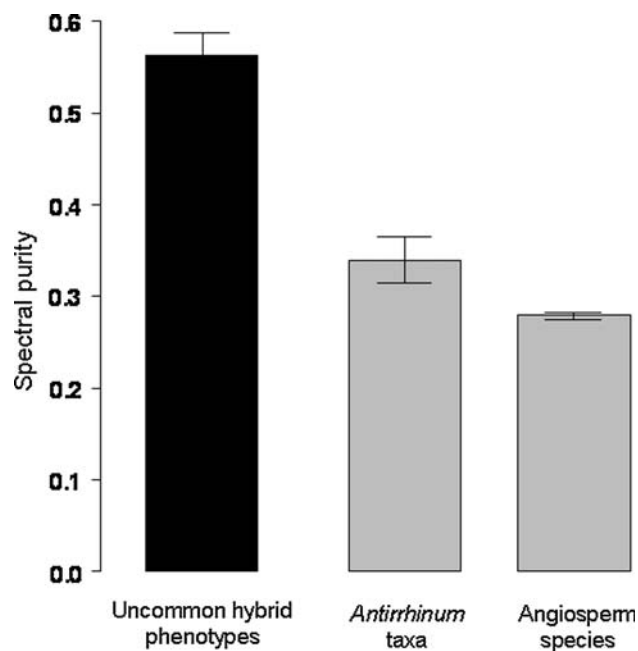


Fig. 4 Differences in spectral purity between plants from the hybrid zone whose flower colours were not found in other *Antirrhinum* taxa, other *Antirrhinum* taxa, and 573 angiosperm species analyzed by Chittka et al. (1994, Table 1). Data are mean values (\pm SE)

its functional significance in terms of plant-pollinator interactions. We found that, in the bee colour perceptual space, the range of flower colours encountered in an

Fig. 5 Dendrogram for clustering colour loci of plants from the hybrid zone. Branch lengths (in hexagon units) correspond to distances between clusters in the bee colour perceptual space. Asterisks indicate colour loci that were not found in other *Antirrhinum* taxa

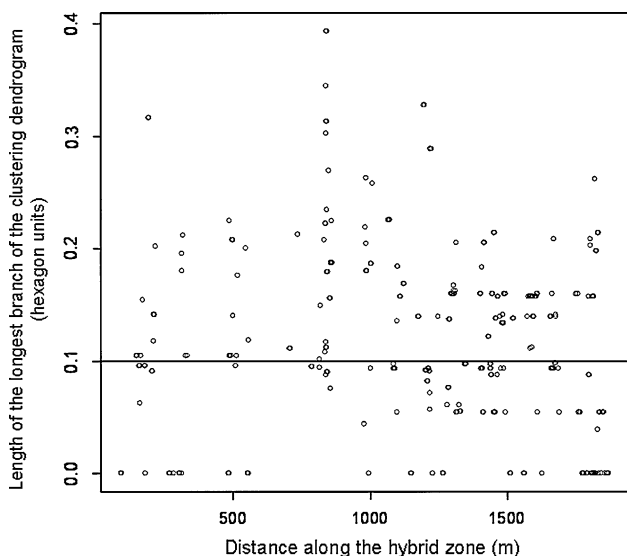
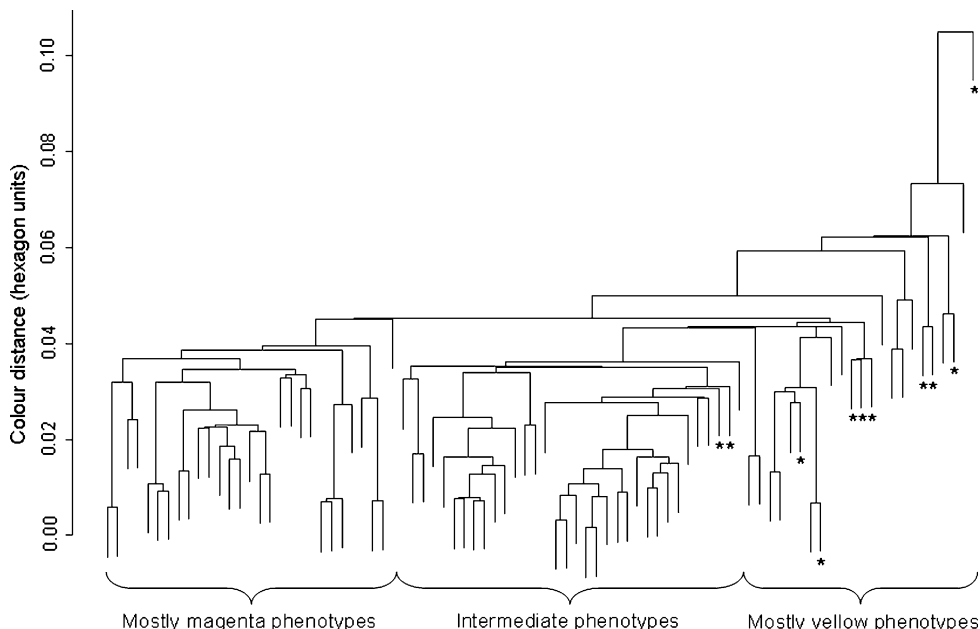


Fig. 6 Local flower colour discontinuity in the *Antirrhinum* hybrid zone. The *x* axis gives the position of focal plants along the hybrid zone transect (distance from the westernmost plant, in metres) whereas the *y* axis gives the length (in hexagon units) of the longest branch for the clustering dendrogram of colour loci found in the neighbourhood of the focal plant (within 6 m). The horizontal line represents the discrimination threshold (0.1 hexagon units). Points found above this line indicate a discontinuity in flower colour variation at a local scale

Antirrhinum hybrid zone was nearly as large as that estimated for the *Antirrhinum* lineage. Moreover, some of these colours were not observed in other *Antirrhinum* taxa and are also uncommon among angiosperms. This very large phenotypic diversity is the consequence of pigmentation gene segregation in hybrids, as shown by Whibley et al. (2006). Extreme hybrid flower colours have also been

found in tomato (Rick and Smith 1953), and in irises (Bouck et al. 2007). However, in all these studies, the functional significance of colour variation was never assessed.

Bee pollinators are often flower-constant and tend to forage on familiar floral types as long as they provide nectar and/or pollen reward (Chittka et al. 1999). If no such flower is available, they are more likely to generalize to a similar flower than to visit a more different one (Gumbert 2000). To what extent two individuals produce similar (or different) flowers depends on pollinator perception. Similarity can be assessed in several perceptual dimensions such as colours or odours but colours serve as detection and recognition signals at further spatial distances. Therefore, if pollinators are flower constant, hybrid plants displaying uncommon flower colours with respect to other neighbouring plants should receive fewer visits and might be poorly pollinated given the low generalization levels they would promote. It could be argued that such a pattern of visitations could result in less frequently visited flowers becoming more rewarding as time passes because these flowers will tend to accumulate nectar. This could entail a breakdown of floral constancy and allow increased gene flow between parental populations. However, the frequency of pollinator visitations appears to be low in the *Antirrhinum* hybrid zone and emptied flowers usually refill quickly (E. Tastard, unpublished data), making this mechanism unlikely.

Some hybrid flower colours had a particularly high spectral purity. This could be due to the formation, by recombination, of pigment combinations that are unusual in angiosperms, probably because of evolutionary constraints (Chittka et al. 1994). Since bees' photoreceptor set is

optimal for the discrimination of flower colours, flowers having such colour purity are however unlikely to be less conspicuous or discriminable than others for bee pollinators (Chittka 1997). In fact, high spectral purity could even increase flower attractiveness and make them more conspicuous to pollinators against spectrally less pure natural backgrounds such as green foliage or stone (Lunau et al. 1996). Such extreme hybrid colours may promote better learning and retention in pollinators because more salient cues are always better learned. Thus, hybrids with unusually pure colours could receive more visits of pollinators if such mechanisms were at play. However, two kinds of cost could temper these benefits. First, because high colour purity implies a higher concentration of pigments (Saks et al. 2003) and increased fitness of non-pigmented plants was observed in some environmental conditions (Atanassova et al. 1997; Warren and Mackenzie 2001), producing a corolla with highly pure colours may incur some fitness costs. Second, even if bumblebees are attracted by pure corolla colours, they apparently need an even purer and therefore contrasting colour in the coloured guides that on petals directly signal the presence of pollen or nectar reward within the flower (Lunau 1990). Consequently, flowers with pure corolla colours could be disadvantageous for the plant because coloured guides look less conspicuous to the bee against the corolla background. Such a reduction in the level of advertising may limit the pollinators' ability to find the rewarding site of the flower (Lunau 1990).

Hybridization can generate a set of colours that builds a continuum connecting rare hybrid colours to the most abundant parental ones. This seems to be the case in the hybrid zone described here because the number of hybrid colours is high and the difference between most similar hybrid colours is so low that they are undistinguishable for pollinators. In other words, flower colour variation appears continuous at the scale of the entire hybrid zone. Such continuum in flower colour could, in principle, favour generalization behaviour in bumblebees and favour gene mixing between parental taxa. However, at the spatial scale at which pollinators move from one plant to the next, particularly in the centre of the hybrid zone, there were often individual plants displaying colours that were distinguishable by bees from the colours displayed by their neighbours. Thus, at a local scale, such plants represent rare colour phenotypes. Because pollinators foraging on rewarding plants usually have a positive frequency-dependent behaviour (Smithson and Macnair 1996, 1997), these plants are likely to be discriminated against (Clegg and Durbin 2000).

A possible limitation in our study arises from the fact that, for many individual plants, we predicted locations in the bee colour perceptual space using visual scores. Even

though we showed that the uncertainty associated with our approach had little influence on the results of the study, spectrophotometer measurements of flower colours would be highly desirable in future studies of this sort when logistic constraints can be overcome.

In conclusion, even if colour variation is continuous at the scale of the whole hybrid zone, the characteristics of some hybrid colours and the local discontinuity perceived by pollinators suggest that a proportion of hybrids may suffer low pollination rate, which may contribute to the hybrid zone stability. These expectations are based on known bumblebees' behaviour. Behavioural experiments involving bumblebees and mixtures of parental and hybrid flower colours will be necessary to ascertain that our interpretation is correct. Further insights are likely to come from future ecological work on *Antirrhinum*, today one of the most promising model systems to dissect the interaction between floral morphology and pollinator behaviour (Whitney and Glover 2007).

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Annexe: List of *Antirrhinum* taxa used in the analysis

Antirrhinum australe
Antirrhinum barrelieri
Antirrhinum boissieri
Antirrhinum braun-blanquetii
Antirrhinum hispanicum
Antirrhinum latifolium
Antirrhinum majus cirhigerium
Antirrhinum majus litigosum
Antirrhinum meonanthum
Antirrhinum molle
Antirrhinum mollissimum
Antirrhinum pertegasii
Antirrhinum sempervirens
Antirrhinum siculum
Misopates calycinum

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Supplementary material: Accuracy assessment of the prediction of colour locations in the bee colour perceptual space based on human visual scoring

1- Comparison of the locations of plants with identical colour scores in the colour perceptual space

Plants for which no reflectance measurements were available were assigned the mean colour location obtained from plants with the same magenta and yellow human visual scores for which colour reflectance spectra were measured. In order to assess the accuracy of this assignment, we examined the variability in the colour location, using only the plants for which we had reflectance data, sorted by magenta and yellow scores. For each combination of magenta and yellow scores, we calculated the distances between the colour location of individual plants and the mean location of the plants of that combination. Short colour distances indicate that the prediction is rather accurate. Mean and standard deviation of the colour distances are shown for each combination of colour scores in Table 1.

Table1. Mean and standard deviation (in hexagon units) of the colour distances between each plant location and the mean location for each combination of colour scores. N indicates the number of colour distances. Mean colour distances exceeding the discrimination threshold value (0.1 hexagon units) are highlighted in grey.

magenta score	yellow score	mean	standard deviation	N
1	1	0.078	0.061	3
1	1.5	0.055	0.000	2
1	2	0.145	0.000	2
1	3	0.103	0.031	11
1.5	1	0.028	0.000	2
2	1	0.036	0.035	10
2	1.5	0.041	0.000	2
2	2	0.089	0.025	4
2	2.5	0.055	0.000	2
2	3	0.102	0.059	6
2.5	1	0.098	0.000	2
3	1	0.103	0.077	7
3	2	0.078	0.031	6
3	2.5	0.077	0.000	2
3	3	0.067	0.021	4
3.5	1	0.068	0.000	2
3.5	2.5	0.046	0.000	2
4	1	0.078	0.030	10
4	1.5	0.034	0.000	2
4	2	0.090	0.034	3
4	2.5	0.071	0.025	4
4.5	1	0.079	0.049	4
4.5	1.5	0.021	0.009	3
4.5	2	0.079	0.017	6
4.5	3	0.088	0.040	9
5	1	0.015	0.009	4

For most combinations of magenta and yellow scores, the mean colour distance between individual plant locations and the mean location was between 0.05 and 0.1 hexagon units, thus below the perception threshold of bees. The mean colour distance between individual plant locations and the mean location exceeded the threshold value for four combinations of colour scores (Table 1). In these cases, deviation above the threshold was rather low (0.002 to 0.003 hexagon units) in 3 combinations, but was higher in the last one

(magenta score: 1, yellow score: 2): 0.045 hexagon units. Thus, the prediction of colour locations was not accurate for this combination, perhaps in relation with the particular pigment patterning in this category of plants. However, only one plant having the corresponding colour scores had its location predicted in the study. Thus, the impact of this lack of accuracy on the results of the study must be very limited.

2- Simulation of new data sets taking into account the uncertainty of the prediction and assessment of the influence on the results of the study

We performed simulations to assess if the uncertainty of the prediction of plant locations in the colour perceptual space could have an influence on the results of our study. All the plants for which we had no reflectance data were assigned a new location in the colour perceptual space, taking into account the uncertainty of the prediction. We then performed analyses on the simulated data sets, and compared the results with those obtained with the original data set.

For each plant for which we had no reflectance data, instead of using solely the mean location of the corresponding colour score combination, we modelled the dispersion of colour locations around this mean by a 2D-Gaussian distribution to build our simulated data sets. Specifically, for each plant, we computed a random distance r to the mean location, sampled in a Gaussian distribution with the mean and the standard deviation calculated for the corresponding colour score combination (Table 1), and a random angle value θ , sampled between 0 and 2π radians in a uniform distribution. The location (X', Y') assigned to the plant was the point of the r -radius circle centred on the mean location (X, Y) of the corresponding combination of colour scores which made a θ angle with the X-axis:

$$X' = X + r \cdot \cos(\theta) \text{ and } Y' = Y + r \cdot \sin(\theta).$$

We performed 100 simulations. For each of them, we assessed the continuity of flower colour variation at the scale of the hybrid zone and in the neighbourhood of each plant. At the scale of the hybrid zone, we performed a clustering analysis for the loci in the colour perceptual space. The length of the longest branch was smaller than the value obtained with the original data set (0.105 hexagon units) in 82 out of 100 simulations and the length of the second longest branch was smaller than this value in 97 out of 100 simulations. Therefore, the results were equivalent to those obtained with the original data set in 82% of the simulations and similar to them, with nearly continuous colour variation, in 97% of the simulations. We then performed a clustering analysis in a 6-m radius circle around each plant. In all 100 simulations, the length of the longest branch was above the discrimination threshold (0.1 hexagon units) in the neighbourhood of 70 to 90% of the plants, compared to 58% with the original data set. Therefore, we found a higher level of discontinuity at a local scale in the simulations than in the original data set, indicating that our results are conservative; in fact, we may underestimate the level of local discontinuity in our study, but we certainly do not overestimate it. In conclusion, the results of the simulations show that the error associated with the prediction of plant locations in the colour perceptual space had no influence on the fundamental results of our study.