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Locally asymmetric introgressions between subspecies suggest circular range expansion at the *Antirrhinum majus* global scale

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genetic introgression;
haplotype sharing.

Abstract

Assessing processes of geographic expansion in contact zones is a crucial step towards an accurate prediction of the evolution of species genetic diversity. The geographic distribution of cytonuclear discordance often reflects genetic introgression patterns across a species geographic range. *Antirrhinum majus pseudomajus* and *A. m. striatum* are two interfertile subspecies that occupy nonoverlapping areas but enter in contact in many locations at the margin of their geographic distribution. We found that genetic introgression between both subspecies was asymmetric at the local scale and geographically oriented in opposite directions at both ends of their contact zone perimeter in the Pyrenees. Our results suggest that the geographic expansion of *A. majus* subspecies was circular around the perimeter of their contact zone and pinpoint the need to integrate different spatial scales to unravel complex patterns of species geographic expansion.

Introduction

Species range expansion is a key mechanism that shapes the genetic diversity of species (Hewitt, 2000; Excoffier *et al.*, 2009) and modifies their evolutionary potential (Lavergne & Molofsky, 2007; Pujol & Pannell, 2008; Pujol *et al.*, 2009). Range expansion frequently leads to the formation of contact zones between populations of differentiated species (Anderson, 1949; Grant, 1971; Arnold, 1997; Barton, 2001). In such cases, genes from a foreign species might replace genes of, and therefore introgress, the gene pool of the native species (Potts & Reid, 1988, 1990; Schemske & Morgan, 1990). Genetic introgression (i.e. the transfer of genetic material in the genome of another species) is often rendered possible by fertile hybrids that occupy the contact zones and act as 'bridges to gene flow', therefore allowing gene exchange between species to occur (Broyles, 2002). Recurrent pollen exchanges between species and recurrent backcrossing between hybrids and parental species are then likely to generate large geographic areas of genetic introgression inside and outside contact zones (Campbell *et al.*, 1998; Leebens-Mack & Milligan, 1998). The characterization of genetic introgression is a key step towards a better understanding of the expansion dynamics of species in contact and of the evolution of their diversity (Currat *et al.*, 2008; Excoffier *et al.*, 2009).

Recent work on *Antirrhinum majus* showed that floral trait segregation, in combination with pollinator behaviour, can explain, at least partly, the maintenance of flower colour polymorphism in one particularly narrow hybrid zone between *A. majus pseudomajus* and *A. m. striatum* subspecies (Whibley *et al.*, 2006; Tastard *et al.*, 2008). It is, however, currently unknown whether contact zones and gene exchanges between *A. m. pseudomajus* and *A. m. striatum* are widespread across the geographic range of both these subspecies. The general aim of our study is to understand the biogeography of these two interfertile subspecies. We expect that contact might be frequent across the species range if one subspecies progressively expands its range into the range

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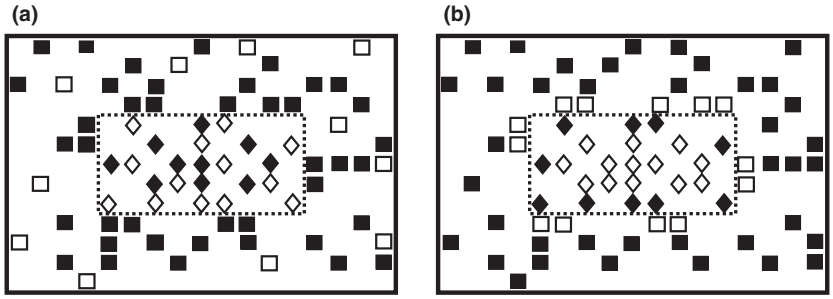

Fig. 1 Expected patterns of chloroplast sharing between species. Black and white colours represent Species 1 and Species 2, respectively. The contact zone perimeter between both species is symbolized by a dotted line. Squares represent the chloroplast haplotype 1 most often associated with Species 1, and diamonds represent the chloroplast haplotype 2 most often associated with Species 2. Under the hypothesis that black squares and white diamonds reflect the ancestral state, then the two less frequent associations (i.e. black diamonds and white squares) are called 'discordant'. (a) Random geographic distribution of discordant associations within each species range expected that results from the retention of ancestral polymorphism or convergence. (b) Geographic structure of discordant associations found only around the contact zone perimeter that results from local introgression between both species where genes can be exchanged.

occupied formerly by the other subspecies because the boundary between *A. majus* subspecies is not linear. *A. m. pseudomajus* is distributed around the range of *A. m. striatum*. Ultimately, moving boundaries sometimes result in the local replacement of the invaded species by the species pushing off the contact zone on its front of colonization (Buggs & Pannell, 2007; Pannell & Pujol, 2009). Alternatively, stable boundaries between both taxa can be maintained at equilibrium between migration and selection (Barton & Hewitt, 1989; Bull, 1991). When the contact zone between two species ranges is not linear, one could expect geographically complex expansion patterns and/or reciprocal gene exchanges to occur and result in multiple sites of genetic introgression. The detection of such widespread pattern is important because it might result in the long term in the genetic admixture of their formerly differentiated genomes. Evidence to determine whether taxa replacement, maintenance of species boundaries or admixture are the most likely evolutionary outcomes can be provided by the analysis of the geographic distribution of relict uniparentally inherited DNA (i.e. mitochondrial or chloroplastic DNA) where the nuclear genome is being replaced (Potts & Reid, 1988, 1990; Schemske & Morgan, 1990).

To establish whether parapatric boundaries between *A. majus* subspecies are moving following a complex geographic expansion pattern, we studied the geographic distribution of the association between chloroplast haplotypes (maternally inherited) and a nuclear gene (biparentally inherited) regulating the main taxonomic criterion, which is the magenta flower colour for *A. m. pseudomajus* and the yellow flower colour for *A. m. striatum* (see the study system section for details on the taxonomy of the species; Rothmaler, 1956 and Sutton, 1988) over the range of the species. We then searched for evidence of genetic introgression between *A. m. pseudomajus* and *A. m. striatum*. Although chloroplast haplotype sharing across taxa boundaries is often the outcome of genetic introgression (Rieseberg & Soltis, 1991; Wendel & Doyle, 1998; Linder & Rieseberg, 2004), caution must be taken when interpreting patterns of haplotype sharing because convergence or incomplete sorting of ancestral polymorphism might generate similar patterns (Muir & Schlotterer, 2005; Lexer *et al.*, 2006). In cases of retention of ancestral polymorphism or convergence, we would expect cytonuclear associations to be randomly distributed in a mosaic pattern over the species geographic range (Fig. 1a). In contrast, if chloroplast sharing between both subspecies is the result of introgression, we would expect discordant cytonuclear associations to be located close to the perimeter zone formed by the contact between subspecies (Fig. 1b). Geographic sectors characterized by the high frequency of one cytonuclear association are also expected if heterogeneous selection spatially structured *A. majus* ancestral polymorphism. In this article, we confront those hypotheses to establish the most likely scenario of evolutionary history that can explain the observed geographic distribution of cytonuclear associations in *A. majus*. Our investigation of those scenarios was rendered possible by the broad scale at which our study was conducted, i.e. the species geographic range, which allowed us to uncover the geographic direction of genetic introgression between both subspecies around their geographic boundaries.

Materials and methods

Study system

Antirrhinum majus (Scrophulariaceae) is a herbaceous short-lived perennial plant characterized by a patchy distribution in southern Europe. Its geographic distribution is centred over the Pyrenees, between north-eastern Spain and south-western France. The two subspecies *A. m. striatum* and *A. m. pseudomajus* occupy largely parapatric geographic regions. The geographic area

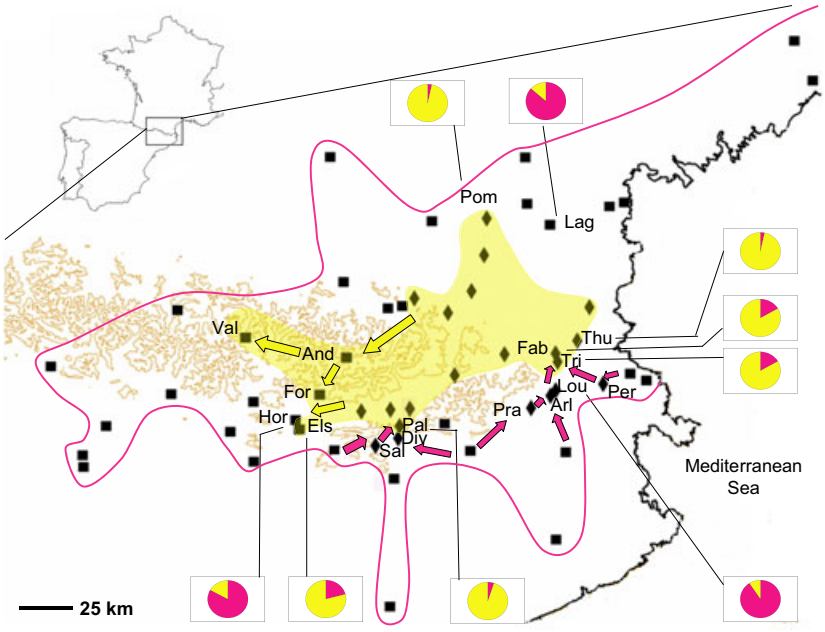

Fig. 2 Geographic distribution of *A. majus* cytonuclear associations. Magenta and yellow layers represent, respectively, *Antirrhinum majus pseudomajus* and *A. m. striatum* geographic ranges. Within each subspecies, black symbols represent populations sampled for this study. Squares and diamonds represent populations characterized by Haplotype I and Haplotype II, respectively. Pie charts are only presented for populations that are polymorphic at the *ROS1* locus. The magenta and the yellow proportion of the pie charts represent the respective frequencies of *ROS1-M* and *ROS1-Y* alleles in the population. Magenta and yellow arrows indicate the hypothetical scenario of range expansion followed by *A. m. pseudomajus* and *A. m. striatum* that is supported by our data. Brown lines represent elevation isoclines above 1800 m.

occupied by *A. m. striatum* is surrounded by the geographic area occupied by *A. m. pseudomajus* (Fig. 2). Taxonomic determination of *A. majus* subspecies is mostly based on the colour of flower corolla. *A. m. pseudomajus* is characterized by magenta flowers. It is referred to interchangeably in the literature as *A. m.* ssp. *majus* and *A. m.* ssp. *linkianum*. Some authors include the ssp. *cirrhigerum* as a variety of *A. m.* ssp. *linkianum*. *A. m. striatum* is characterized by yellow flowers. It is referred to interchangeably in the literature as *A. latifolium* ssp. *striatum*, *A. huetii* and *A. braun-blanquetii* (Rothmaler, 1956; Sutton, 1988). It is important to note that *A. m. pseudomajus* and *A. m. striatum* are interfertile and share pollinators (Whibley *et al.*, 2006; Andalo *et al.*, 2010).

Plant material sampling strategy

A total of 685 plants were sampled from 2002 to 2007 in 55 allopatric or parapatric populations distributed over the geographic range of the species. Geographic coordinates of populations were recorded by using a GPS device (Garmin, Olathe, KS, USA). A numerical scoring system was used to rank magenta and yellow flower colour phenotypes visually, following methods developed by Whibley *et al.* (2006). Obviously, plants that displayed yellow flowers were classified as *A. m. striatum* whereas plants that displayed magenta flowers were classified as *A. m. pseudomajus*. Population characteristics are summarized in Table S1a and S1b. For each individual, young leaves and shoot tips were collected and stored at −20 °C until DNA was extracted by using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany).

Molecular analyses

ROSEA genotyping

The *ROSEA* locus is made of 2 MYB – myeloblastosis – regulatory genes controlling floral pigmentation intensity, out of which *ROS1* has the main role in flower colour variation (Schwinn *et al.*, 2006). *ROS1* sequences can be grouped in three main haplotypes *ROS1*-Ma, *ROS1*-Mb and *ROS1*-Y (Whibley, 2004). *ROS1*-Ma and *ROS1*-Mb haplotypes are diagnostic of *A. m. pseudomajus* and are grouped under the name of *ROS1*-M whereas the *ROS1*-Y haplotype is diagnostic of *A. m. striatum* (Whibley, 2004). *ROS1* genotypic data were available for the 14 populations ($n = 166$ plants) that were previously examined by Whibley *et al.* (2006). We obtained *ROS1* genotypic data for the remaining 41 populations ($n = 519$ plants) using the RG4/RR21, RG6/RR21 and RG1/RR21 primers in a single PCR, following the protocol established by Whibley (2004).

PCR–RFLP analysis of chloroplast DNA

Maternal lineages were determined in the 55 populations ($n = 685$ plants) by genotyping the 1.6-kb *psbC* [*psII*

44-kDa protein] – *trnS* [*tRNA-Ser*(*UGA*)] intergenic region, using the CS universal primers (Demesure *et al.*, 1995). Sequencing of this chloroplast region revealed two haplotypes that differed at two SNP loci, one of which was included in a *MseI* restriction site. We therefore obtained two different haplotypes after digestion of the *psbC-trnS* fragment by the *Mse I* enzyme. Haplotype I was characterized by eight *Mse I* restriction sites that generated a nine-band profile on agarose gel. Haplotype II was characterized by a 10-band profile. The PCR amplification protocol is presented in the supplementary online material.

Data analyses

To examine cytonuclear associations, we calculated *ROS1* allelic frequencies and chloroplast haplotype frequencies within each population and mapped these frequencies using ArcGis (ESRI, Redlands, CA, USA) software. To determine whether subspecific patterns of chloroplast haplotype sharing were the result of evolutionary convergence, incomplete sorting of ancestral polymorphism or introgression, we assessed the role of the geographic distance between populations of different subspecies on the geographic distribution of chloroplast haplotypes. To do so, we calculated the Euclidian geographic distance to the closest population of the other subspecies for every population within each subspecies. We then tested whether this geographic distance differed between populations that share chloroplast haplotypes with the other subspecies and populations that do not share chloroplast haplotypes with the other subspecies. We performed a two-sample *t*-test built on the basis of 2000 permutations (Good, 2000) in R (R Development Core Team, 2007, Vienna, Austria).

Results

Relationship between flower colour and *ROS1* genotype

At the population level, 67% of the 55 populations were assigned to *A. m. pseudomajus* and 33% to *A. m. striatum* on the basis of their flower colour phenotype (n = 37 *A. m. pseudomajus* populations and n = 18 *A. m. striatum* populations). Forty-six populations out of 55 were monomorphic at the *ROS1* locus. All plants in those populations presented the same homozygote genotype at the *ROS1* locus, being either *ROS1-M/ROS1-M* in 34 *A. m. pseudomajus* populations or *ROS1-Y/ROS1-Y* in 12 *A. m. striatum* populations (Table 1). Six *A. m. striatum* populations (*Els, Fab, Pal, Pom, Thu* and *Tri*) and three *A. m. pseudomajus* populations (*Hor, Lag* and *Lou*) were polymorphic at the *ROS1* locus (allelic frequencies are presented on Fig. 2).

Over all individuals, plants that displayed yellow flowers were characterized by the genotypes *ROS1-Y/ROS1-Y, ROS1-Y/ROS1-M* or *ROS1-M/ROS1-M* at the respective frequencies of 94%, 4.5% and 1.5%. Plants that displayed magenta flowers were characterized by the genotypes *ROS1-Y/ROS1-Y, ROS1-Y/ROS1-M* or *ROS1-M/ROS1-M* at respective frequencies of 0.8%, 2.2% and 97%. Such correlation between *ROS1-Y* and the yellow colour and between *ROS1-M* and the magenta colour is in agreement with the previous study conducted by Whibley *et al.* (2006).

Distribution of chloroplast DNA genotypes

Each particular population was characterized by a unique *psbC-trnS* chloroplast haplotype. The geographic distribution of population haplotypes was nonoverlapping across the geographic range of the species (Fig. 2). Haplotype I was found in 79% of *A. m. pseudomajus* populations and in 20% of *A. m. striatum* populations. Haplotype II was found in the remaining 21% of *A. m. pseudomajus* populations and 80% of *A. m. striatum* populations (Table 1).

Among *A. m. pseudomajus* populations, the chloroplast haplotype depended significantly on whether populations were located closely to *A. m. striatum* populations. Most of the *A. m pseudomajus* populations that were characterized by Haplotype I were distant from *A. m. striatum* populations (Fig. 2). In contrast, *A. m. pseudomajus* characterized by Haplotype II could only be found in populations located closely to the contact zone perimeter. The mean distance between *A. m. pseudomajus* popula-

Table 1 Cytonuclear associations.

Chloroplast haplotype	Population subspecies	ROS-1 genotypes
Haplotype I (35)	*Antirrhinum majus pseudomajus* (31)	**ROS1-M/ROS1-M** (29)
		ROS1-M/ROS1-M; ROS1-M/ROS1-Y; ROS1-Y/ROS1-Y (2)
	A. m. striatum (4)	**ROS1-Y/ROS1-Y** (3)
		ROS1-M/ROS1-Y; **ROS1-Y/ROS1-Y** (1)
Haplotype II (20)	*A. m. pseudomajus* (6)	**ROS1-M/ROS1-M** (5)
		ROS1-M/ROS1M; ROS1-M/ROS1-Y (1)
	A. m. striatum (14)	**ROS1-Y/ROS1-Y** (9)
		ROS1-M/ROS1-M; ROS1-M/ROS1-Y; **ROS1-Y/ROS1-Y** (5)

*Most frequent genotype in bold. The number of populations is indicated between parentheses.

tions characterized by Haplotype I and the closest *A. m. striatum* population (mean distance ± SD = 40.3 ± 30.1 km) was significantly larger (using a permutation *t*-test $P < 0.05$) than the mean distance between *A. m. pseudomajus* populations characterized by Haplotype II and the closest *A. m. striatum* population (mean distance ± SD = 12.9 ± 5.7 km). Within the *A. m. striatum* geographic range, no correlation between the occurrence of a chloroplast haplotype and the distance to the nearest *A. m. pseudomajus* population was detected. The mean distance between *A. m. striatum* populations characterized by Haplotype II and the closest *A. m. pseudomajus* population (mean distance ± SD = 16.6 ± 7.0 km) was not significantly different (using a permutation *t*-test $P > 0.05$) than the mean distance between *A. m. striatum* populations characterized by Haplotype I and the closest *A. m. pseudomajus* population (mean distance ± SD = 18.3 ± 11.1 km). It is important to note that the few *A. m. striatum* populations characterized by chloroplast Haplotype I ($n = 4$) were all grouped on the west border of *A. m. striatum* geographic distribution (see Fig. 2). It is also important to note that such analysis in *A. m. striatum* was limited by the small number of *A. m. striatum* populations that are located far from the contact zone perimeter, which is a direct consequence of the narrower geographic area occupied by *A. m. striatum*. Furthermore, *A. m. pseudomajus* populations characterized by Haplotype II were located in the east of the contact zone perimeter whereas *A. m. striatum* populations characterized by Haplotype I were located in the west of the contact zone perimeter (Fig. 2). The direction of the geographic gradient formed by *ROS1* allele frequencies in the east was different from the one in the west of the contact zone.

Cytonuclear association

Because of the correlation between *ROS1* and flower colour, the overall pattern of cytonuclear association was very similar to the pattern presented above. Most of the *A. majus* populations were characterized either by the association of chloroplast Haplotype I and the *ROS1*-M allele or by the association of chloroplast Haplotype II and the *ROS1*-Y allele. Among populations characterized by Haplotype I, most of them (83%) were also characterized by the fixation of the *ROS1*-M allele whereas the remaining populations were characterized either by polymorphism at the *ROS1* locus (8.5%) or by the fixation of the *ROS1*-Y (8.5%). Among populations characterized by Haplotype II, only 45% were characterized by the fixation of the *ROS1*-Y allele whereas the other populations were characterized either by polymorphism at the *ROS1* locus (30%) or by the fixation of the *ROS1*-M allele (25%) (Table 1).

In most of the *A. m. pseudomajus* populations ($n = 29$ out of 37), all individuals were characterized by the cytonuclear association of chloroplast Haplotype I and *ROS1*-M. This includes all the *A. m. pseudomajus* populations that were distant from the contact zone perimeter (Fig. 2). The most frequent cytonuclear association that characterized *A. m. striatum* populations was found in 50% of *A. m. striatum* populations ($n = 9$). In those populations, all individuals were characterized by the same cytonuclear association (chloroplast Haplotype II and *ROS1*-Y). Around the contact zone perimeter (see Fig. 2), we found five *A. m. pseudomajus* populations (*Arl*, *Div*, *Per*, *Pra* and *Sal*) where all individuals were characterized by the cytonuclear association of chloroplast Haplotype II and *ROS1*-M. Those populations were located at the eastern side of the contact zone perimeter (Fig. 2). Around the contact zone perimeter, we also found three *A. m. striatum* populations (*And*, *For* and *Val*) where all individuals were characterized by the association of the chloroplast Haplotype I and *ROS1*-Y. Those populations were located at the western side of the contact zone perimeter (Fig. 2).

In two populations of the three *A. m. pseudomajus* populations that were polymorphic at the *ROS1* locus, Haplotype I was associated with a high frequency of *ROS1*-M. Similarly, in five populations of the six *A. m. striatum* populations that were polymorphic at the *ROS1* locus, Haplotype II was associated with a high frequency of *ROS1*-Y alleles (Table 1). Interestingly, such populations at an intermediary stage of genetic introgression were always very close to the contact zone perimeter (Fig. 2).

Discussion

Heterogeneous selection of ancestral polymorphism vs. genetic introgression

One hypothesis explaining that chloroplast haplotypes are shared between *A. m. pseudomajus* and *A. m. striatum* is that such pattern results from the retention of ancestral polymorphism without selection being involved. Under such scenario, we would expect cytonuclear associations to be widespread across the entire range of *A. majus* (Fig. 1). This was however not the case. We found them to be grouped in four discrete geographic areas. We therefore discarded this hypothesis (Fig. 2). Another hypothesis that can be invoked is that local heterogeneous selection is responsible for the geographic distribution of the four cytonuclear associations in four discrete geographic sectors in the absence of interspecific introgression. Under such scenario, natural selection would have differently advantaged four ancestral cytonuclear associations between the chloroplast Haplotypes I and II and *ROS1* alleles in four regions. In populations located between those four regions where cytonuclear associations were fixed, we found populations that were polymorphic for *ROS1* alleles but not for chloroplast haplotypes (Fig. 2). These polymorphic populations formed geographically orientated gradients in *ROS1* allele

frequencies that were all located onto the contact zone perimeter between *A. m. pseudomajus* and *A. m. striatum*. Gradients were found on the east side and on the west side of the contact zone perimeter. Because the contact zone perimeter is where we expect gene exchanges between both subspecies to occur, the geographic distribution of chloroplast haplotypes between subspecies and the gradients of *ROS1* allele frequencies that we found in the contact zone perimeter are more likely reflecting genetic introgression between subspecies than geographically heterogeneous selection on cytonuclear ancestral polymorphism.

Local patterns of genetic introgression reflect a circular range expansion scenario

The geographic distribution of cytonuclear associations suggests that chloroplast Haplotype I was historically associated with *A. m. pseudomajus*. This is because chloroplast Haplotype I was more frequent in *A. m. pseudomajus* populations, especially those that were geographically isolated from *A. m. striatum* populations (i.e. allopatric populations) whereas Haplotype II was only found in *A. m. pseudomajus* populations located in the contact zone perimeter (i.e. parapatric populations). The distribution of chloroplast Haplotypes was less strikingly structured among *A. m. striatum* populations. It would seem nevertheless logical, in regard of Haplotypes I and II distribution in *A. m. pseudomajus*, that Haplotype II was historically associated with *A. m. striatum*. Under the assumption that Haplotype I and Haplotype II were originally associated specifically with *A. m. pseudomajus* and *A. m. striatum*, respectively, the geographic distribution of subspecies, chloroplast haplotypes and nuclear *ROS1* alleles revealed areas of cytonuclear discordance. In those areas, chloroplast haplotypes were not associated with the expected subspecies. This was the case on the east side of the contact zone perimeter for six *A. m. pseudomajus* populations characterized by chloroplast Haplotype II and a high frequency of *ROS1*-M alleles that had often reached fixation. This is probably because *A. m. striatum* plants were previously occupying the sites where those *A. m. pseudomajus* populations are nowadays found. Historically, those six populations were probably displaying yellow flowers and were characterized by matching chloroplast nuclear genotypes, i.e. Haplotype II and *ROS1*-Y. It is plausible that cytonuclear discordance emerged because nuclear genes of foreign populations were dispersed and introgressed the gene pool of local populations. The exact inverse scenario can be observed on the west side of the contact zone perimeter in four populations of *A. m. striatum*, which habitat was probably occupied previously by *A. m. pseudomajus* populations. Such geographic distribution of cytonuclear associations could be interpreted as reflecting asymmetric introgression between subspecies at a local scale, i.e. unidirectional introgression of *ROS1* alleles of one subspecies into the gene pool of the second subspecies. At the broad scale of the species geographic distribution, such directional genetic introgression, however, appeared to be inverted between the east side and the west side of the contact zone perimeter. Because the genotype at the *ROS1* locus determines whether a plant belongs to *A. m. pseudomajus* or to *A. m. striatum*, the spread of *ROS1* alleles reflects the spread of the corresponding subspecies. Our results therefore reflect a progressive shift in the geographic range of both *A. m. pseudomajus* and *A. m. striatum*. Under such scenario, both subspecies expanded and/or still expand their ranges in opposite directions on the east and the west side of the contact zone perimeter, which ultimately results in their global range expansion being articulated around each other into a circular pattern (Fig. 2).

The relative role of selection and dispersal in the spread of *ROS1* alleles

Either selection or dispersal can generate and maintain genetic introgression patterns, such as those detected in our study (Currat *et al.*, 2008). Local selection might explain the local asymmetry in the introgression pattern, even in the presence of bidirectional gene flow. In such case, we would expect cytonuclear discordant associations 'Haplotype II/*ROS1*-M' and 'Haplotype I/*ROS1*-Y' to provide a selective advantage, respectively, on the east side and on the west side of the contact zone perimeter. When patterns of introgression are asymmetric, they might result from intrinsic attributes of species, such as prezygotic asymmetric barriers [e.g. asymmetric pollen-style incompatibilities (Cruzan & Arnold, 1994)], sex-biased dispersal (Petit *et al.*, 2003) or post-zygotic asymmetric barriers [e.g. partial hybrid sterility (Shuker *et al.*, 2005), which are commonly attributed to cytonuclear interactions (Levin, 1971; Tiffin *et al.*, 2001)]. The hypothesis of one subspecies having an intrinsic advantage over the other subspecies when introgressing a foreign gene pool can be discarded because reciprocal patterns of introgressive hybridization between subspecies were detected on the west and the east side of the contact zone perimeter. Our results bring evidence that genes of each subspecies have the potential to introgress the other subspecies. They therefore corroborate the absence of intrinsic post-pollination barriers to reproduction between both subspecies previously found in an experimental study by Andalo *et al.* (2010). Local selection might also be driven by extrinsic factors. Environmental conditions might exert selective pressures on the *ROS1* locus that vary between regions where genetic introgression was found. Such selective pressures might also target nuclear genes that are linked with ROSEA. We acknowledge the limits of our genetic assay based only on the single-locus ROSEA, which is responsible for the taxonomic criterion determining to which subspecies a plant belongs. Investigating more markers would bring

a more complete picture about the extent of genetic introgression between both subspecies and would be informative on the role played by local selection on the spread of *ROS1* alleles. Local asymmetric introgression patterns might also be explained by recurrent unidirectional gene exchanges. Because asymmetric introgression was restricted to specific geographic areas, local environmental barriers to gene flow (valleys, mountains, etc.) might be responsible for local unidirectional gene flow. Our study therefore calls for testing whether specific environmental or physical conditions on each side of the contact zone might exert directional constraints to gene flow. Finally, biotic interactions might also be involved in the spread of *ROS1* alleles at the local scale. Experimental pollination studies brought evidence of a constancy phenomenon in the pollinating behaviour of bumblebees that was driven by *A. majus* flower colour, i.e. pollinators visited preferentially the same morph during a foraging sequence (Jones & Reithel, 2001; Tastard, 2009). Such pollinator behaviour was already shown to affect the evolution of a floral trait coded by a single locus (Jones & Reithel, 2001). In our case, such behaviour might result in positive frequency-dependent selection on flower colour that would ultimately reinforce or accelerate the spread of *ROS1* alleles. Such process would counteract the spread of rare variants in a population and is therefore not expected to be at the origin of the asymmetric introgression of *ROS1* alleles. It might, however, participate to the fixation of a new variant in a population that is submitted to massive unidirectional gene flow from the other subspecies.

Cytonuclear discordance as a result of pollen flow

Genetic introgression patterns such as those detected between *A. m. pseudomajus* and *A. m. striatum* are a common outcome when invading populations can spread their nuclear genes at a long distance by means of pollen flow and seed dispersal is limited (Petit *et al.*, 2003). Such hypothesis is not exclusive because the geographic distribution of cytonuclear associations that we observed could also be explained by demographic expansion through seed dispersal. In such case, the demographic imbalance between invaders and residents would result in the asymmetric introgression of genes from the resident species genome into the invader genome (Currat *et al.*, 2008). Dispersal characteristics of *A. majus*, however, bring support to the first hypothesis, i.e. genetic introgression by pollen flow. Indeed, *A. majus* seeds are very small and light [<15 mg (Andalo *et al.*, 2010)] and can mostly be dispersed at a short distance of the maternal plant by gravity. In contrast, *A. majus* pollen is transported by bumblebees (several *Bombus* species) and carpenter bees (*Xylocopa* sp.) and is therefore likely to migrate across long distances (Whibley, 2004). Indeed, distance covered by carpenter bees of the species *Xylocopa violacea* can reach 1.2 km (Molitor, 1937) whereas bumblebees of the species *Bombus terrestris* can cover up to 2.8 km (Chapman *et al.*, 2003; Darvill *et al.*, 2004). In the light of such dispersal characteristics, the geographic scale at which we observed the signature of genetic introgression reinforces our view that the spread of nuclear genes across subspecies boundaries in *A. majus* was/is progressive. Such progressive spread certainly involved populations, either disappeared or still present, that were separated by close distances suitable for pollinator browsing. Such populations would then play the role of a relay for pollinators and act as directional bridges to gene flow.

Conclusion

Documented examples of species geographic expansion in a contact zone generally imply a unique geographic direction at the scale of the species (Martinsen *et al.*, 2001; Rohwer *et al.*, 2001; Melo-Ferreira *et al.*, 2005). Here, we found that *A. m. pseudomajus* invaded what was previously the habitat of *A. m. striatum* by expanding its range northward on the east side of the contact zone perimeter whereas *A. m. striatum* expanded its range southward within the initial habitat of *A. m. pseudomajus* on the west side of the contact zone perimeter. Both subspecies appear thus to replace each other in a rotation movement at the scale of the species geographic range. Ultimately, this circular mode of geographic expansion might result in the global admixture of both subspecies nuclear genomes. Evolutionary consequences of genetic admixture in *A. majus* might therefore be expected to influence the evolutionary dynamics of the species at a global scale. This system, because it integrates reciprocal gradients of range expansion and genetic admixture in the two subspecies, constitutes a unique opportunity to evaluate their relative impact on the evolutionary potential of a species. It was possible to detect this surprising geographic pattern because we evaluated the geographic distribution of few but spatially structured chloroplastic and nuclear loci in multiple populations from geographically distinct sectors of the whole contact zone perimeter between *A. m. pseudomajus* and *A. m. striatum*. Our study therefore reinforces the current view that direction and speed of hybrid zone displacement can vary across replicates (Hairston *et al.*, 1992; Britch *et al.*, 2001; Buggs & Pannell, 2007). It also pinpoints the need to take into account multiple sites when studying contact zones between species because a broad geographic scope might reveal different patterns than those observed at a local scale. Indeed, focusing on a restricted area of the contact zone might shed light on species geographic range expansion patterns that are not representative of the whole species expansion dynamics.

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References

Andalo, C., Cruzan, M.B., Cazettes, C., Pujol, B., Burrus, M. & Thebaud, C. 2010. Post-pollination barriers do not explain the persistence of two distinct *Antirrhinum* subspecies with parapatric distribution. *Plant Syst. Evol.* **286**: 223–234.

Anderson, E. 1949. *Introgressive Hybridization*. Whiley & Sons, New York.

Arnold, M.L. 1997. *Natural Hybridization and Evolution*. Oxford University Press, Oxford.

Barton, N.H. 2001. The role of hybridization in evolution. *Mol. Ecol.* **10**: 551–568.

Barton, N.H. & Hewitt, G.M. 1989. Adaptation, speciation and hybrid zones. *Nature* **341**: 497–503.

Britch, S.C., Cain, M.L. & Howard, D.J. 2001. Spatio-temporal dynamics of the *Allonemobius fasciatus-A. socius* mosaic hybrid zone: a 14-year perspective. *Mol. Ecol.* **10**: 627–638.

Broyles, S.B. 2002. Hybrid bridges to gene flow: a case study in milkweeds (*Asclepias*). *Evolution* **56**: 1943–1953.

Buggs, R.J.A. & Pannell, J.R. 2007. Ecological differentiation and diploid superiority across a moving ploidy contact zone. *Evolution* **61**: 125–140.

Bull, C.M. 1991. Ecology of parapatric distributions. *Annu. Rev. Ecol. Syst.* **22**: 19–36.

Campbell, D.R., Waser, N.M. & Wolf, P.G. 1998. Pollen transfer by natural hybrids and parental species in an *Ipomopsis* hybrid zone. *Evolution* **52**: 1602–1611.

Chapman, R.E., Wang, J. & Bourke, A.F.G. 2003. Genetic analysis of spatial foraging patterns and resource sharing in bumble bee pollinators. *Mol. Ecol.* **12**: 2801–2808.

Cruzan, M.B. & Arnold, M.L. 1994. Assortative mating and natural selection in an iris hybrid zone. *Evolution* **48**: 1946–1958.

Currat, M., Ruedi, M., Petit, R.J. & Excoffier, L. 2008. The hidden side of invasions: massive introgression by local genes. *Evolution* **62**: 1908–1920.

Darvill, B., Knight, M.E. & Goulson, D. 2004. Use of genetic markers to quantify bumblebee foraging range and nest density. *Oikos* **107**: 471–478.

Demesure, B., Sodzi, N. & Petit, R.J. 1995. A set of universal primers for amplification of polymorphic noncoding regions of mitochondrial and chloroplast DNA in plants. *Mol. Ecol.* **4**: 129–131.

Excoffier, L., Foll, M. & Petit, R.J. 2009. Genetic consequences of range expansions. *Annu. Rev. Ecol. Evol. Syst.* **40**: 481–501.

Good, P. 2000. *Permutation Tests*. Springer, New York.

Grant, V. 1971. *Plant Speciation*. Columbia University Press, New York.

Hairston, N.G., Wiley, R.H., Smith, C.K. & Kneidel, K.A. 1992. The dynamics of 2 hybrid zones in Appalachian salamanders of the genus *Plethodon*. *Evolution* **46**: 930–938.

Hewitt, G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405**: 907–913.

Jones, K.N. & Reithel, J.S. 2001. Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *Am. J. Bot.* **88**: 447–454.

Lavergne, S. & Molofsky, J. 2007. Increased genetic variation and evolutionary potential drive the success of an invasive grass. *Proc. Natl. Acad. Sci. USA* **104**: 3883–3888.

Leebens-Mack, J. & Milligan, B.G. 1998. Pollination biology in hybridizing *Baptisia* (*Fabaceae*) populations. *Am. J. Bot.* **85**: 500.

Levin, D.A. 1971. The origin of reproductive isolating mechanisms in flowering plants. *Taxon* **20**: 91–113.

Lexer, C., Kremer, A. & Petit, R.J. 2006. Shared alleles in sympatric oaks: recurrent gene flow is a more parsimonious explanation than ancestral polymorphism. *Mol. Ecol.* **15**: 2007–2012.

Linder, C.R. & Rieseberg, L.H. 2004. Reconstructing patterns of reticulate evolution in plants. *Am. J. Bot.* **91**: 1700–1708.

Martinsen, G.D., Whitham, T.G., Turek, R.J. & Keim, P. 2001. Hybrid populations selectively filter gene introgression between species. *Evolution* **55**: 1325–1335.

Melo-Ferreira, J., Boursot, P., Suchentrunk, F., Ferrand, N. & Alves, P.C. 2005. Invasion from the cold past: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia. *Mol. Ecol.* **14**: 2459–2464.

Molitor, A. 1937. Zur vergleichenden Psychobiologie der akuleaten Hymenopteren auf experimenteller Grundlage. *Biologia Generalis.* **13**: 294–333.

Muir, G. & Schlotterer, C. 2005. Evidence for shared ancestral polymorphism rather than recurrent gene flow at microsatellite loci differentiating two hybridizing oaks (*Quercus* spp.). *Mol. Ecol.* **14**: 549–561.

Pannell, J.R. & Pujol, B. 2009. The paradoxical spread of a new Y chromosome – a novel explanation. *Trends Ecol. Evol.* **24**: 59–63.

Petit, R.J., Bodenes, C., Ducousso, A., Roussel, G. & Kremer, A. 2003. Hybridization as a mechanism of invasion in oaks. *New Phytol.* **161**: 151–164.

Potts, B.M. & Reid, J.B. 1988. Hybridization as a dispersal mechanism. *Evolution* **42**: 1245–1255.

Potts, B.M. & Reid, J.B. 1990. The evolutionary significance of hybridization in *Eucalyptus*. *Evolution* **44**: 2151–2152.

Pujol, B. & Pannell, J.R. 2008. Reduced responses to selection after species range expansion. *Science* **321**: 96.

Pujol, B., Zhou, S.R., Vilas, J.S. & Pannell, J.R. 2009. Reduced inbreeding depression after species range expansion. *Proc. Natl. Acad. Sci. USA* **106**: 15379–15383.

Rieseberg, L.H. & Soltis, D.E. 1991. Phylogenetic consequences of cytoplasmic gene flow in plants. *Evol. Trends Plants* **5**: 65–84.

Rohwer, S., Bermingham, E. & Wood, C. 2001. Plumage and mitochondrial DNA haplotype variation across a moving hybrid zone. *Evolution* **55**: 405–422.

Rothmaler, W. 1956. *Taxonomische monographie der Gattung Antirrhinum*. Akademie-Verlag, Berlin.

Schemske, D.W. & Morgan, M.T. 1990. The evolutionary significance of hybridization in eucalyptus. *Evolution* **44**: 2150–2151.

Schwinn, K., Venail, J., Shang, Y.J., Mackay, S., Alm, V., Butelli, E. *et al.* 2006. A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *Plant Cell* **18**: 831–851.

Shuker, D.M., Underwood, K., King, T.M. & Butlin, R.K. 2005. Patterns of male sterility in a grasshopper hybrid zone imply

accumulation of hybrid incompatibilities without selection. *Proc. Biol. Sci.* **272**: 2491–2497.
Sutton, D.A. 1988. *A revision of the Tribe Antirrhineae*. British Museum (Natural History), London and New York.
Tastard, E. 2009. *Maintien d'une zone hybride de gueules de loup (Antirrhinum majus): role de quelques intéractions biologiques*. PhD dissertation of the University of Toulouse Paul Sabatier, Toulouse.
Tastard, E., Andalo, C., Giurfa, M., Burrus, M. & Thebaud, C. 2008. Flower colour variation across a hybrid zone in *Antirrhinum* as perceived by bumblebee pollinators. *APIS* **2**: 237–246.
Tiffin, P., Olson, M.S. & Moyle, L.C. 2001. Asymmetrical crossing barriers in angiosperms. *Proc. R. Soc. Lond. B Biol. Sci.* **268**: 861–867.
Wendel, J. & Doyle, J. 1998. *Phylogenetic Incongruence: Window into Genome History and Molecular Evolution in Molecular Systematics of Plants*. Kluwer Academic Publishers, Boston.
Whibley, A.C. 2004. *Molecular and Genetic Variation Underlying the Evolution of Flower Colour in Antirrhinum*. PhD dissertation of the University of East Anglia, Norwich.
Whibley, A.C., Langlade, N.B., Andalo, C., Hanna, A.I., Bangham, A., Thebaud, C. *et al.* 2006. Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science* **313**: 963–966.

Supporting information

Additional Supporting Information may be found in the online version of this article:
Table S1a *Antirrhinum majus pseudomajus* population characteristics.
Table S1b *Antirrhinum majus striatum* population characteristics.

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