

Do floral odor profiles geographically vary with the degree of specificity for pollinators? Investigation in two sapromyphilous *Arum* species (Araceae)

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Abstract. We compared floral odour profiles among populations of two *Arum* species which show different degrees of specificity for their fly pollinators. Insects were collected from inflorescences in four populations of *Arum italicum* and two populations of *Arum maculatum*. In six *Arum* populations, we compared inflorescences odour profiles collected by Solid Phase Micro Extraction (SPME) and analysed by gas chromatography. We confirmed that from a pollination point of view, *A. italicum* is an opportunist species, as it is mainly pollinated by insects of the families Psychodidae, Chironomidae and Sciaridae, whereas *A. maculatum* is a specialist species, as it is 90% pollinated by Psychodidae. In all populations, *Arum italicum* was less attractive to pollinators than *Arum maculatum*. Floral odour profiles of *A. italicum* were not geographically structured among populations, suggesting a high gene flow or adaptation to a fluctuant guild of pollinators. On the contrary, odour profiles of *A. maculatum* varied between the two populations studied suggesting a lower gene flow or adaptation to different local pollinator preferences

Résumé. Les profils d'odeurs florales varient-ils géographiquement avec le degré de spécificité pour les pollinisateurs ? Recherche sur deux espèces d'*Arum* (Araceae) sapromyophiles. Nous avons comparé les profils d'odeurs florales de deux espèces d'*Arum* qui présentent différents degrés de spécificité pour leurs mouches pollinisatrices. Les insectes ont été collectés dans les inflorescences de quatre populations d'*Arum italicum* et deux d'*Arum maculatum*. Dans ces six populations d'*Arum*, nous avons comparé les profils d'odeur d'inflorescence récoltés par micro extraction sur phase solide (SPME) et analysés par chromatographie en phase gazeuse. Nous avons confirmé que, du point de vue de la pollinisation, *A. italicum* est une espèce opportuniste, principalement pollinisée par des espèces de Psychodidae, Chronomidae et Sciaridae. De son côté, *A. maculatum* est une espèce spécialiste, pollinisée à 90% par des Psychodidae. Dans toutes les populations étudiées, *Arum italicum* était moins attractif pour les pollinisateurs qu'*A. maculatum*. Les profils d'odeur florale d'*A. italicum* ne présentaient pas de structuration géographique entre les populations, ce qui suggère un fort flux de gènes ou une adaptation à une guilde fluctuante de pollinisateurs. Au contraire, les profils d'odeur d'*A. maculatum* étaient fortement structurés géographiquement. Cela suggère des flux de gènes plus faibles ou des adaptations locales aux préférences des pollinisateurs.

Keywords: Psychodidae, *Arum italicum*, *Arum maculatum*, floral scent, specificity.

Insects have played a primordial role in diversification of angiosperms *via* pollination, as their attraction and capacity of pollen transfer affects directly plant reproductive success (reviewed by Johnson 2006 & Herrera *et al.* 2006). Floral traits directly dedicated to pollinators' attraction and rewarding (which are called "pollination syndromes") have thus been strongly selected in plants. These traits may be flower colour, display, odour, size and shape, but also rewards like edible floral tissues, nectar or other floral secretions (Stebbins 1970; Fenster *et al.* 2004). Pollinators and

pollination syndromes can vary among populations from the same species, resulting in populations under different selective pressures where plants and pollinators coevolve in a geographic mosaic of coevolution (Thompson 2005; Gomez *et al.* 2009). These microevolutionary processes, when aggregated, lead to macroevolutionary processes, and can in extreme cases be key factors of the speciation of plants through pollinator shifts (Gould & Johnston 1972; Kiester *et al.* 1985; Bradshaw & Schemske 2003). Studies on geographical variations of floral traits linked to pollinator variations among populations are thus of great importance to the understanding of the dynamics of angiosperms evolution. Geographical variations of floral traits may result from different factors such as phenotypic plasticity in response to spatially variable

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Accepté le 31 mai 2010

environments (e.g. Alonso *et al.* 2007), or neutral phenotypic variations arising from genetic drift or divergent natural selection (e.g. Gomez *et al.* 2008). Such geographical variations may be more easily expressed in species with broad distribution areas.

The common lord-and-ladies, *Arum maculatum* L., and the closely related species *Arum italicum* Mill., illustrate such a case, being distributed throughout Europe from the Middle East to the Atlantic Ocean. These two *Arum* species are sapromyophilous as they deceive their insect pollinators (Diptera) by mimicking the fecal odour of their ovipositing sites (Lack & Diaz 1991; Albre *et al.* 2003; Gibernau *et al.* 2004a). Up to now, *A. maculatum* has been shown to be mainly pollinated by *Psychoda phalaenoides*, a moth fly from the Psychodidae family, even if some other insects have been found in small quantities in the inflorescences (Prime 1960; Rohacek *et al.* 1990; Lack & Diaz 1991; Diaz & Kite 2002). On the contrary, the insect diversity found in the inflorescences of *A. italicum* fluctuates greatly between sites (Gibernau *et al.* 2004a): different Psychodidae species were found in Spain and in the South of France (Mendez & Obeso 1992; Diaz & Kite 2002; Albre *et al.* 2003), as well as diverse Diptera species of the families Ceratopogonidae, Sciaridae and Chironomidae (Mendez & Obeso 1992; Albre *et al.* 2003). In the South of France, Sciaridae and Chironomidae may represent up to 75% of the insects trapped by *A. italicum* (Albre *et al.* 2003). Therefore, pollinators of the “opportunistic” *A. italicum* appear to vary among sites, whereas the main pollinator of the “specialist” *A. maculatum* appears to be *P. phalaenoides*.

In *Arum*, the attractive odour is likely to be linked to the degree of specificity, since it mimics the ovipositing site odour of the deceived pollinators (Gibernau *et al.* 2004a). Odours of *A. italicum* and *A. maculatum* have been studied in England (Kite 1995; Kite *et al.* 1998; Diaz & Kite 2002). In a comparative study in England, Kite *et al.* (1998) found that the odour of the two species clearly present different profiles with different volatile compounds. A preliminary study on floral volatiles of French *A. italicum* has been conducted (Gibernau *et al.* 2004b) but the results need to be confirmed. To our knowledge, no study has yet investigated the floral odour profile variations among natural populations of *A. italicum* and *A. maculatum*. The two species share the same ecological habits and pollinators from the same functional group. Thus we expect that the floral volatile profiles of the two *Arum* species present different patterns of geographical variations, in relation to their opposite degree of specificity for their pollinators (Thompson 2005).

Here we present the geographical variations of pollinators’ diversity for four populations of *A. italicum* and two populations of *A. maculatum*, and provide a first insight into the odour profiles’ variations among plant populations. The specific questions are:

(i) How do pollinator diversity and abundance vary between and within the two species *A. italicum* and *A. maculatum*?

(ii) How do odour profiles vary among the populations of each *Arum* species?

(iii) Do different degrees of pollinator specificity lead to different geographical patterns of odour profile variations?

Material and methods

Ecology of the plant model studied

A. italicum and *A. maculatum* are two species from the Araceae family growing in temperate and warm temperate woodlands, on the forest floor (Mayo *et al.* 1997). Their inflorescences attract Diptera pollinators and sequester them almost a day in a trap (Lack & Diaz 1991; Albre *et al.* 2003; Gibernau *et al.* 2004a). The classical floral cycle lasts about 24 hours over two days. In the afternoon of the first day, the spathe (a modified bract wrapping the inflorescence) begins to open above a constriction, uncovering a sterile organ called the appendix. In the evening, the appendix begins to warm (Bermadinger & Bermadinger-Strabentheiner 1995; Albre *et al.* 2003) and emits a dung odour (Kite 1995). Insects - mainly Diptera - are attracted, land on the spathe and slide into the floral chamber. At this stage, female flowers are receptive and can be pollinated if the insects carry fresh pollen. The insects, throughout the night and morning of the second day, remain captive within the floral chamber due to a sterile hair corona that blocks the exit. In the afternoon of the second day, the pollen is released and the sterile hairs dry, allowing the insects to leave the inflorescence carrying fresh pollen.

Inflorescence visitors

Insects caught in the floral chamber of *A. italicum* and *A. maculatum* were collected in the field, at 5 locations. Inflorescences were sampled in four populations of *A. italicum* in Chantonnay (Vendée, France, 46°40’N 1°06’O), Igeldo (Gipuzkoa, Spain, 43°18’N 2°04’O), Toulouse (Haute-Garonne, France, 43°33’N 1°28’E) and Uzer (Midi-Pyrénées, France, 43°04’N 0°09’E); and in two populations of *A. maculatum* in Uzer (Midi-Pyrénées, France, 43°04’N 0°09’E) and Smarves (Vienne, France, 46°30’N 0°22’E). Note that Uzer is a site of sympatry where *A. maculatum* and *A. italicum* are found in close proximity (i.e. distance between patches of each species <5m).

Inflorescence visitors were collected in each population in the morning of the second day of flowering. At this phenological stage, the insects are captive in the inflorescences. Inflorescence visitors were collected by pouring ethanol 70% into the floral chamber and then opening the spathes with a scalpel. The insects were conserved in 70% ethanol until determination at the family level under a stereomicroscope, with help of a Diptera taxonomist (Prof. Alain Thomas).

Floral scent collection

A. italicum individuals from Chantonay, Igeldo, Pierrelatte (Rhône-Alpes, France, 44°20'N 4°39'E) and Toulouse were grown under equal conditions in a greenhouse at Paul Sabatier University (Toulouse, France). Odours of *A. maculatum* inflorescences were collected in the field in Smarves and Uzer.

Inflorescence odours were collected for both species in the evening, between 6 pm and 1 am, when the spathe is widely open, the appendix is warm and the odour is strong. Each inflorescence (spathe and spadix) was wrapped in an inert plastic bag (Nalophan NA colorless, diameter 90, ETS Charles-Frères, France) in order to create an "open static headspace": the bottom of the bag was closed around the section below the base of the inflorescence with a bond, isolating the inflorescence from the leaves, pot and soil. The top of the bag was kept open 10 cm above the spathe, to avoid any condensation due to the heat of the appendix. Volatile organic compounds (VOCs) were collected by solid phase microextraction (SPME): VOCs were absorbed and desorbed from a fiber attached within the needle of a modified syringe. We used StableFlex™ SPME Fiber, 65 µm Polydimethylsiloxane/Divinylbenzene coating for manual holder (available from Supelco). The fiber was introduced in the nalophan bag through a little slit and maintained 0.5–1.0 cm away from the appendix for 20 min. A nalophan bag containing ambient air from 3–4 m away from the inflorescence was used as control to discard putative VOCs not originating from *Arum* inflorescences.

Gas chromatography (GC) analyses

GC analyses were performed at the Laboratory for Interaction Chemistry and Biochemistry at the Champollion University (Albi, France). Fibers were desorbed 5 sec (injector temperature 250 °C) splitless into a gas chromatograph (Finnigan Polaris Q, Thermo Electron Corporation) with an ion trap system and a Rtx®-5 (Restek) non-polar column (30 m × 0,25 mm ID × 0.25 µm film thickness, (5% phenyl)-methylpolysiloxane). Column temperature was maintained at 50 °C for 2 min after injection, linearly increased to 250 °C at a rate of 5 °C/min, and then maintained at 250 °C for 5 min. Helium was used as a carrier gas at 1 mL/min.

Statistical analyses

Insect abundances and Psychodidae proportions were compared between populations by analysis of variance (non parametric Kruskal-Wallis and Wilcoxon tests).

Chromatograms were treated as follow: peaks corresponding to the different VOCs were integrated and their area values were transformed into area percentages in order to obtain the relative amount of each compound in the blend. Only peaks representing more than 1% of the total chromatogram area were considered. Correspondence between peaks among the different chromatograms was assessed comparing their retention times (RT).

Variations in the odour profiles between the different inflorescences were represented with the method of non-metric multidimensional scaling (non-metric MDS). This non parametric method represents the interrelationships among a set of data objects using a distance matrix (dissimilarity index = Bray Curtis). Data points are placed in a two-dimensional coordinate system preserving the ranked differences between the objects. In this representation, each point represents the odour profile of one inflorescence. The further apart two points are in the score plot, the more distinct are the odour profiles of the two inflorescences. The stress value gives the percentage of difference not optimally represented by the analysis (Buja *et al.* 2008). Inter and intra-population variances were compared using a non parametric multivariate analysis of variance using distance matrices (function Adonis() from the vegan package in R, dissimilarity index = Bray Curtis, see Anderson 2001). All analyses were performed using R 2.10.0 (2009) software. Codes are available from M. Chartier.

Results

Inflorescence visitors

Inflorescence visitors obtained from inflorescences of *A. italicum* and *A. maculatum* belonged mainly to the families Chironomidae, Ceratopogonidae, Psychodidae, Sciaridae (Diptera) and Staphylinidae (Coleoptera). Some individuals (10 %) not determined

Table 1. Number and percentage of insects from the different families/orders captured in the inflorescences of *A. italicum* and *A. maculatum* in the six populations studied.

N=number of inflorescences sampled, Mean insects = Mean number of insects per inflorescence for each population.

Species	<i>A. italicum</i>								<i>A. maculatum</i>			
	Chantonay N 12		Igeldo 35		Toulouse 135		Uzer 25		Uzer 22		Smarves 3	
Psychodidae	124	72.1%	88	19.6%	82	19.1%	239	35.8%	2303	90.6%	85	92.4%
Sciaridae	7	4.1%	7	1.6%	106	24.7%	2	0.3%	2	0.1%	0	0.0%
Brachycera	13	7.6%	48	10.7%	49	11.4%	19	2.8%	92	3.6%	0	0.0%
Chironomidae	23	13.4%	294	65.5%	80	18.6%	401	60.0%	140	5.5%	1	1.1%
Staphylinidae	0	0.0%	0	0.0%	50	11.7%	2	0.3%	0	0.0%	0	0.0%
Ceratopogonidae	4	2.3%	11	2.4%	1	0.2%	4	0.6%	4	0.2%	6	6.5%
Others	1	0.6%	1	0.2%	61	14.2%	1	0.1%	1	0.0%	0	0.0%
Total	172		449		429		668		2542		92	
Mean insects	16.3 ± 10.2		14.7 ± 12.8		2.0 ± 3.2		26.7 ± 52.3		115.6 ± 108.9		32.7 ± 16.0	

at the family level were classified as “Brachyceras” and “others” for other arthropods. Inflorescences of *A. maculatum* trapped more insects than those of *A. italicum* (Wilcoxon test: $p < 10^{-11}$) with a mean of 105.60 ± 105.61 insects ($N = 25$ inflorescences, median = 76.00) for *A. maculatum*, and a mean of 5.93 ± 17.30 insects ($N = 207$ inflorescences, median = 1.00) for *A. italicum*, (fig. 1). The mean number of insects entrapped per inflorescence strongly differs among populations (table 1). *Arum italicum* trapped from 2 ipi (insects per inflorescence) in Toulouse up to 27 in Uzer. *A. maculatum* caught a mean of 33 ipi in Smarves, and 116 in Uzer. In Uzer, where the two *Arum* species are sympatric, *A. italicum* caught a mean of 27 ipi insects per inflorescence, which is significantly less than *A. maculatum* (Wilcoxon test: $p < 10^{-4}$).

The insects attracted to the inflorescences of *A. italicum* appear to be more diverse than to the inflorescences of *A. maculatum* (fig. 2). *A. maculatum* attracts principally Psychodidae within its inflorescences (tab. 1, fig. 2). The proportion of Psychodidae entrapped in the inflorescences of *A. maculatum* (91% at Uzer, 92% at Smarves) is higher than in the inflorescences of *A. italicum* (range: 19–72%). Even in the sympatric

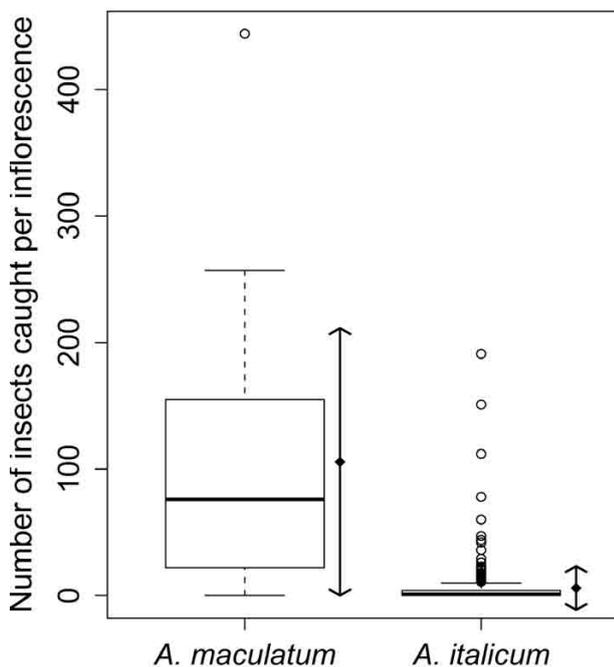


Figure 1
Boxplot of the number of insects caught in the inflorescences of *Arum italicum* and *A. maculatum* in the five studied populations. Quartiles = 0, 22, 76, 155, 257 for *A. maculatum* ($N = 25$) and 0, 0, 1, 4, 10 for *A. italicum* ($N = 207$). Arrows indicate means and standard deviations.

populations of Uzer, *A. maculatum* sequestered a significantly higher proportion of Psychodidae (91%) than did *A. italicum* (36%; Wilcoxon test: $p < 0.05$). In three populations of *A. italicum*, two diptera families (Psychodidae and Chironomidae) represented most (85%) of the insects entrapped (fig. 2) but in different proportions: in Chantonay, Psychodidae were more abundant (72%) than Chironomidae (13%) whereas in Igeldo and Uzer, Chironomidae were more abundant (66% and 60% respectively) than Psychodidae (20% and 36% respectively). Finally, the population of *A. italicum* at the University of Paul Sabatier (Toulouse) appears to be the most diverse in term of insect families (fig. 2), since three diptera families (Chironomidae, Psychodidae, Sciaridae) were caught in similar proportions (respectively 19, 19 and 25%) plus the less abundant beetle family Staphylinidae (12%).

Inter and intra-specific variations of floral odour profiles

Based on the comparison of mass spectra and retention times, we detected a total of 54 different VOCs in the chromatograms of *A. maculatum* (10 inflorescences analysed), and 60 in the chromatograms of *A. italicum* (22 inflorescences analysed).

Among populations of *A. maculatum* and *A. italicum* the odour profiles present differing patterns of geographical variation (fig. 3). The score plot of the four populations of *A. italicum* indicates a large overlap of the odour profiles from these populations (Adonis on the four groups: $p = 0.07$, $r^2 = 0.20$). A significant difference is found between Vendée and Igeldo when the two are considered alone (Adonis: $p < 0.05$, $r^2 = 0.28$), but no significant difference between the other populations. On the contrary, odours of *A. maculatum* appear well differentiated between populations (fig. 3), with a significant difference between the two populations (Adonis: $p < 0.05$, $r^2 = 0.18$). Geographical odour profile variations are thus well differentiated between *A. maculatum* populations, in contrast to the overlapping odour profiles of the *A. italicum* populations.

Discussion

The difference in specificity of the two *Arum* species found in the literature is clearly confirmed here. All insects found were small Diptera living in the same habitat (big insects can not enter the floral chamber closed by bristles). More than 90% of the insects attracted by *A. maculatum* where Psychodidae in the two studied sites, which is consistent with results from Germany and England (Prime 1960; Beck 1983; Rohacek *et al.* 1990; Lack & Diaz 1991; Diaz & Kite 2002).

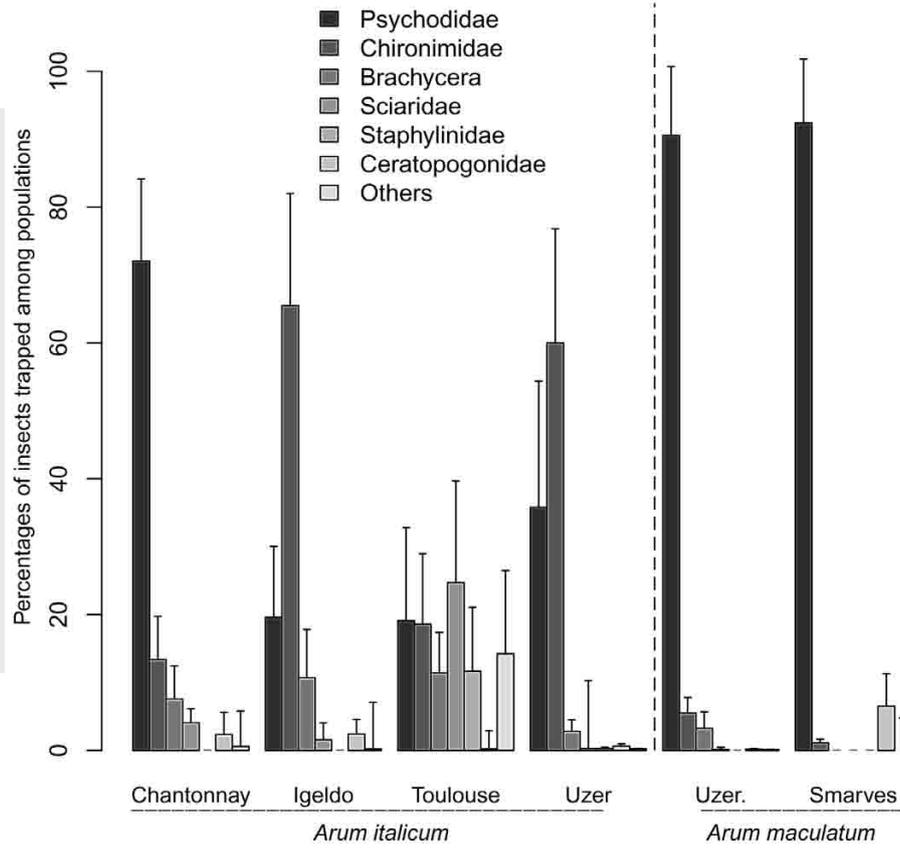


Figure 2 Diversity of the inflorescence visitors of *Arum italicum* and *A. maculatum*. Percentages and standard deviations of the different insect groups caught in the inflorescences from the five studied populations are given. See tab. 1 for the sample size and total number of insects.

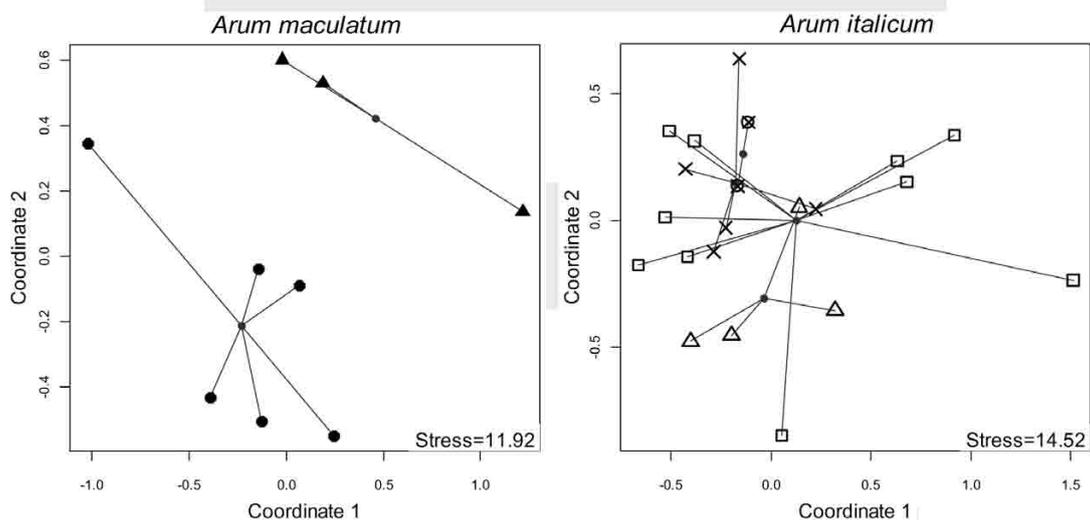


Figure 3 Non-metric MDS representation of the inflorescence odour profiles obtained from *Arum italicum* from Chantonnay (crosses), Igeldo (empty triangles), Toulouse (empty squares), and Uzer (full circles) and *A. maculatum* from Smarves (full triangles) and Uzer (full circles). The distance between symbols represents the difference in inflorescence odour profiles. Black full symbols represent the scent profiles of *A. maculatum*. Empty symbols represent the scent profiles of *A. italicum*.

On the contrary, the dominant insect groups found in the inflorescences of *A. italicum* belong to the families Psychodidae (72% in Chantonay), Chironomidae (respectively 66 and 60% in Igeldo and Uzer) and Sciaridae (25% in Toulouse). Interestingly, in the northernmost population in Chantonay, Psychodidae were the main pollinators of *A. italicum*, as was observed in England (Diaz & Kite 2002). The Southernmost populations (Toulouse, Uzer and Igeldo) were mainly pollinated by Chironomidae and Sciaridae. In conclusion, even if they share pollinators from the same “functional group”, *A. maculatum* can be considered as a specialist, whereas *A. italicum* can be considered as an opportunist. Nevertheless, differences in pollinator diversity exist among populations of *A. italicum*. One or two pollinators represent more than 60% of the pollinators in the populations of Chantonay, Igeldo and Uzer, whereas in Toulouse no insect family dominates. Thus, from a pollination point of view, *A. italicum* may be considered as an opportunist at the species level, but composed of populations which may be specialist or generalist. We underline here the importance of studying several populations when working on coevolutionary interactions.

In the studied populations, *A. maculatum* attracted a higher number of insects per inflorescence than did *A. italicum* (mean insects per inflorescence were respectively 106 and 6). This result appears to be different from that of Diaz & Kite (2002), who found no clear difference among the number of insects caught by the two species in England over four different locations. In our study, the mean insect number per inflorescence differs between populations of both species, and the biggest effective found in the Uzer population of *A. italicum* is approximately the smaller effective found in the Smarves population of *A. maculatum* (respectively 27 and 33 insects per inflorescence). However, in Uzer, where the two species are sympatric, *A. maculatum* attracts four times more insects than *A. italicum* (respectively 116 and 27 insects per inflorescence), confirming its higher attractiveness. Another indication for a stronger attractiveness of *A. maculatum* is that its appendix, which produces the attractive odour, is smaller than the appendix of *A. italicum*, and represents a smaller proportion of the total spadix length (Chartier & Gibernau 2009; Gibernau & Albre 2008).

Such a difference in attractiveness may be explained by at least two non exclusive factors. First, the inflorescence odour of *A. maculatum* might be a better attractant for Psychodidae than the odour of *A. italicum*. Second, populations of *A. maculatum* might grow in particular habitats with higher densities of Psychodidae. In the same way, the dramatic variation of the number of caught pollinators among populations is likely to be related to genetic differences between the

Arum populations, or to different insect availabilities between sites, which has been shown for *Psychoda* in England (Diaz & Kite 2002). In Toulouse, the low level of insects caught by *A. italicum* is consistent with data from Albre & Gibernau (2008) who found a mean of 3.5 insects per inflorescence in the same population. This is likely related to the low number of insects present at this site (Chartier *pers. obs.*). In Uzer, a large quantity of psychodid midges are observable in the field (Chartier *pers. obs.*), which is consistent with the high number of insects found in the inflorescences from the two *Arum* species. In posterior studies, insect availability must be quantified at each site, in order to establish the Diptera abundance of the different sites.

Arum italicum and *A. maculatum* also differ in the geographical intra-specific variation of the scent of their inflorescences. Surprisingly, the odour of *A. maculatum* appears to be geographically structured among populations whereas no strong geographic structure exists among individuals in *A. italicum*. Hence, the two populations of *A. maculatum* have distinct odour profiles (fig. 2) while they are visited mainly by Psychodidae (fig. 1) and the populations of *A. italicum* show overlapping odour profiles and attract a variety of insects (fig. 1). At present, some hypothesis can be proposed to explain these different variations. In *A. maculatum*, gene flow may be weak, leading to the evolution of several, more or less equally attractive odours in each population. Pollinators may also present different local preferences for odours, leading to local adaptations of the *Arum* odours. Also note that in this study we compared the variations of the total VOC emitted by inflorescences, certainly including non biologically active compounds. The variation of biologically active VOCs emitted by the plants could be different from the overall patterns of variations observed. It has been shown in a deceptive orchid, *Ophrys sphegodes*, that scent from individuals can differ less when considering the variation of the biologically active compounds than of the total scents (Ayasse *et al.* 2000). In *A. italicum*, the pattern of geographical variation of odours and pollinators is different. Inflorescences from Chantonay and Igeldo show significantly different odour profiles, and their major pollinators belong respectively to the Psychodidae and Chironomidae, whereas in Toulouse inflorescences appear to have the most diverse odour profiles and no dominant insect visitors as it is the case in the other populations (see fig. 2 and 3). Therefore, the high variation of scent profiles in *A. italicum* could be a response to fluctuations of pollinator guilds over space and time, with more or less locally specialized populations. In Chantonay and Igeldo, if pollinators remain the same from year to year, the difference of odours between these two groups may result from different selective pressures exerted by two different main

pollinators (resp. Psychodidae and Chironomidae; see fig. 2), leading to local specialization (Fenster *et al.* 2004). Contrastingly, in Toulouse there is no main pollinator and insect families' proportions may vary from year to year (eg. Tollsten & Bergströme 1993; Petanidou *et al.* 2008). This may result in fewer selective pressures and a more diverse attractive odour (Geber & Moeller 2006, Herrera *et al.* 2006). Record of the insects caught in the inflorescences over several years, as well as insect biotests to determine which compounds are attractive/repulsive will be necessary to infirm or confirm this hypothesis.

In conclusion, we describe two patterns of geographic differentiation linked to the degree of pollinator specificity and the variation patterns of the odour profiles. Further investigations have to be conducted to better understand, within the frame of the geographic mosaic of coevolution, the mechanisms leading to such variations at the species level.

Acknowledgments. We wish to thank Michel Treilhou for having given us the opportunity to use the GC-MS of the Interactions Chemistry and Biochemistry Laboratory (Albi, France), and Natan Tene for his assistance during the GC-MS analyses. We are grateful to Professor Alain Thomas and Quentin Laurent for their precious help at determining the insects. We thank Josselin Cornuault, Julien Rimour, and Guillaume Chartier for their help in the field, and Jean-Marie Bessière, Candida Shin and Artur Maia for their advises during the manuscript redaction. We are grateful to the financial and intellectual support of the GDR Ecologie Chimique.

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