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À mes parents et grands parents.

À mes six frères et sœurs.

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Première partie

INTRODUCTION GÉNÉRALE

0.1 INTRODUCTION

La pollinisation est le mécanisme par lequel les plantes acheminent le pollen (gamètes mâles) vers les sigmates sur lesquels ils vont germer pour atteindre et féconder les ovules (gamètes femelles). Les plantes étant des organismes fixes (du moins à l'échelle de temps nécessaire à la pollinisation), la dissémination des grains de pollen peut se faire entre les parties florales d'une même fleur (on parle d'auto-pollinisation ou autogamie), mais dans la majorité des cas nécessite l'utilisation d'un vecteur pour atteindre les autres fleurs d'une même plante (geitonogamie), ou de plantes différentes (allogamie). Ce vecteur peut être le vent, l'eau ou les animaux (Ackerman 2000, Proctor *et al.* 1996).

La majorité des plantes à fleurs sont pollinisées par des insectes...

La pollinisation des plantes par les animaux, et notamment par les insectes (on parle alors de plantes entomophiles), concerne environs 90% des plantes à fleurs, et un tiers des cultures mondiales d'intérêt agronomique (Aizen *et al.* 2009). Elle nécessite des adaptations importantes des plantes, pour amener les insectes à visiter la partie mâle des fleurs et à en récolter le pollen, puis la partie femelle pour l'y déposer. Dans le cas extrême, une plante peut dépendre strictement de l'activité de pollinisation d'un insecte pour se reproduire. Cette observation a été la source de nombreux questionnements scientifiques et de théories supposant que les interactions plantes-pollinisateurs soient à l'origine de pressions de sélection importantes pesant sur l'évolution des traits floraux, et que la compréhension de ces mécanismes soit primordiale dans un contexte de préservation et/ou de restauration de la biodiversité (Stebbins 1970, Grant 1994, Barrett 2006).

... les insectes seraient ainsi à l'origine des pressions de sélection sur les traits floraux...

Ces pressions de sélection imposées aux plantes entomophiles par la nécessité d'attirer des pollinisateurs constituent un des facteurs importants de diversification des formes florales, au même titre, par exemples, que la sélection due aux contraintes physiologiques ou morphologiques imposées à la plante par les conditions abiotiques, ou par les prédateurs (révisé par Galen 1999, Johnson 2006, Herrera *et al.* 2006). Dans le cas de la pollinisation, ces pressions sont d'autant plus fortes que la plante dépend de la fécondation croisée (allofécondation), et qu'elle est spécialisée à un type de pollinisateurs (Fenster *et al.* 1994, Ollerton *et al.* 2007).

. . . ce qui implique la notion de syndromes de pollinisation . . .

Ainsi, les botanistes et biologistes ont depuis longtemps observé l'apparition de formes florales caractéristiques d'un pollinisateur ou d'un mode de pollinisation. L'ensemble de ces traits floraux, adaptés à un type de pollinisateur, forme un « syndrome de pollinisation » (Stebbins 1970, Faegri et Van der Pijl 1979) : ainsi, Frederico Delpino (1833-1905), premier botaniste à discuter du concept de syndromes de pollinisation, avait prédit que l'étonnante fleur de Rafflésia, d'après sa morphologie et son odeur fétide, devait être pollinisée par des mouches nécrophages, ce qui fut précisément décrit un siècle plus tard par Beaman (1988). Les traits floraux constituant les syndromes de pollinisation sont liés aux différents aspects de la pollinisation : certains ont trait à la reconnaissance de la plante par le pollinisateur (odeur, couleur, texture, disposition et forme), d'autres à la récompense induisant la venue répétée du pollinisateur (tissus floraux, nectar, sécrétions nutritives) ou encore à augmenter l'efficacité de la pollinisation en étant adaptés à la morphologie du pollinisateur (profondeur de la corolle, position des étamines, taille des stigmates). Dans le cas de la duperie, certaines plantes développent d'étonnantes structures pour mimer un partenaire sexuel, comme par exemples certaines orchidées mimant la forme, la couleur et l'odeur des phéromones de bourdons femelles pour attirer des bourdons mâles (Schiestl et Schlüter 2009). D'autres plantes, dites saprophiles, attirent des insectes nécrophages ou coprophages en mimant l'odeur, la forme et/ou l'odeur d'animaux, de bouses ou de plantes en décomposition (Stensmyr *et al.* 2002, Renner 2006).

. . . variant selon le type d'interaction et son degré de spécificité.

La pertinence de l'utilisation des syndromes de pollinisation pour prédire le type d'interaction existant entre les plantes et leurs pollinisateurs a longtemps été (et est encore aujourd'hui) débattue par les scientifiques (Waser 1997, Ollerton *et al.* 2007, Fenster *et al.* 2004, DeWitt Smith *et al.* 2009). Ainsi, du fait de ces différences de spécificité, le lien entre un pollinisateur et des traits floraux peut-être plus compliqué en réalité qu'à première vue. Par exemple, des plantes différentes peuvent évoluer vers des formes équivalentes adaptées au même pollinisateur (i.e. convergence évolutive), et à l'inverse, des pollinisateurs différents peuvent exercer les mêmes pressions de sélection sur certains traits floraux (Fenster *et al.* 2004). Ainsi, les plantes adaptées au pollinisateur le plus efficace (parce qu'il transporte mieux le pollen, ou parce qu'il est présent en plus grande quantité), ou a un groupe de pollinisateurs partageant les mêmes caractéristiques écologiques, comportementales et morphologiques (on parle alors de groupe fonctionnel), sont différentiables du point de vue des caractères liés à la reproduction de manière bien plus nette que

les plantes généralistes, pollinisées par le vent, ou par l'eau (Grant 1994, Faegri and van der Pijl 1971, Fenster *et al.* 2004). La pollinisation spécialisée, contrairement à ce que l'on admet souvent intuitivement, n'est pas forcément le cas général. Par exemple, une pollinisation non spécifique, généraliste, peut être très adaptative dans le cas où les insectes disponibles varient au cours du temps et dans l'espace et dans ce cas on ne distingue pas de syndrome net de pollinisation (Ollerton *et al.* 2007, Waser *et al.* 1996). Quoiqu'il en soit, il est accepté que les insectes, par les adaptations qu'ils ont engendrées sur les plantes à fleurs, ont constitué un important facteur de leur diversification, notamment lors de radiations adaptatives, c'est-à-dire l'apparition d'espèces à partir d'un ancêtre commun qui vont s'adapter chacune à un environnement différent (Stebbins 1970, Glor 2010).

La coévolution entre plantes et pollinisateurs varie géographiquement...

Comprendre l'évolution des interactions plantes-pollinisateurs permet donc de comprendre une grande part de l'évolution des traits floraux, et la façon dont les espèces s'adaptent à leur milieu. Si une interaction entre une ou plusieurs espèces est conservée de génération en génération, qu'elle est soumise aux pressions de sélections réciproques et évolue dans le temps et l'espace, on parle de coévolution. L'existence de la coévolution à long et à court terme implique notamment que les espèces considérées constituent des populations génétiquement différentes, qu'elles se spécialisent à un nombre restreint d'espèces et que le résultat de ces interactions diffère entre et dans les communautés (Thompson 2005). Ces différences géographiques sont à l'origine de l'existence de populations différentes car soumises aux pressions de milieux différents. Ainsi une espèce peut-elle être vue comme un ensemble de populations connectées par des flux de gènes, mais soumises à des pressions de sélection variant dans le temps et dans l'espace, créant une mosaïque géographique de co-évolution. Le cas d'une divergence trop importante d'une population est l'une des manières de créer une nouvelle espèce (Levin 2000, Thompson 2005, Kay et Sargent 2009).

... et s'étudie à différentes échelles taxonomiques.

Le type de pollinisateurs, le type d'interaction et l'ensemble des traits floraux liés à la pollinisation d'une plante sont des caractéristiques plus ou moins variables non seulement entre populations d'une espèce (dans le temps et l'espace) mais aussi à plus grande échelle phylogénétique au sein d'une famille, voire d'un genre ou d'une espèce. Le but de ma thèse est d'étudier les contraintes qui régissent la mise en place et le maintien des interactions plantes-pollinisateurs

en me basant sur l'exemple de la famille des Aracées. Pour cela, un premier travail a été effectué à l'échelle de la famille, pour étudier la part des contraintes phylogénétiques pesant sur l'évolution des interactions Aracées-pollinisateurs (PARTIE 2). Dans un second temps, les variations géographiques de l'interaction *Arum*-diptères sur deux espèces européennes (*A. italicum* et *A. maculatum*) ont permis d'étudier plusieurs aspects des pressions de sélection locales qui régissent l'évolution de ces interactions au niveau de la population (PARTIE 3).

L'objectif de ma thèse est de répondre aux questions spécifiques suivantes :

A l'échelle de la famille (contraintes phylogénétiques) :

(1) L'utilisation des syndromes de pollinisation est-elle pertinente chez lez Aracées pour prédire le mode de pollinisation (CHAPITRE 2) ? (2) Comment les traits floraux et les interactions plantes-pollinisateurs ont-ils évolué chez les Aracées (CHAPITRES 3 et 4) ? (3) De quelle manière les pollinisateurs ont-ils influé sur l'apparition et la diversification des différentes formes de l'inflorescence chez les Aracées (CHAPITRE 4) ?

A l'échelle de la population (contraintes écologiques) :

(4) Y a-t-il des pressions de sélection exercées par les pollinisateurs sur l'odeur attractive pour le pollinisateur chez *Arum*, et sont-elles liées au degré de spécificité de l'interaction (CHAPITRE 6) ? (5) Ces pressions de sélection varient-elles dans une mosaïque géographique de la co-évolution, comme prédite par Thompson (2005) (CHAPITRE 7) ? (6) Si oui, ces variations de l'odeur florale attractive pour les pollinisateurs résultent-elles d'adaptations locales des plantes (CHAPITRE 7) ? (6) Quels facteurs écologiques locaux peuvent influer sur ces variations (CHAPITRE 8) ?

0.2 DESCRIPTION DU MODÈLE D'ÉTUDE : LES ARACÉES

Article de vulgarisation scientifique publié dans ***Le Courrier de la Nature*** .

Auteurs : Marc GIBERNAU, Marion CHARTIER

Photo Marc Gibernau

Les aracées

Une diversité d'arômes ou les différentes stratégies de la séduction

Marc Gibernau* et Marion Chartier*

« On n'attrape pas les mouches avec du vinaigre », les plantes l'ont bien compris. Pour attirer les insectes pollinisateurs, elles produisent une vaste gamme de substances odorantes allant de parfums sucrés à des odeurs de viande avariée !

Qu'elles soient grandes ou petites, flamboyantes ou ternes, les fleurs ont comme fonction première d'assurer la reproduction sexuée des plantes grâce à la production de graines *via* la pollinisation. Certaines fleurs comptent sur le vent pour assurer leur pollinisation (on parle alors d'anémophilie) ; d'autres sur les insectes (entomophilie). Dans le premier cas, les plantes n'ont aucun moyen d'agir sur le mode de transport du pollen : elles sont complètement dépendantes des conditions environnementales. En revanche, les plantes entomophiles disposent de plusieurs stimuli – principalement les couleurs et les odeurs – pour attirer les insectes (cf. encadré p. 27). Les odeurs (florales) n'étaient pas à l'origine destinées à attirer les insectes pollinisateurs, mais avaient chez les premiers angiospermes – telles les pinales – une fonction de défense chimique (cf. encadré p. 28).

Contrairement à ce que l'on pourrait croire, les odeurs florales ne sont pas toutes agréables. Si l'œillet, le muguet ou la rose dégagent des substances aromatiques, il en va autrement

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L'arum mange-mouches* (Helicodiceros muscivorus) *attirant dans sa chambre florale, obscure et puant le cadavre, les mouches des cadavres.

des plantes comme l'arum mange-mouches, les rafflesias ou le chou puant dont les fleurs produisent des odeurs nauséabondes. Même parmi les splendides orchidées, on trouve des fleurs dont le « parfum » est loin d'être agréable comme chez *Bulbophyllum robustum*. Le type de substances odorantes produites par les fleurs est étroitement lié au type d'insecte pollinisateur qu'elles tentent d'attirer.

Les aracées

Les aracées forment une famille de plantes dont on connaît actuellement quelque 122 genres et 3 300 espèces réparties sur toute la surface du globe, principalement dans les zones intertropicales. Au-delà d'une immense diversité de formes, de modes de vies et d'habitats, les aracées ont en commun – à quelques exceptions près – d'être entomophiles. Pour attirer et retenir les insectes qui les pollinisent, elles ont développé des inflorescences très particulières (cf. encadré p. 29). De plus, les aracées représentent de bons modèles pour étudier l'attraction olfactive des pollinisateurs, car certaines espèces sont pollinisées par des mouches, d'autres par des abeilles, et d'autres encore par des coléoptères. Ces insectes ayant des sensibilités olfactives très différentes, les odeurs florales qui les attirent sont aussi très contrastées.

La pollinisation par les abeilles : une récompense

Dans les forêts d'Amérique tropicale, il existe un mode unique de pollinisation chez les aracées : celle par les abeilles. Selon le type d'abeilles, la récompense donnée en échange du service de pollinisation est différente. Il s'agira de pollen ou alors d'une huile, ou encore d'une cire parfumée.

Certaines inflorescences du genre *Spathiphyllum* comme *S. friedrichsthalii* sont pollinisées par des abeilles sans aiguillon, des mélipones du genre *Trigona*. Les spathiphyllums sont aussi des plantes d'appartement : leur axe florifère ou spadice est simple et constitué de fleurs bisexuées et d'une bractée modifiée (la spathe) dressée comme un étendard (cf. encadré p. 29). La floraison dure entre dix jours et un mois, les fleurs devenant matures progressivement le long de l'axe. Pendant la période de floraison, les fleurs émettent une forte odeur sucrée tôt le matin qui attire les trigones. Les trigones se posent sur les inflorescences et récoltent du pollen, émis en abondance, qu'ils transportent d'une inflorescence à l'autre, assurant la pollinisation. En fin de matinée, il n'y a presque plus de pollen.

D'autres espèces de spathiphyllum (*S. wallisii*) exhalent plutôt une forte odeur épicée et sont pollinisées par les mâles d'abeilles euglossines. Ces fleurs émettent des composés irrésistibles pour ces abeilles nécessaires à leur propre reproduction : 1,8-cinéole, méthyl-eugénol, α-farnésène et des esters benzyliques. Il en est de même pour de nombreux anthuriums. Par exemple, *Anthurium rubrinervium*, espèce

Odeurs simples et odeurs complexes

Les senteurs florales sont un mélange de molécules odorantes volatiles plus ou moins complexes. Les odeurs « simples » (Acacallis, orchidacées) comportent une dizaine de composés, avec souvent deux ou trois molécules dominantes, alors que les odeurs « complexes » (Ophrys, orchidacées) peuvent être des mélanges d'une centaine de composés ! Ceux-ci appartiennent à quatre classes « chimiques » : les dérivés d'acides gras, les composés aromatiques, les terpènes et les composés azotés. Ces différentes classes impliquent des voies de biosynthèse distinctes.

Les mots écrits en vert dans le texte renvoient au lexique page 65.

Photo Marc Gibernau

Inflorescence de* Spathiphyllum sp.** ***La spathe dressée, comme un étendard vert, contraste avec l'axe florifère blanc, le spadice d'où sont émises les odeurs qui vont attirer les abeilles pollinisatrices.

Les parfums sont apparus avant les fleurs

Il existe deux groupes majeurs de plantes : les plantes possédant des organes reproducteurs souvent en forme de cônes, et chez lesquels les futures graines ne sont pas protégées, anciennement appelées gymnospermes, comme les pinales (avec les conifères), les cycadales ou les gnétales, et les plantes possédant des organes reproducteurs enveloppant la future graine, les plantes à fleurs, ou angiospermes.

Les pinales sont apparues bien avant les plantes à fleurs, et produisaient déjà des parfums pour attirer leurs pollinisateurs. A cette époque, toutes les lignées majeures d'insectes pollinisateurs (à l'exception des papillons) existaient déjà, comme en font foi des fossiles d'insectes âgés de 250 à 240 millions d'années. Les fougères à graines ou les bennetitales (cycadophytes) étaient donc déjà sûrement pollinisées par des insectes. En fait, une hypothèse très probable est qu'à l'origine les parfums étaient des « armes » chimiques produites entre autres par les organes reproducteurs des plantes pour se défendre contre les insectes herbivores en les repoussant ou en les empoisonnant. Au cours de l'évolution, en même temps que les insectes devenaient des agents pollinisateurs des fleurs, les senteurs florales se sont transformées en signaux attractifs pour ces nouveaux vecteurs. Si de nouvelles molécules sont apparues, la majorité des senteurs florales actuelles restent constituées de molécules qui, à l'origine, servaient de défense chimique.

De nombreuses pinales actuelles émettent des parfums variés à partir de leurs organes reproducteurs (cônes…) pour attirer leurs insectes pollinisateurs. C'est aussi le cas de nombreuses cycadées (odeur de fruit avarié) et des gnétales (Gnetum, Ephedra) *qui émettent une agréable odeur fruitée. Par ailleurs, chez les conifères (pins, sapins et autres résineux), l'odeur résineuse que nous connaissons bien a toujours un rôle de protection contre les prédateurs des graines nues situées dans les cônes. Chez ces espèces, la pollinisation se fait par le vent.*

guyanaise, est pollinisé par des abeilles solitaires mâles du groupe des euglossines (*Euglossa piliventris* et *E. viridis*). Ses inflorescences sont de même type que celles des *Spathiphyllum* (cf. encadré p. 29). Elles émettent une forte odeur, plaisante et assez complexe, constituée de 34 composés dont six majeurs (qui représentent de 75 à 90 % des émissions totales) : (Z)-8-heptadecène, méthyl salicylate, 1,8-cinéole, benzoate de benzyle, (E)-ocimène et linalol. L'axe florifère est recouvert d'une cire odorante précieuse pour les mâles. Attirés par l'odeur, ceux-ci se posent sur les inflorescences qu'ils parcourent de bas en haut pendant de longues périodes, jusqu'à une heure. Tels des parfumeurs pratiquant la technique de l'enfleurage, ils sécrètent un liquide gras produit par leur glande labiale qu'ils mélangent à la cire à l'aide d'une brosse située sur les tarses antérieurs. Ils transfèrent ensuite cette pâte dans un réservoir situé sur les tibias postérieurs et reprennent leur collecte. On pense que ce mélange leur sert de « parfum » pour attirer les femelles lors de la danse nuptiale et/ou à repousser les mâles rivaux. C'est en arpentant les inflorescences que les insectes, en frottant leur abdomen contre les parties mâles et femelles des fleurs, assurent ainsi la pollinisation. Ces mêmes composés volatils se retrouvent dans les odeurs de fleurs d'orchidées pollinisées aussi par des mâles d'abeilles euglossines. Il s'agit là d'un phénomène de convergence des odeurs florales dans deux familles de plantes qui sont pourtant éloignées d'un point de vue phylogénétique, c'est-à-dire de leur filiation.

Photo Heiko Hentrich

***Abeilles euglossines* (Euglossa piliventris) *arpentant une inflorescence d'*Anthurium rubrinervium.**

Le genre *Anthurium* compte quelques 1 000 espèces et montre une grande diversité d'odeurs florales : certaines très agréables (fleuries), d'autres carrément fétides ! Les odeurs agréables proviennent principalement des composés de la famille chimique des terpènes, comme le linalol et le 1,8-cinéole. D'autres composés de la même famille donnent à certains *Anthurium* une odeur de menthe (sabinène, menthol, carvone) ou de pin (α et β pinènes, myrcène). La pollinisation de ces espèces n'a pas encore été documentée ! Par ailleurs, une espèce, *A. salvadorense*, possède une odeur plutôt rance de fruits mûrs, qui renferme des esters d'acides gras caractéristiques de ce type d'odeur (éthyl-2-méthyl butyrate, 2-hexyl acétate, 6-méthyl-

5-heptèn-2-yl acétate, éthyl valérate), et une pollinisation par des drosophiles et autres mouches des fruits n'est pas exclue.

La pollinisation par les coléoptères – des rencontres

De nombreuses aracées tropicales sont pollinisées par des coléoptères appartenant principalement aux scarabéidés et aux nitidulidés. Ces insectes, attirés par les odeurs florales, visitent les inflorescences d'aracées non pas seulement pour trouver une ressource alimentaire (tissus végétaux riches, pollen) et un abri, mais aussi des partenaires sexuels. Ainsi, les inflorescences deviennent des zones d'accouplements et font partie du cycle biologique des coléoptères.

On observe un tel mode de pollinisation chez les philodendrons pollinisés par des coléoptères nocturnes du genre *Cyclocephala* (scarabéidés). La floraison dure en tout seulement deux jours. Durant le premier jour, la spathe s'ouvre, découvrant des fleurs mâles situées à l'apex du spadice. A la base, elle forme une chambre florale autour des fleurs femelles (cf. encadré ci-dessous). À la tombée de la nuit, les fleurs mâles chauffent et émettent une odeur fruitée entêtante pendant environ une heure, avec parfois des traces de solvant ou de moisissures selon les espèces. Cette odeur attire les coléoptères, qui entrent alors dans la chambre florale dans laquelle ils vont copuler et se nourrir des parties stériles situées entre les fleurs. S'ils portent du pollen, ils assurent ainsi la pollinisation des fleurs femelles alors réceptives. Les insectes, photophobes, restent dans l'inflorescence jusqu'au soir du lendemain, où se produit l'émission du pollen. Les coléoptères quittent l'inflorescence, emportant avec eux du pollen, au moment où les nouvelles inflorescences réceptives de premier jour sont prêtes à les accueillir. Les aracées pollinisées de cette manière émettent de fortes odeurs perceptibles à plusieurs mètres et de composition en général assez simple (rarement plus de 12 composés). Les émissions volatiles sont dans tous les cas dominées par deux à quatre composés. Par exemple, l'odeur de *Philodendron acutatum* ne renferme que deux composés : un terpène irrégulier (dihydro-béta-ionone) et un dérivé d'acide gras (2-hydroxy-5-méthyl-3-hexanone). Pour *Montrichardia linifera*, une autre aracée pollinisée par des *Cyclocephala*, l'odeur est dominée par un dérivé

Photo Marc Gibernau

***Coléoptères* Cyclocephala *sur une inflorescence de* Philodendron solimoesense.**

Les inflorescences d'aracées

Les aracées ne produisent pas de fleurs simples, mais des inflorescences, structures composées de fleurs collées les unes contre les autres.

Les inflorescences de type Anthurium *sont constituées d'une spathe (bractée modifiée) dressée en étendard, et d'un spadice (axe florifère) composé de fleurs bisexuées toutes identiques (constituées d'un ovaire et d'étamines).*

Chez les inflorescences de type Philodendron, *la spathe n'est pas dressée, mais entoure l'axe florifère, le spadice. Elle forme souvent à sa base un espace fermé, nommé chambre florale, sa partie supérieure gardant la fonction d'étendard. Les fleurs sont différenciées, les fleurs femelles (contenant les ovaires) sont situées à la base de l'axe et sont surmontées par des fleurs mâles (étamines). On peut également trouver des fleurs stériles soit entre les fleurs femelles et mâles soit au-dessus des fleurs mâles. Ces fleurs stériles assurent différentes fonctions telles que tissu nourricier pour les pollinisateurs. Parfois, l'inflorescence est surmontée d'un organe responsable du stockage et de l'émission des composés volatils : l'appendice (chez le genre* Arum *par exemple).*

*Inflorescence d'une espèce d'***Homalomena** *asiatique, genre proche des* **Philodendron** *américains, pollinisée par des scarabées mais qui attire aussi des mouches drosophiles et des coléoptères* **chrysomélidés.**

de cyclopentenone (le jasmone) et par quelques dérivés aromatiques (1,3,5 triméthoxy benzène, méthyl benzoate et méthyl salicylate). Pour finir, *Homalomena propinqua* est une aracée du sud-est asiatique pollinisée par un scarabée local. Son odeur florale est dominée par cinq composés issus de trois voies de biosynthèse différentes (2-butanol, 1,2-diméthoxy-benzène, alpha-pinène, acide 2,4-décadiénoïque méthyl ester, 2-méthyl-3-butèn-2-ol).

Il est intéressant de noter que les scarabéidés en général et les *Cyclocephala* en particulier pollinisent d'autres familles d'angiospermes telles que certains palmiers, les cyclanthacées, les nénuphars, les magnoliacées ou encore les annonacées. Les odeurs florales dans ces différentes familles sont très différentes les unes des autres, mais sont constituées d'un nombre de composés volatils relativement faible (en général moins de 20) et le bouquet floral n'est dominé que par quelques molécules. Une étude comparative de ces différentes odeurs impliquées dans l'attraction des coléoptères pollinisateurs est une piste de recherche intéressante.

La pollinisation par les mouches : un site d'oviposition

Un système de pollinisation moins commun existe chez des aracées d'Asie tropicale et chez une aracée d'Amérique du Nord. Il s'agit de pollinisation par des mouches qui pondent sur l'inflorescence. Par exemple, les *Alocasia* émettent une odeur agréable qui attire des mouches du genre *Colocasiomyia* (drosophilidés). Chaque espèce est pollinisée par une espèce différente de mouche : la relation est donc très spécifique. Le cycle floral est court et dure de deux à cinq jours. Les mouches sont attirées par l'odeur spécifique émise lors du début du cycle floral. Elles restent au contact de l'inflorescence et pondent sur le spadice. Puis, lors de la libération du pollen, elles en consomment une partie et s'envolent chargées de pollen à la recherche d'une nouvelle inflorescence où pondre d'autres œufs. Les larves mangent les parties végétales en décomposition avant de finir leur cycle de développement dans le sol.

Chez *Alocasia odora*, l'odeur florale est composée de trois molécules communément trouvées dans les senteurs florales (diméthyl 1,3,7 nonatriène, méthyl benzoate, méthyl salicylate). Mais l'originalité du message olfactif réside dans leur association en un bouquet (combinaison) unique qui assure la reconnaissance et la spécificité de l'interaction avec les pollinisateurs.

Ainsi, le peltandre de Virginie (*Peltandra virginica*), une espèce indigène de l'est de l'Amérique du Nord, est pollinisé spécifiquement par une mouche chloropidés (*Elachiptera formosa*). Cette espèce émet une odeur de moisi et de résine due à un composé aliphatique original et unique au monde : le 1,3,6 triméthyl-2,5-dioxabicyclo [3,2,1] nonane accompagnée de quatre isomères. La spécificité de la reconnaissance de la plante hôte par la mouche choloropide est donc sans aucun doute

Photo Marc Gibernau

Caladium bicolor *est une espèce pollinisée par des coléoptères scarabées (***Cyclocephala sp.***) sur le même modèle que les* **Philodendron,** *l'inflorescence représentant le lieu de rencontres des coléoptères qui effectuent alors la pollinisation croisée.*

Photo : Marc Gibernau

Mouches drosophilidés sur une inflorescence d'*Alocasia robusta *qui représente leur site d'oviposition.

basée sur cette molécule complexe et unique dont la fonction s'apparente plus à la phéromone sexuelle qu'à un parfum floral.

La pollinisation par duperie (mouches ou coléoptères) : un piège

Certaines espèces d'aracées ont évolué vers un mode de pollinisation nettement moins favorable aux insectes. Il s'agit alors pour la plante d'attirer les insectes en les dupant, puis de les piéger. L'inflorescence est constituée d'une spathe cirée pour être glissante et formant à sa base une chambre florale renfermant les ovaires et les étamines dont l'ouverture est obstruée par une couronne de poils. A l'extérieur de la chambre florale, l'axe florifère se termine par une partie stérile exposée à l'air libre, l'appendice. Le piège est en place.

Le premier soir de floraison, à la tombée du jour, l'appendice chauffe et émet une odeur désagréable qui, selon les espèces d'aracées, sent la bouse, l'urine, la matière végétale en décomposition, le fruit pourri ou le cadavre. Cette odeur attire les diptères et les coléoptères en recherche d'un lieu de ponte correspondant à l'odeur. Ils se posent sur la spathe et glissent à l'intérieur de la chambre florale. Les poils et les parois glissantes empêchent les insectes de sortir de la chambre. Pas de récompense pour ces pollinisateurs dupés ! S'ils portent du pollen, ils polliniseront les fleurs femelles en voletant à la recherche de la sortie. Ce n'est que le lendemain, en début d'après-midi, que les fleurs mâles libèrent du pollen en grande quantité et saupoudrent ainsi les insectes captifs. Les poils sèchent alors, permettant aux insectes piégés de grimper le long de l'axe florifère et de sortir enfin du piège. Ils sont alors prêts à féconder une autre inflorescence, s'ils se font à nouveau avoir…

Ce système de pollinisation antagoniste nécessite la capture d'un même insecte à deux reprises et son maintien en captivité pour qu'il puisse repartir avec le précieux pollen : la chambre florale de ces aracées possède donc une paroi poreuse qui lui permet de rester aérée et de garder un taux d'humidité adéquat à la survie de ces petits insectes !

Les inflorescences de nombreux *Arum* (un genre réparti dans le pourtour méditerranéen et en Europe de l'Ouest) émettent une odeur putride ou d'urine à base de cétones (2-heptanone), d'indole (un composé azoté), de quelques terpènes (citronellène, germacrène, *p*-crésol) et ses quiterpènes (caryophyllène). Cette odeur attire selon les espèces des moucherons psychodidés (diptères, nématocères) mais aussi des moustiques (chironomidés), des mouches (sciaridés ou sphaeroceridés) et des coléoptères (staphylinidés) qui pondent dans les excréments ou dans la matière organique en décomposition. C'est le cas par exemple d'*Arum italicum* (le gouet d'Italie), *A. maculatum*, *A. nigrum*, *A. dioscoridis* et *A. pictum*. Une espèce du Moyen-Orient, *Arum palaestinum*, émet une odeur de fruit fermenté à base de composés dérivés des acides gras (acétate d'éthyle, éthanol, acide acétique) et est pollinisée par les mouches des fruits (*Drosophila sp.*) qui sont à la recherche d'un fruit pourri pour y pondre leurs œufs.

D'autres exemples de pollinisation par duperie bien connus concernent des espèces des genres *Amorphophallus* et *Sauromatum*, ou

encore l'arum mange-mouches (*Helicodiceros muscivorus*). Ces aracées sont pollinisées par des insectes nécrophages (mouches et coléoptères) qui pondent leurs œufs dans les cadavres de mammifères. Les inflorescences de ces aracées produisent des odeurs nauséabondes de viande ou de poisson avariés grâce à des composés soufrés, les sulfides (diméthyl di- ou tri-sulfides), ou des amines (triméthyl amine) et des dérivés d'acide gras (acide iso-caproïque, iso-amyle d'acétate, éthyl d'acétate).

Conclusions

Les aracées, de par leurs interactions de pollinisation variées, non seulement en termes d'agents pollinisateurs (abeilles, mouches, coléoptères), mais aussi en termes de nature de l'interaction (récompenses nutritives ou reproductrices, duperie) ont développé une grande diversité d'odeurs florales. Ces odeurs constituent le médiateur chimique dans l'attraction des insectes pollinisateurs. Elles permettent la reconnaissance par les pollinisateurs des aracées (identification olfactive) et donc leur reproduction sexuée. Ainsi, ce message volatil est « honnête » dans les cas d'interactions mutualistes, puisqu'il annonce une ressource que l'insecte obtiendra en échange de son comportement de pollinisateur. En revanche, ce message est « malhonnête » dans les cas d'interactions antagonistes, où seule la plante obtient un bénéfice de cette interaction.

La diversité des odeurs florales d'aracées ne s'exprime pas seulement en termes de types d'odeurs (fruitée, sucrée, aigre, pourrie…), mais aussi dans la nature du bouquet odorant. Nous avons vu qu'il existe chez les aracées deux « stratégies » pour obtenir une odeur florale originale : soit c'est la combinaison de quelques molécules assez communes (trois à cinq) qui est unique, soit l'odeur florale est due à une unique molécule que l'on ne retrouve chez aucune autre fleur. L'originalité chimique est alors totale.

Les études sur les odeurs florales des inflorescences d'aracées sont encore peu nombreuses. Actuellement, elles ne sont connues que dans 11 genres soit environ 60 espèces, ce qui est faible en comparaison avec la diversité de la famille des aracées (122 genres et 3 300 espèces). De plus, la majorité des composés chimiques jouant un rôle dans l'attraction des pollinisateurs ne sont pas encore identifiés. Étant donnée la variété de types de pollinisation rencontrée chez les aracées, ce domaine de recherche semble prometteur pour une meilleure compréhension de l'évolution de la diversité des odeurs florales en relation avec la diversité des agents pollinisateurs.

M. G. et M. C.

Photo Marion Chartier

Le gouet tacheté* (Arum maculatum) *ayant piégé dans sa chambre florale, sous la couronne de fleurs stériles modifiées en « poils », un grand nombre de moucherons.

Pour en savoir plus :

Chartier M., Maia A. and Gibernau M. 2009. La pollinisation des aracées. Insectes, *154 (3): 5-7.*

Debroise, A. 2001. Le parfum pour signature. Science & Vie, *vol. 13 (Hors Série), p. 72-73.*

Gibernau M. and Barabé D. 2007. Des fleurs à « sang chaud ». Pour la Science, *N°359, Septembre, p. 50-56.*

www.aroidpictures.fr *: magnifiques photos d'aracées de David Scherberich.*

Deuxième partie

ÉTUDE À GRANDE ECHELLE DE L'ÉVOLUTION DE LA POLLINISATION CHEZ LES ARACÉES

Chapitre 1

Résumé des articles

Le CHAPITRE 2 est une étude corrélative qui a permis de mettre en évidence des correspondances entre les traits floraux et les types de pollinisateurs chez les Aracées. Elle fait suite à une première étude (Chouteau *et al.* 2008) dont le nombre d'espèces étudiées a été augmenté, et dont les statistiques corrélatives ont été refaites, et a fait l'objet d'un chapitre de livre.

Les espèces d'Aracées sont quasiment toutes pollinisées par des insectes, et ces interactions sont dans la majorité des cas spécialisées (Mayo *et al.* 1997, Gibernau *et al.* 2003, 2011). Les pollinisateurs des Aracées impliquées dans des interactions spécialisées sont répartis dans trois ordres qui sont les hyménoptères (Hymenoptera), les diptères (Diptera), et les coléoptères (Coleoptera). De plus, il existe quatre types d'interactions de pollinisation chez les Aracées. Le premier type de pollinisation est néotropical et caractéristique du genre *Anthurium*. Les espèces de ce genre sont pollinisées par des abeilles euglossines qui parcourent les inflorescences pour en récolter une résine ou des substances odorantes dont elles se servent pour créer un parfum attirant les femelles (Schwerdtfeger *et al.* 2002). Chez le genre *Monstera*, phylogénétiquement proche, ce sont des abeilles mélipones (sans dard) qui parcourent les inflorescences à la recherche de gommes ou de fluides stigmatiques, les Aracées ne produisant pas de nectar (Gibernau 2003). Le deuxième type de pollinisation spécialisée est assuré par des insectes de l'ordre des coléoptères, selon une interaction mutualiste qui est toujours la même, et qui implique une imbrication des cycles de reproduction de la plante et de l'insecte. Ce type d'interaction a beaucoup été étudié chez le genre *Philodendron* (Gibernau 2003). Ainsi, des coléoptères passent-ils une nuit et une journée consécutives à l'intérieur d'une chambre florale formée par la spathe de l'inflorescence, dans laquelle ils vont copuler et se nourrir, et dont ils ressortiront couverts de pollen (Gibernau 2003). Le troisième type d'interaction spécialisée est la pollinisation par des diptères, qui comme

les coléoptères, se retrouvent dans les inflorescences pour copuler et se nourrir, mais qui en plus pondent dans les inflorescences. Les larves de ces insectes se développent ensuite dans les parties pourrissantes des inflorescences. C'est le cas par exemple des espèces asiatiques du genre *Colocasia*, pollinisé par des mouches (Drosophilides) du genre *Colocasiomyia* (Sultana *et al.* 2006). Si les trois cas précédents sont des interactions mutualistes, le dernier type de pollinisation chez les Aracées est un antagonisme, la pollinisation par duperie. Les inflorescences imitent (olfactivement et visuellement) le site d'oviposition des insectes qui se font capturer dans un piège floral pour assurer le cycle de pollinisation (Gibernau *et al.* 2004).

La spécificité de ces interactions rend probable l'émergence de schémas d'adaptation des traits floraux aux différents pollinisateurs (syndromes de pollinisation), impliquant que l'on puisse discriminer les espèces pollinisées par des insectes appartenant à des ordres différents sur la base des traits floraux seulement. Cette discrimination n'était pas parfaite lors de l'étude de Chouteau *et al.* (2008). Nous avons donc ajouté des espèces à leur échantillonnage, et refait l'analyse discriminante avec des données supplémentaires. Nous avons par ailleurs essayé de déterminer si la pollinisation par duperie et la pollinisation généraliste (effectuée par des insectes d'ordres différents attirés par la même inflorescence) pouvaient aussi être caractérisés par des traits floraux particuliers.

Cette étude visait donc à répondre à deux questions principales : certains traits floraux correspondent-ils à certains types de pollinisateurs ? Si oui, ces traits floraux suffisent-ils à classer les espèces d'Aracées dans des groupes différents correspondant à leur mode de pollinisation ?

Pour répondre à ces questions, 17 traits floraux tels que le nombre de fleurs mâles et femelles, le nombre d'ovules et de grains de pollen produits par fleurs, la surface stigmatique ou le ratio pollen/ovule ont été mesurés sur 22 nouvelles espèces appartenant à 19 genres différents. Les ordres des différents pollinisateurs de ces espèces ont ensuite été répertoriés dans la littérature, et une analyse discriminante et des tests de comparaisons ont permis de sélectionner les traits floraux qui contribuaient le plus à différencier les espèces d'Aracées selon leurs types de pollinisateurs, et de déterminer les caractéristiques de chaque groupe. Ainsi, les espèces pollinisées par des hyménoptères se sont avéré produire un grand nombre de fleurs et une faible quantité de pollen par rapport aux autres espèces. Les espèces pollinisées par des diptères possédant des fleurs unisexuées, sont pérennes, et produisent un faible nombre de fleurs, tandis que les espèces pollinisées par des coléoptères possédent des fleurs unisexuées, produisent un nombre intermédiaire de fleurs et des grains de pollen ayant un volume plus gros que la moyenne. Une représentation graphique en deux dimensions des différences morphologiques,

ainsi que des tests de comparaisons des traits floraux entre les groupes d'Aracées ont ensuite permis de montrer que les espèces d'Aracées pollinisées par des hyménoptères étaient clairement différentes des espèces pollinisées par des diptères ou des coléoptères, ces deux derniers groupes se chevauchant plus ou moins. La discrimination imparfaite entre les Aracées pollinisées par des diptères ou des coléoptères a plusieurs explications. Elle peut évidemment être due à des observations incomplètes sur le terrain, ayant menées à de fausses interprétations du pollinisateur majoritaire de ces espèces, et donc exerçant le plus de pressions sélectives sur les traits floraux. Il se peut également que les espèces intermédiaires entre les deux groupes soient pollinisées par des insectes des deux ordres (pollinisation mixte), ou bien que les coléoptères et les diptères exercent les mêmes pressions de sélections sur les traits étudiés. Dans ce cas, expliquer les changements de pollinisateurs au cours de l'évolution nécessiterait l'utilisation de traits supplémentaires.

Par ailleurs, il est intéressant de noter que les espèces appartenant aux sous-familles des Lasioideae et des Orontioideae ont été groupées ensembles sans relation avec les groupes pollinisés par les hyménoptères, les coléoptères, ou les diptères, et pouvaient représenter un système de pollinisation généraliste. Enfin, les espèces qui dupent leurs pollinisateurs sont principalement pollinisées par des diptères et ne diffèrent pas significativement des espèces pollinisées par des diptères dans des interactions mutualistes.

L'étude du CHAPITRE 2 a permis de montrer que l'on pouvait classer les Aracées selon certains traits floraux dans différents groupes correspondant à des espèces pollinisées par des insectes appartenant à trois ordres différents. Cependant, les groupes pollinisés par les coléoptères et ceux pollinisés par les diptères ne sont pas totalement discriminés par ces traits.

Les CHAPITRES 3 et 4 (deux articles en préparation) ont donc consisté à rajouter des traits morphologiques qualitatifs à cette étude et à y inclure une dimension phylogénétique. Ceci a permis, d'une part, de prendre en compte les liens de parenté entre les espèces lors du calcul des corrélations entre les traits floraux et le mode de pollinisation, d'autre part de décrire l'histoire évolutive de la pollinisation chez les Aracées.

Pour cela, un premier travail a tout d'abord consisté à obtenir une phylogénie robuste de la famille des Aracées (CHAPITRE 3). Une phylogénie des Aracées a récemment été proposée sur la base de six marqueurs chloroplastiques (Cabrera *et al.* 2008, Cusimano *et al.* 2011). Cependant, certains embranchements profonds de l'arbre phylogénétique n'étaient toujours pas résolus, empêchant de comprendre le déroulement de la transition entre les clades d'espèces produisant

des fleurs bisexuées (clades basaux) et le clade dérivés d'espèces produisant des fleurs unisexuées ("Clade à fleurs unisexuées" *sensu* Cusimano *et al.* 2011). Ce problème semblait en partie lié à la mauvaise position dans la phylogénie d'un genre monospécifique, *Calla*, présentant des fleurs biséxuées, classé à la transition entre les clades à fleurs bisexuées et unisexuées par Cabrera *et al.* (2008) puis au milieu du clade à fleurs unisexuées par Cusimano *et al.* (2011). L'ajout pour la première fois d'un marqueur nucléaire (*PhyC*, Mathews et Donoghue 1999) à l'alignement de marqueurs chloroplastiques de Cusimano *et al.* (2011) a permis de résoudre de façon plus robuste deux nœuds profonds de la phylogénie : celui formant le "Clade à fleurs unisexuées", ainsi qu'un deuxième clade à fleurs unisexuées, que nous proposons de nommer le "clade *Cercestis*". Par ailleurs, pour la première fois à partir de données moléculaires, le genre *Calla* est placé dans un clade assez bien soutenu à la base du clade des espèces à fleurs unisexuées, avec les genres *Montrichardia* et *Anubias*. Bien que toujours en partie peu satisfaisante du point de vue morphologique, cette nouvelle position de *Calla* dans la phylogénie des Aracées est beaucoup plus logique que la précédente (Cusimano *et al.* 2011), car plus proche phylogénétiquement des clades à fleurs bisexuées.

La nouvelle phylogénie obtenue a ensuite servi de support à la cartographie des caractères floraux utilisés par Gibernau *et al.* (2010, CHAPITRE 1) auxquels ont été ajoutés de nouveaux traits potentiellement liés à la pollinisation, quantitatifs cette fois, tels que le type de fleurs, l'organisation du spadice, la forme de la spathe, ou l'ornementation de la surface des grains de pollen (Mayo *et al.* 1997, Cusimano *et al.* 2011). En tout, 19 traits floraux ont été répertoriés, auxquels ont été ajoutées des données sur l'ordre des pollinisateurs (généraliste, pollinisation par des diptères, hyménoptères ou coléoptères) ainsi que sur le type de pollinisation (pollinisation mutualiste par récompense, par imbrication des cycles de reproduction avec ou sans ponte des insectes, et pollinisation par duperie). Cette nouvelle classification permettait de prendre en compte la duperie et la pollinisation généraliste en tant que telles et non en tant que sous-catégories d'un type de pollinisation.

Dans le CHAPITRE 4, l'utilisation de la méthode de cartographie stochastique bayésienne, et de méthodes bayésiennes de tests de corrélations des traits sur l'arbre phylogénétique, ont permis de reconstruire l'histoire évolutive de la pollinisation chez les Aracées, et de mettre en évidence des corrélations entre l'apparition de certains traits morphologiques floraux et certaines modalités de pollinisation. Ainsi, l'ancêtre des Aracées présentait probablement une inflorescence plutôt simple, formée d'un spadice portant des fleurs bisexuées et une spathe peu différenciée, pollinisée

selon un mutualisme par récompense, et selon une interaction plutôt généraliste. L'inflorescence des Aracées a ensuite évolué en corrélation avec le mode de pollinisation, vers des interactions de plus en plus spécialisées. Chez les Pothoideae et Monsteroideae d'Amérique du sud, un système de récompense se serait spécialisé en relation avec des abeilles mélipones ou euglossines. Les modes de pollinisation ont été peu étudiés dans le clade suivant, les Lasioideae, qui constituent peut-être un clade de transition entre la pollinisation par les diptères et les coléoptères, et entre des espèces généralistes et spécialistes du point de vue de la pollinisation (Gibernau *et al.* 2003). Les changements majeurs des modalités de pollinisation sont apparus avec le clade des espèces à fleurs unisexuées, accompagnés de spécialisations des structures florales, comme par exemple la fermeture de la spathe en une chambre florale, accompagnée parfois d'une constriction. L'ancêtre de ce clade était probablement pollinisé par des coléoptères selon un mutualisme avec imbrication des cycles de reproduction, ayant évolué deux fois vers un mutualisme avec ponte des insectes (diptères) au sein du clade des Rhéophytes et du clade *Ambrosina*, ou ayant évolué vers de la pollinisation par des diptères selon un mutualisme avec ponte et ayant subit une réversion à la pollinisation par des coléoptères au sein du clade des Caladieae (hypothèse la plus probable).

Enfin, la pollinisation par duperie est apparue quatre fois au sein des Aracées, trois fois à partir de systèmes de pollinisation mutualiste avec ponte (diptères, *Dracontium*, *Cryptocoryne* et le clade terminal *Alocasia*) et une fois à partir d'un système de pollinisation par des coléoptères (*Peudodracontium*).

Les corrélations entre l'évolution des traits floraux et les modalités de pollinisation chez les Aracées, ainsi qu'entre l'évolution de traits floraux entre eux, permettent de suggérer que chez les Aracées, l'unité évolutive liée à la pollinisation est l'inflorescence (et non la fleur), et qu'il existe bien des syndromes de pollinisation probablement apparus au cours de l'évolution sous des pressions de sélection exercées par des pollinisateurs (comme par exemple la résine sécrétée par les inflorescences de certaines Aracées pollinisées par des coléoptères pour coller les grains de pollen sur le dos lisse des insectes). A l'inverse, l'apparition de certains modes de pollinisation, comme par exemple la pollinisation par duperie avec séquestration des insectes dans une chambre florale, a probablement été conditionnée par la présence préalable de certains traits, comme par exemple une spathe refermée à la base autour du spadice portant les fleurs.

Chapitre 2

Avancées récentes vers une compréhension de l'évolution de la pollinisation chez les Aracées

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Recent Advances Towards an Evolutionary Comprehension of Araceae Pollination

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Abstract—A correspondence between floral traits and pollinator types is found in Araceae. Hence different complexes of floral traits are associated with bee-, fly- and beetle-pollinated aroids. Using the method of non-metric multidimensional scaling (NMDS) bee-pollinated species appear to have very different floral traits from fly- and beetle-pollinated taxa, these two groups showing some overlapping. This imperfect discrimination between fly- and beetle-pollinated aroids may have several explanations which are discussed. Interestingly the species belonging to the Lasioideae and Orontioideae subfamilies are grouped together not in relation to fly- and bee- pollinated groups and may represent a generalist pollination system. Finally, floral traits of fly deceptive species appear to be characteristic of the fly-pollinated taxa and not clearly different from fly-mutualistic species.

Keywords—bee, beetle, co-adaptation, floral traits, fly, pollination syndrome.

In all pollination systems, pollinators visit flowers looking for a resource, which can be alimentary (stigmatic fluid, pollen, floral parts, etc.) or reproductive (mating and/or laying site). Flowers in return have developed adaptations to transform this pollinator behaviour (e.g., resource seeking) into a 'pollinating act', and thus ensure their reproduction.

In insect-pollinated taxa, it is assumed that floral traits have evolved in relation to the interaction between the flower and the 'most efficient' pollinator in order to increase the frequency of this interaction and thus of plant

reproductive success (Stebbins 1970; Cruden 2000; Fenster et al. 2004). The 'most efficient' pollinator will be the agent among the different flower visitors whose visits ensure a higher seed production due to its particular behaviour or its high frequency/abundance. In consequence, pollinators become selective agents of floral traits associated with attraction, but also of fertile/sexual parts of the flowers leading to adaptations that increase visits by efficient pollinators (Fenster et al. 2004).

Thus, in specialized pollination systems, we expect to find some kind of correlation or association between the most efficient pollinator and the floral traits associated with it. Consequently, the selective pressure of the different types of pollinators has led to pollination syndromes (reviewed in Fenster et al. 2004): adaptive floral character complexes resulting in different types of floral architecture adapted to particular groups of pollinators. On the contrary, in non-specialized pollination systems, no such association or correlation will be found as different pollinators are likely to exert different even opposite selective pressures on floral traits (Waser et al. 1996).

Araceae is a monocotyledon family mainly pollinated by insects and this type of interaction may lead to a process of specialization/adaptation of aroid inflorescences (Mayo et al. 1997; Gibernau 2003). Grayum (1986) was the first author to suggest the existence of such adaptive traits in relation to the pollinator in his palynological study. He grouped pollen grains into 5 groups according to their exine sculpturing: psilate, striate, verrucate(+tuberculate), foveolate (+reticulate), and spinose. He found that "*psilate pollen is intimately and almost exclusively associated with beetle pollination and spinose pollen is equally closely associated with fly pollination…Verrucate and tuberculate pollen in Araceae also seems to be fairly well correlated with beetle pollination….Striate and foveolate exines…being less extreme, might be adaptable to a wider range of vectors. Sculpturing, not size, is thus the overriding factor*". This first study showed clearly that some floral characters, here the exine sculpturing of pollen grains, may be adapted to the type of pollinator. In fact, most of the Araceae seem to have quite a specific or specialized pollination system which can be divided into three types: bee, beetle, and fly (Gibernau 2003).

The first type occurs in the Neotropics within the genera *Anthurium* Schott and *Spathiphyllum* Schott (Montalvo and Ackerman 1986; Schwerdtfeger et al. 2002), which are pollinated by male euglossine bees in a way very similar to orchids (Dressler 1982). The male euglossines visit the inflorescences to collect resin and/or odoriferous substances used in the building of their nests. In the neotropical genus *Monstera* Adanson, typically considered a bee-pollinated genus, the data are contradictory. Several species are described as pollinated by stingless bees *Trigona* Jurine, which collect stigmatic and gums (Madison 1977; Ramirez and Gomez 1978; Ramirez 1980) whereas *M. obliqua* has been found to be beetle-pollinated (Chouteau et al. 2007).

The second type of specialized system is beetle-pollination. Even if the different genera and families of beetles are implicated (review in Gibernau 2003),

the interaction is functionally the same. Pollinating beetles are attracted to the receptive inflorescences in which they mate and eat various floral parts (sterile flowers or tissue, pollen grains). This pollination system is widespread within the Araceae and present in five subfamilies: Orontiodieae, Pothoideae, Lasioideae, Philodendroideae, and Aroideae (Gibernau 2003).

The third type of specialized system is fly-pollination. This interaction is mainly represented by one Asian fly genus, *Colocasiomyia* de Meijere (Drosophilidae), which pollinates Asian genera from the subfamilies Monsteroideae, Schismatoglottideae, Philodendroideae, and Aroideae (Sultana et al. 2006). The notable exceptions are a chloropid fly which pollinates *Peltandra virginica* Rafinesque in North-America (Patt et al. 1995) and unidentified drosophilids in the African genus *Culcasia* Palisot de Beauvois (Knecht 1983). Here again, the interactions follow the same functional schema. Flies visit the receptive inflorescences in order to mate and females oviposit their eggs on the inflorescences surface. The adults feed on the stigmatic secretions and on pollen whereas larvae eat decomposing matter and rotting flowers such as the stamens after pollen release. The inflorescence becomes part of the reproductive cycle of the pollinator. Insects visit Araceae inflorescences not only for food rewards (nectar, pollen, or floral tissue) but also to meet sexual congeners, achieve copulations, and sometimes lay their eggs.

In some genera of Araceae; depending on the genus, generalist or deceit systems can be found. Generalist pollination systems are rare and only documented in two species, *Lysichiton camtschatcense* Schott (Tanaka 2004) and *Symplocarpus renifolius* Schott (Uemera et al. 1993), both belonging to the basal Orontioideae subfamily. In these cases, various insects, a few of them documented as efficient pollinators, can be found in low frequencies. One interesting point is that not a single aroid species is known to offer nectar as a reward (Schwerdtfeger et al. 2002). In the deceit pollination system, the inflorescence dupes the pollinators by mimicking its laying sites (faeces, mushrooms, dead animal, etc.). Hence, the insects visit the inflorescence in order to complete their reproductive cycle. Through this deceptive attraction, the insects perform pollination but without actually receiving any reward (e.g., *Arum* L., *Helicodiceros* K. Koch, *Amorphophallus* Decaisne). This pollination system has only been documented in the Araceae family for some genera of the Aroideae subfamily.

In a previous study of the Araceae family, it has been shown that some specific floral traits were associated with pollination by bees, beetles, and flies. However, the discrimination between the three types of pollination was not perfect (Chouteau et al. 2008). In order to further understand the relationship between aroid floral traits and the type of pollinator, the floral characters of new species were measured and a new discriminant analysis was performed. For example, we added *Monstera obliqua* Miquel, a beetle-pollinated species belonging to a bee-pollinated genus, to our analysis. Moreover, we investigated whether deceit pollination systems were characterized by unique floral traits.

More precisely, the purposes of this study were: 1) to see if the different types of pollination in the Araceae family are correlated with specific floral traits and 2) to see if the boundaries between different types of pollination are clearly delimited.

Material and Methods

This study was conducted on 22 species belonging to 19 genera of *Araceae* sampled from the living collections of the Botanical Garden of the Montet (Nancy, France) as well as from the field in Corsica, French Guiana, Japan, and Sarawak (Borneo, Malay) (Table 1). Data from 46 species belonging to 27 genera taken from Chouteau et al. (2008) were added to our new data set. Note that five genera are common to both sets of data. Overall, the new analysis included 68 species belonging to 41 genera.

The same floral traits which have proven to discriminate among the types of pollinators in Araceae were measured (Chouteau et al. 2008). In order to count the pollen grains, inflorescences were collected during the first day of their flowering cycle, before the pollen is released (e.g., lost) in order to be able to calculate the pollen/ovule ratio. Floral ratio can vary according to the pollinator, thus for each inflorescence, the total numbers of flowers (female and male) were counted. In some cases, the number of male flowers was estimated by counting the number of male flowers on a 5 mm slice cut in the middle of the male zone. The total number of stamens was obtained by multiplying the number of stamens on the slice by the total length of the male zone and dividing by 5 (for details see Chouteau et al. 2008). The mean number of stamens per flower was counted on 10 flowers from at least three separate inflorescences. Different types of pollinator have different level of pollination efficiency which may affect female flowers. The number of ovules per flower was estimated by counting the number of locules on 10 flowers and the number of ovules per locule on these 10 flowers for each inflorescence collected. Ovule number per inflorescence was obtained by multiplying the mean number of ovules per flower by the mean number of flowers per inflorescence bearing ovules.

To estimate the number of pollen grains per inflorescence, three stamens were dissolved individually in 300 µl of 95% sulphuric acid, for 3-5 days at 24° C. The solution was homogenized every day. The last day, 1 µl was collected and carefully placed on a microscope slide. The number of pollen grains was counted for three independent replicates of 1 µl. The number of pollen grains per stamen was obtained by multiplying the mean of the triplicate by 300 (for details see Chouteau et al. 2008). For two species *Aridarum nicolsonii* Bogner and *Piptospatha elongata* (Engl.) N.E.Br., no pollen grains were observed after the acid digestion, thus pollen grains per flower were directly counted from three fresh stamens squashed between microscope slides and cover glasses. Pollen grains per inflorescence were obtained by multiplying the mean number of pollen

Species	Location of collection	Sample number	Date of collection
Symplocarpus renifolius Schott	Katashina-mura Tone-gun, Japan	3	April 2007
Lysichiton camtchatcense Schott	Katashina-mura Tone-gun, Japan	3	April 2007
Monstera oblique Miquel	Petit Saut Dam (French Guiana)	8	June 2007
Philodendron acutatum Schott	Petit Saut Dam (French Guiana)	3	June 2007
Aglaonema commutatum Schott	Nancy Botanical Garden(France)	3	August 2007
Callopsis volkensis Engl.	Nancy Botanical Garden(France)	3	August 2007
Chlorospatha longipoda (K. Krause) Madison	Nancy Botanical Garden(France)	3	August 2007
Dracontioides descicens (Schott) Engl.	Nancy Botanical Garden(France)	3	August 2007
Nephthytis hallaei (Bogner) Bogner	Nancy Botanical Garden(France)	3	August 2007
Schismatoglottis neo-guineensis (Linden ex André) N. E. Br.	Nancy Botanical Garden(France)	3	August 2007
Scindapsus hederaceus Schott	Nancy Botanical Garden(France)	3	August 2007
Spathicarpa hastifolia Hook.	Nancy Botanical Garden(France)	3	August 2007
Ulearum sagittatum Engl.	Nancy Botanical Garden(France)	3	August 2007
Aridarum nicolsonii Bogner	Sarawak (Borneo, Malay)	9	December 2004
Dieffenbachia seguine Schott	Nouragues (French Guiana)	7	July 2006
Dieffenbachia paludicola N.E. Br. ex Gleason	Nouragues (French Guiana)	7	July 2006
Helicodiceros muscivorus (L.f.) Engl.	Corsica (France)	12	April 2004
Arum concinnatum Schott	Crete (Greece)	9	April 2007
Homalomena hostiifolia Engl.	Sarawak (Borneo, Malay)	3	December 2004
Homalomena sp.	Sarawak (Borneo, Malay)	3	December 2004
Piptospatha elongata (Engl.) N.E.Br.	Sarawak (Borneo, Malay)	3	December 2004
Piptospatha grabowski (Engl.) Engl.	Sarawak (Borneo, Malay)	3	December 2004

Table 1. List of species, locations and dates of collection, and sample numbers studied.

grains per flower by the mean number of flowers bearing pollen. In the same way, the pollen grain volume per inflorescence was obtained by multiplying the mean number of pollen grains per inflorescence by the mean pollen volume of the species concerned (see below).

The size of elongate pollen grains was estimated by measuring the diameter of the polar and equatorial axes of the grains. The volume of a single pollen grain was estimated using the formula $\pi PE^2/6$ (Harder 1998), where P is the polar axis and E the equatorial axis diameter. For globose pollen, the diameter D was measured and the volume calculated with the formula $(4/3)\pi(D/2)^3$. Generally, 10 pollen grains per inflorescence were measured from three independent inflorescences (generally $N = 30$).

The pollen-ovule ratio was calculated for the inflorescence by dividing the mean number of pollen grains per inflorescence by the mean number of ovules per inflorescence.

For each inflorescence studied, the stigma area (estimated as a circle) of 10 flowers was calculated using the diameter (0.01mm resolution) of the stigmas measured at 20× magnification under a dissecting microscope equipped with an ocular micrometer and using the formula $\pi D^2/4$ where D is the measured diameter. To obtain the total stigmatic area of the inflorescences, the mean stigma area was multiplied by the mean number of flowers bearing stigmas for each species. Life form, growth mode, and climatic region were obtained using Mayo et al. (1997) and from personal observations (for details see Chouteau et al. 2008).

The discriminant analysis was conducted for three types of pollinating insects (grouping variable) – bee, beetle and fly – according to the data available in the literature (see Gibernau 2003 for a review). Twenty-three species were coded as beetle-pollinated, 21 as fly-pollinated, seven as bee-pollinated, and 17 as unknown. The 17 floral traits (variables) available for all species were selected in order to test any discrimination among the three pollinator groups: stigma area per flower and per inflorescence, mean volume of a pollen grain, pollen volume per flower and per inflorescence, pollen number per flower and per inflorescence, number of locules per female flower, number of ovules per locule, per flower and per inflorescence, pollen-ovule ratio, number of male and female flowers, sexual type of the flower, growth mode, and life form. A preliminary step consists in performing a stepwise backward discriminant analysis which allowed us to reduce the number of discriminant floral traits (Systat 8.0). The results of the discriminant analysis are not totally reliable because both categorical and continuous data were used. Consequently, differences among the six floral traits (e.g., resulting from the discriminant analysis) were represented with the method of non-metric multidimensional scaling (NMDS) using the Gower similarity measure which allows using both categorical and continuous data (PAST 1.74). This non parametric method represents the studied species in a two-dimensional coordinate system preserving the ranked differences between the species. The more two points are separated in the score plot, the more dis-

tinct the floral traits are. The stress value gives the percentage of difference not optimally represented by the analysis. In order to visualize the type of pollinator, each species has been coded by a coloured symbol representing the different types of pollinator. Differences in floral traits between the different types of pollinators were tested using ANOVA (Systat 8.0).

Results

Table 2 summarizes the floral traits, life form, growth mode, and pollinator type for the 22 newly studied aroid species.

The stepwise backward discriminant analysis of the 68 species held back six variables, even if some other variables showed significant differences between pollinator types (Table 3): pollen volume per inflorescence, pollen number per inflorescence, number of female and male flowers, sexual type of the flower, and growth mode. The NMDS plotting distinguishes the three pollinator groups although some overlapping occurs (see Fig. 1) and some species were misclassified (see below). The bee-pollinated group is characterized by species with bisexual flowers, an evergreen life form type, and a high number of male and female flowers (Table 3). Beetle-pollinated species are characterized by a high pollen volume per inflorescence, a medium number of male and female flowers, and almost always bearing unisexual flowers (see Table 3). Fly pollination is associated with species with low numbers of male and female flowers and a relatively low number of pollen grains per inflorescence (Table 3). The P/O was much higher in beetle- (mean: 44,004) and fly-pollinated species (mean: 32,511) than in bee-pollinated species (mean: 10,605), but these differences were not significant (Table 3). Pollen grain volume in relation to pollinator class displayed the same kind of difference, with pollen volume of beetle-pollinated species being significantly larger (mean: 419,828 μm^3) than related fly- (mean: 28,903 μm^3) and bee-pollinated (mean: 15,145 μm^3) species (Table 3). In the same way, the flower stigma surface was significantly larger in beetle-pollinated species (mean: 3.85 mm^2) than in fly- (mean: 0.90 mm^2) or bee-pollinated (mean: 0.64 mm^2) taxa (Table 3).

Now we shall consider species classification according to pollinator type since some species had been misclassified – in fact, 12 out of 51. Four beetle-pollinated species were classified among fly-pollinated species, namely: *Anubias barteri* Engl. and *A. heterophylla* Engl., *Caladium bicolor* (Ait.) Vent. and *Xanthosoma conspurcatum* Schott, and two among bee-pollinated taxa, *Monstera obliqua* and *Rhaphidiphora schottii* Hook. f. (Fig. 1). Inversely, *Alocasia amazonica* Andre., *A. portei* Becc. & Engl., *A. macrorrhizos* (L.) G.Don, *Aglaonema commutatum* Schott, *Schismatoglottis neo-guineensis* (Linden ex André) N. E. Br., and *Homalomena* sp. fly-pollinated species were classified among beetle-pollinated species (Fig. 1).

The fly-pollinated *Dracontium polyphyllum* L. appears close to *Anaphyllopsis americana* (Engl.) A. Hay and *Dracontioides descicens* (Schott) Engl., two 'unknown taxa'. These species are clearly not related to fly- and bee-pollinated groups

Species	Life form	Growth mode	Pollinator	Stigma area (mm^3)	Pollen grains volume (μm^3)	Number of male flowers per inflorescence	Number of female flowers per inflorescence	Number of pollen grains per flower	Number of pollen grains per inflorescence	Number of ovules per flower	Number of ovules per inflorescence	Ratio pollen/ ovule per inflorescence
Symplocarpus reniifolius	S	T	fly	0.18 ± 0.1	75,584 ± 17,155	145 ± 18	145 ± 18	108,667 ±78,630	14,620,667 ± 8,594,082	1.0 ± 0.0	145 ± 18	108,667 ± 78,630
Lysichiton camtchatcense	S	H	fly	0.06 ± 0.01	161,540 ± 8,759	236 ± 118	236 ± 118	23,156 ± 8,365	4,798,578 ± 1,319,381	2.0 ± 0.0	471 ± 237	11,578 ± 4,183
Monstera obliqua	E	HE	beetle	0.3 ± 0.2	177,594 ± 73,014	56 ± 10	56 ± 10	26,850 ± 7,522	1,528,067 ± 536,866	4.0 ± 0.0	226 ± 38	6,713 ± 1,881
Philodendron acutatum	E	HE	beetle	1.4 ± 0.4	319,477 ± 67,866	2,982 ± 92	746 ± 4	8,910 ± 7,623	25,166,460 ± 23,404,527	62.8 ± 3.6	46,851 ± 2,456	525 ± 472
Aglaonema commutatum	E	T	fly	0.03 ± 0.01	91,774 ± 7,492	181 ± 15	13 ± 7	31,175 ± 13,965	5,895,750 ± 3,159,736	1.0 ± 0.0	13 ± 7	430,252 ± 349,413
Callopsis volkensis	E	G		0.13 ± 0.02	34,306 ± 8,431	121 ± 1	11 ± 1	1,356 ± 317	164,136 ± 39,191	1.0 ± 0.0	11 ± 1	15,222 ± 4,968
Chlorospatha longipoda	E	T	beetle	0.002 ± 0.001	12,287 ± 3,186	64 ± 4	33 ± 3	2,600 ± 521	165,557 ± 28,892	19.5 ± 3.5	632 ± 81	269 ± 80
Dracontioides descicens	S	H		0.16 ± 0.04	4,797 ± 2,307	141 ± 4	141 ± 4	153,500 ± 13,763	21,620,766 ± 2,458,006	1.9 ± 0.1	272 ± 9	79,583 ± 11,912
Nephthytis hallaei	E	G		0.19 ± 0.03	30,346 ± 10,570	58 ± 12	9 ± 3	1,089 ± 504	58,903 ± 13,916	1.0 ± 0.0	9 ± 3	6,963 ± 2,591
Schismatoglottis neoguineensis	E	T	fly	0.07 ± 0.02	2,212 ± 1,029	198 ± 3	277 ± 48	59,490 ± 9,666	11,808,060 ± 2,014,416	21.6 ± 1.0	5,942 ± 775	2,041 ± 640
Scindapsus hederaceus	E	HE		0.004 ± 0.001	121,876 ± 8,295	76 ± 3	76 ± 3	54,400 ± 15,546	4,132,233 ± 1,105,668	1.0 ± 0.0	76 ± 3	54,400 ± 15,546
Spathicarpa hastifolia	S	G		0.07 ± 0.01	12,286 ± 3,185	12 ± 3	17 ± 2	4,793 ± 2,387	58,593 ± 37,755	1.0 ± 0.0	17 ± 2	3,610 ± 2,644
Ulearum sagittatum	E	G		0.28 ± 0.09	17,121 ± 2,824	48 ± 5	22 ± 1	480 ± 480	19,583 ± 5,916	1.0 ± 0.0	22 ± 1	450 ± 163
Dieffenbachia seguine	E	H	beetle	4.47 ± 0.4	2,509,040 ± 418,007	235 ± 26	46 ± 8	6,760 ± 2,056	1,531,040 ± 558,747	1.97 ± 0.08	90 ± 18	18,444 ± 5,938
Dieffenbachia paludicola	E	H	beetle	21.01 ± 6.02	4,462,440 ± 341,046	169 ± 18	10 ± 1	2,703 ± 1,787	497,366 ± 341,956	7.17 ± 1.63	69 ± 18	6,223 ± 3,532
Helicodiceros muscivorus	S	G	fly	0.02 ± 0.01	32,459 ± 14,424	261 ± 46	158 ± 28	10,446 ± 1,899	2,700,942 ± 559,726	4.5 ± 0.7	726 ± 212	3,944 ± 1,066
Aridarum nicolsonii	E	R	fly	0.99 ± 0.29	4,849*	353 ± 113	98 ± 17	20 ± 10	6,724 ± 3,003	14.5 ± 2.7	1,385 ± 372	5 ± 2
Arum concinnatum	S	G	fly	0.21 ± 0.06	17,157*	114 ± 26	57 ± 14	2,588 ± 2,835	302,870 ± 359,358	7.4 ± 3.3	437 ± 237	755 ± 831
Homalomena hostiifolia	E	T	beetle	2.43 ± 0.30	3,054*	210 ± 29	145 ± 27	7,850 ± 4,621	1,716,310 ± 1,111,200	255.6 ± 50.9	37,942 ± 14,934	43 ± 24
Homalomena sp	E	T	fly	3.21 ± 0.38	3,054*	289 ± 45	169 ± 19	37,461 ± 17,081	10,474,250 ± 4,154,627	207.7 ± 34.6	35,327 ± 9,359	300 ± 117
Piptospatha elongata	E	R	fly	1.11 ± 0.05	8,181*	233 ± 53	129 ± 20	58 ± 24	13,471 ± 7,153	19.4 ± 3.0	2,555 ± 744	5 ± 2
Piptospatha grabowski	E	R	fly	2.42 ± 0.43	8,181*	317 ± 97	110 ± 25	15,778 ± 14,879	4,071,111 ± 2,747,889	44.8 ± 3.3	4,969 ± 1,388	970 ± 925

Table 2. Life form, growth mode, pollinator, and floral traits measured for 22 aroid species in 19 genera. Life form was coded: E = Evergreen, S = Seasonally dormant. Growth mode was coded: T = Terrestrial, H = Helophyte, G = Geophyte, E = epiphyte, HE = Hemiepiphyte, FF = Free floating.

(Fig. 1). Interestingly, these three species belong to the Lasioideae subfamily and are grouped with *Lysichiton camtschatcense* and *Symplocarpus renifolius* two examples of a generalist pollination system. The other species with unknown pollinators were tentatively classified as follows: *Stenospermation sessile* Engl., *S. longipetiolatum* Engl., and *Scindapsus hederaceus* Schott may be bee-pollinated. *Homalomena philippinensis* Engl. and *H. rubescens* Kunth may be among the beetle-pollinated species. *Pistia stratiotes* L. is rather fly-pollinated, but its outstanding position may suggest an original unknown pollination system (Fig. 1). All the other taxa with unknown pollinators, *Synandrospadix vermitoxicus* Engl., *Pseudodracontium fallax* Serebr., *Gonatopus boivinii* (Decne) Engl., *G. angustus* N.E. Br., *Zamioculcas zamiifolia* (Loddiges) Engl., *Callopsis volkensis* Engl., *Nephthytis hallaei* (Bogner) Bogner, *Spathicarpa hastifolia* Hook., and *Ulearum sagittatum* Engl. were

Floral character	Beetle pollination (N = 23)	Fly pollination (N = 21)	Bee pollination (N = 7)	Statistic values $F_{2,48}$ =
Flower sexual type [1]	1.91 ± 0.29	1.85 ± 0.36	1 ± 0	26.08***
Growth mode [2]	3.04 ± 1.06	1.86 ± 0.85	4 ± 1	15.52***
Pollen volume per inflorescence	14.6 ± 20.6 x 10^{11}	1.84 ± 2.95 x 10^{11}	2.22 ± 2.73 x 10^{11}	5.06*
Pollen number per inflorescence	11.6 ± 21.2 x 10^{6}	5.60 ± 5.55 x 10^{6}	23.97 ± 29.84 x 10^{6}	2.72
Male flower number	496.5 ± 650.5	210.5 ± 123.6	710.6 ± 881.2	2.75
Female flower number	254.6 ± 333.8	119.2 ± 69.5	853.4 ± 849.7	9.97***
Life form [3]	1.09 ± 0.29	1.57 ± 0.51	1 ± 0	10.98***
Flower stigma area	3.85 ± 5.55	0.90 ± 1.01	0.64 ± 0.23	3.95*
Mean pollen grain volume	420 ± 1,022 x 10^{3}	28.9 ± 38.4 x 10^{3}	15.1 ± 17.1 x 10^{3}	2.04
Ovule number per flower	22.81 ± 53.11	22.83 ± 45.3	5.28 ± 3.54	0.43
Nb of locules per flower	2.96 ± 2.75	1.61 ± 1.06	2.43 ± 0.53	2.51
Nb of ovules per locule	7.39 ± 17.61	13.87 ± 19.79	2 ± 1	1.46
Pollen-ovule ratio	44,004 ± 90,220	32,511 ± 94,616	10,605 ± 7,131	0.41

1 The flower sexual type was coded: 1 = bisexual, 2 = unisexual.

2 The growth mode was coded: 1 = geophyte, 2 = helophyte, 3 = ground, 4 = hemiepiphyte, 5 = epiphyte.

3 The life form was coded: 1 = evergreen, 2 = seasonally dormant.

Table 3. Group means (± standard deviation) used in the discriminant analysis for the different floral characters according to type of pollinator in 51 species of Araceae. The first six variables were selected by the discriminant analysis. Note that some variables showing statistically significant differences were not included in the analysis. The level of significance of the ANOVA results is coded as follows: * P < 0.05, ** P < 0.01, *** P < 0.001.

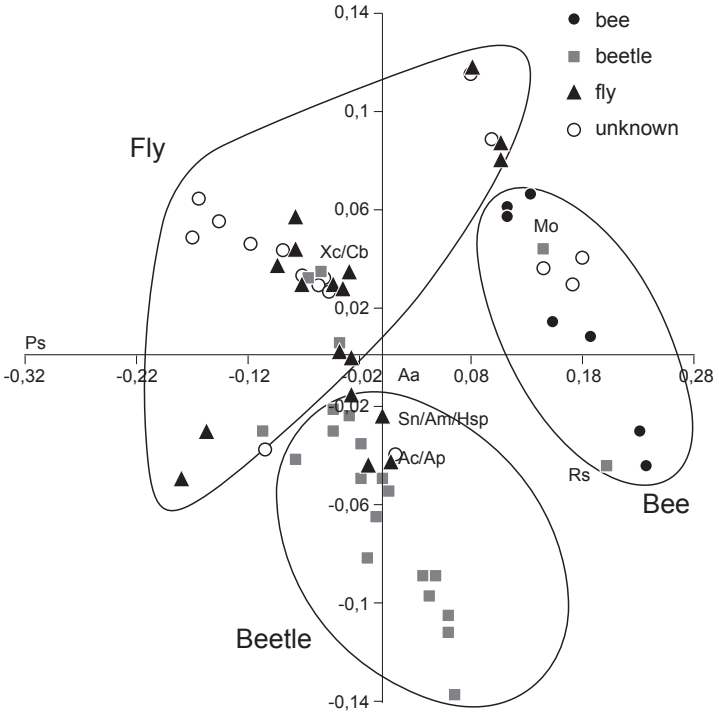

Fig. 1. Non-metric multidimensional scaling (NMDS) representation of the six selected floral traits (see results section) comprising overall data (48 genera, 68 species). Stress value = 0.12. The Letter Codes are 'misclassified' species which floral characters do not correspond to their type of pollinator. Among fly-pollinated species Xc: *Xanthosoma conspurcatum*, Cb: *Caladium bicolor*, Ab: *Anubias barteri* and Ah: *Anubias heterophylla*. Among bee-pollinated species Mo: *Monstera oblique* and Rs: *Rhaphidophora schottii*. Among beetle-pollinate species: Aa: *Alocasia amazonica*, Sn: *Schismatoglottis neo-guineensis*, Am: *Alocasia macrrorhizos*, Hsp: *Homalomena* sp., Ac: *Aglaonema commutatum* and Ap: *Alocasia portei*. Outstanding species on the left Ps: *Pistia stratiotes*.

clearly classified as fly-pollinated (Fig. 1). Note that the classifications of the unknown species must be considered as hypotheses to validate in the field.

Finally, the deceit pollination systems is not clearly defined in this analysis since fly-deceptive taxa such as *Arum italicum* Mill. or *Helicodiceros muscivorus* (L.f.) Engl. are close to two mutualistic fly-pollinated species like *Colocasia esculenta* (L.) Schott or *C. fallax* Schott and the two beetle-pollinated species *Caladium* Ventenat and *Xanthosoma* Schott within the fly-pollinated 'cloud' (Fig. 1).

Discussion

We confirm a generally good correspondence between floral traits and pollinator types. Hence, different complexes of floral traits are associated with bee-, fly-, and beetle-pollinated aroids (Fenster et al. 2004; Chouteau et al. 2008). It appears that bee-pollinated taxa have very different floral traits from fly- and beetle-pollinated ones, these two latter groups showing some overlapping. This

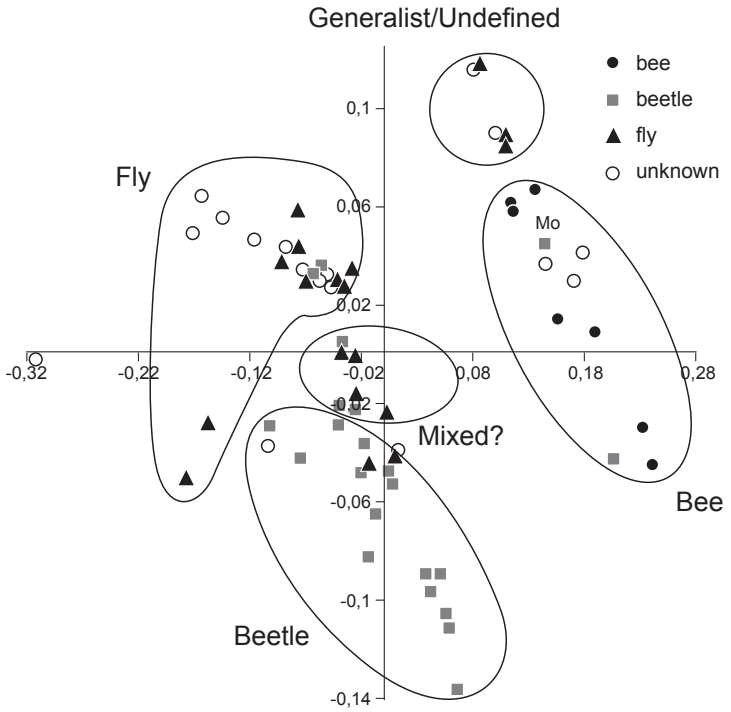

Fig. 2. Interpretation of the NMDS representation represented in figure 1 with respect to the floral traits and pollination types already known in the Araceae family.

imperfect discrimination between fly- and beetle-pollinated aroids may have several explanations. The first is that some of the species misclassified by the discriminant analysis may have been wrongly labeled as beetle- or fly-pollinated due to incomplete observations. Thus, field validations are needed for these 'intermediate'/misclassified species. Second, fly- and beetle-pollination may represent partially similar selective pressure on flowers leading to a convergence of some floral traits and thus an incomplete discrimination. In such a case, the shift from one type of pollination to another might be due to a change in a single character that is not included in our analysis, for example the odour or the colour of the spathe. Third, some of the misclassified species may indeed be pollinated by both flies and beetles. Consequently, floral traits are under a double selective pressure leading to the evolution of floral traits with characteristics intermediate between fly and beetle-pollinated floral traits. These intermediate species may represent a mixed pollinated system (Fig. 2), which needs to be validated by further field studies.

Interestingly the three species belonging to the Lasioideae subfamily are grouped with *Lysichiton camtschatcense* and *Symplocarpus renifolius*, two examples of a generalist pollination system in an intermediate position between fly- and bee-pollinated groups. We hypothesize that this group in an intermediate position between fly- and bee-pollination systems may represent a generalist pol-

lination system (Fig. 2). This hypothesis needs to be validated in the field and through study of other potentially generalist species, such as *Calla palustris* L..

Floral traits of fly-deceptive species appear to be characteristic of the fly-pollinated taxa with no clear discrimination. However, more data are needed in order to increase the sample size of deceptive species ($N = 6$). Moreover it could be interesting to include some beetle-deceptive pollinated species such as *Amorphophallus* species to test whether they will be grouped close to beetle-pollinated species or not.

Pistia stratiotes, an aquatic species, appears to be related to fly-pollination even if its pollinators are still unknown. But its outstanding position on the NMDS representation may suggest an original pollination system may be linked to its aquatic habit and the extreme reduction of its inflorescence which is functionally one male and one female flower. Further studies on this very common aquatic tropical species are needed.

Surprisingly, *Monstera obliqua* a species pollinated by small nitidulid scarabs (Chouteau et al. 2007), appears within the bee-pollinated group. We proposed several explanations. First, *M. obliqua*, belonging to the Monsteroideae subfamily, may be phylogenetically constrained against any drastic floral changes from bee-pollination characteristics which are present in other genera of this subfamily. Second, the pollination of *M. obliqua* by nitidulid scarabs may be recent from an evolutionary point of view and the selective pressure of the scarabs on the floral trait characteristics still be ongoing. Third, pollination by nitidulid beetles may not require different floral traits, and those associated with bee-pollination may also be adapted to nitidulid beetle pollination leading to a reduced selective pressure on floral traits by beetles. Fourth, *M. obliqua* may be pollinated by bees and the conclusions of Chouteau et al. (2007) could be erroneous because nitidulid scarabs may not be efficient pollinators. Further studies of pollination in the genus *Monstera* are needed to explain the pattern resulting from the discriminant analysis.

In order to fully understand the evolution of floral traits in relation to pollinators in Araceae further work is needed. In particular, more species should be studied in order to document all the different aroid taxonomical groups, but also to increase the sample sizes of the different pollination systems, particularly the newly proposed ones (generalist, mixed) and deceit pollination. More floral traits could be added, most obviously, the types of exine sculpturing of pollen grains, since large data sets are available (Thanikaimoni 1969; Grayum 1992; Hesse 2006). However, in some cases, the exine sculpturing of pollen grains varies within a genus, as in *Syngonium* Schott (Grayum 1986), and thus one must be carefully assign a type of pollen exine to a given genus particularly when the same species has not been studied. Finally, the recent molecular phylogeny of the aroid genera will permit the mapping of discriminant floral traits on the phylogenetic tree in order to study their changes and (co-)evolution in relation to phylogeny and pollinator type (Cabrera et al. 2008).

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Chapitre 3

Relations phylogénétiques entre les Aracées : avancées à partir d'un marqueur moléculaire nucléaire

Soumission prévue dans ***TAXON*** .

Auteurs : Marion CHARTIER, Natalie CUSIMANO, Martina SILBER, Marc GIBERNAU.

Phylogenetic relationships within Araceae: insights from one nuclear gene marker

3.1 ABSTRACT

Araceae is a plant family from the early-diverging monocotyledon Alismatales, composed of 117 genera including about 3400 species. The most recent phylogeny of the Araceae family has been inferred from a set of six chloroplast markers, but some deep nodes of the phylogeny remained to be resolved. One reason for such result is the uncertainty of the position in the phylogeny of one monospecific genus bearing bisexual flowers, *Calla*. This genus has so far been classified with molecular data at different positions within the unisexual flowers clade, the most derived aroid clade, which appears to be improbable from a morphological point of view.

Here, a nuclear marker, *Phytochrome C*, (*PhyC*), was added to the last chloroplast alignment of the family of 53 genera of the Araceae and three outgroup taxa. Data were obtained from amplified sequences, and from GenkBank data. Maximum Likelihood and Bayesian analyses were then performed to infer a new phylogeny of Araceae.

Our results were congruent with the former phylogeny, and allowed to improve two of the deep nodes, and to place *Calla* for the first time into a well supported clade (support values = 46/0.98) at the base of the Unisexual Flower clade. The phylogenetic relationships of the different clades are discussed and one new clade tentatively named "*Cercestis* clade" is proposed to be recognized.

3.2 INTRODUCTION

Araceae is a plant family from the early-diverging monocotyledon Alismatales. This family is composed of 117 genera including about 3 400 species (CATE Araceae 2011), mainly distributed under tropical latitudes (Mayo *et al.* 1997). Araceae species show a great variety of growing forms and vegetative shapes (Croat 1985, Mayo *et al.* 1997). Flowers are disposed together onto a cylindrical axis, the spadix, surrounded by a modified bract, the spathe, forming striking inflorescences that can show great variations (Mayo *et al.* 1997).

Araceae classification (reviewed in Nicolson 1987, Grayum 1990, Mayo *et al.* 1997, Cabrera *et al.* 2008) began in the seventieth century, with the important monograph and classification of Heinrich Wilhem Schott (1784-1865) mostly based on floral characteristics in a pre-Darwinian fixed view (Nicolson 1987). The next important work was the classification of Adolf Engler and his assistant Kurt Kraus (e.g. 1876, 1884, cited in Nicolson 1987), who added morphological and anatomical vegetative characters to Schott's work in an evolutionary view. Their work served as support for most of the following classifications based on morphological inference (Nicolson 1987, Hooker 1883, Bogner 1979, Grayum 1990, Bogner and Nicolson 1991), the latest of which was constructed by Mayo *et al.* (1997), and classified the Araceae into seven subfamilies. The first subfamilies, Gymnostachydoideae (one genus) and Orontioideae (three genera) formed the Proto-Araceae. In the next group called True Araceae, the subfamilies Pothoideae (four genera), Monsteroideae (12 genera), Lasioideae (10 genera), and Calloideae (one species) bear bisexual flowers, like Proto-Araceae. The last diverging subfamily Aroideae was the most important in size and comprises 74 genera bearing unisexual flowers (Mayo *et al.* 1997).

The development of phylogenetic analyses based on molecular data allowed to construct new phylogenies, at first from chloroplast DNA restriction-site data (French *et al.* 1995), and then from chloroplast and mitochondrial sequences (Barabé *et al.* 2002, Renner and Zang 2004, Renner *et al.* 2004, Tam *et al.* 2004, Barabé *et al.* 2004, Gonçalves *et al.* 2007, Cabrera *et al.* 2008, Cusimano *et al.* 2011). *Acorus* was excluded from the Araceae and placed in its own family, Acoraceae, as sister to the remainder of monocotyledon.The Lemnaceae were in addition included into the Araceae (Cabrera *et al.* 2008). Cusimano *et al.* (2011) completed and reanalyzed a set of six chloroplast markers (*rbcL*, *matK*, partial *trnK* intron, partial *tRNA-Leu* gene, *trnL-trnF* spacer, and partial *tRNA-Phe* gene) generated by Cabrera *et al.* (2008) on 113 genera. They used the resulting phylogeny to propose a new formal classification of the family, based on comparison of the phylogeny to morphological and anatomical data taken

from Mayo *et al.* (1997), Grayum (1984, 1990, 1992) and Keating (2002). Finally, in this most recent work, the Araceae were divided into 44 clades, in which remained the Proto-Araceae, Pothoideae, Monsteroideae, Lasioideae sensu Mayo *et al.* (1997), plus the Lemnoideae as a basal subfamily diverging next to the Proto-Araceae. The Aroideae sensu Mayo *et al.* (1997) was renamed Unisexual Flower clade, comprising the Stylochaeton clade at its base, followed by the Aroideae clade sensu Cusimano *et al.* (2011), composed of five main supported clades previously forming the aperigoniate Aroideae clade sensu Mayo *et al.* (1997). The terminology of Cusimano *et al.* (2011), also still informal, will be used in the following text to avoid confusion. Although the last phylogenetic inference was well resolved and strongly supported, some relationships remained to be resolved at the subfamily level, especially within the Aroideae clade, as well as the branching of the genera *Callopsis*, *Anubias*, *Montrichardia*, *Calla*, *Alocasia*, *Protarum* and *Pistia*.

Among these genera, one has attracted the attention for several decades: *Calla*. The different positions of this monospecific genus according to the phylogenetic analyses prevent a clear understanding of the relationships at the base of the Aroideae (or Unisexual Flower Clade) which coincides with the transition from bisexual to unisexual flowers. *Calla palustris* L. is a circomboreal species growing in swamps and bogs. This seasonnaly dormant herb presents rhizomatous stems, rampant or submersed. Its flowers are bisexual, aperigoniate and spirally arranged on a spadix surrounded by a white fully expanded spathe (Fig. 1, Mayo *et al.* 1997). Engler (1876) grouped *Calla* with the early divergingl subfamily Orontioideae, with which it shares a unilocular ovule, a Northern Hemisphere distribution and stamens at the apex of spadix. Grayum (1990) placed *Calla* at the base of the unisexual Philodendroideae, but in its most "primitive" group, as its bears distic leaves, aperturate pollen and bisexual flowers. According to Mayo *et al.* (1997), *Calla* constitutes an independent clade, but rather basal according to its morphological characteristics. The three phylogenies including *Calla* and based on chloroplast markers diverged from the morphological inferences in placing *Calla* with species bearing unisexual flowers in the last diverging unisexual Aroideae, but with poor support (Barabé *et al.* 2004, Cabrera *et al.* 2008, Cusimano *et al.* 2011): whereas it was placed at the transition between species bearing unisexual and bisexual flowers in Cabrera *et al.* (2008), it was recently embedded in the middle of Aroideae by Cusimano *et al.* (2011).

Here, we added a nuclear marker, *Phytochrome C* (*PhyC*, Mathews and Donoghue 1999) to the last alignment of Cusimano *et al.* (2011), and ran new analyses which allowed to improve two deep nodes of the phylogeny, and to place *Calla* for the first time into a well supported

clade at the base of the Aroideae (sensu Cusimano *et al.* 2011). The different new phylogenetic groups and the phylogenetic relationships within the Araceae are also discussed.

3.3 MATERIALS AND METHODS

Taxonomic sampling Forty two species were sampled from 41 genera representative of the Araceae subfamilies, and also used in the last published phylogeny of the Araceae (Cusimano *et al.* 2011), plus the species *Ooia grabowskii* (Engl.) Engl.. One coding nuclear marker, the *Phytochrome C* partial sequence (*PhyC*, Mathews and Donoghue 1999), was amplified in the perspective of getting more variable character information in comparison to chloroplast markers.

Leaf material was harvested in the living collections of the Botanical Gardens of Lyon (France), the Montet (Nancy, France) and Munich (Germany) in 2008 and 2009. Two samples of *Calla palustris* were analysed, one from Nancy and the second one from Munich, as *Calla* position appeared to be problematic in previous published phylogenies. Fourteen sequences from GenBank were added: *Arisaema speciosum, A. tortuosum, Arum hygrophilum, A. italicum, A. nigrum, Biarum davisii, B. ditschianum, Dracunculus vulgaris, Eminium spiculatum, Helicodiceros muscivorus, Lemna gibba, Sauromatum diversifolium, Theriophonum infaustum* and *Thyphonium trilobatum*. As outgroup taxa, two Toefieldiaceae sequences (*Tofieldia calyculata* and *Pleea tenuifolia*) and one Acoraceae sequence (*Acorus gramineus*) were also obtained from GenBank resulting in a total data set of 60 species and 56 genera. See Table 1 for the list of all the taxa, GenBank accessions and voucher information.

DNA extraction, amplification and sequencing Genomic DNA was extracted from silica-gel-dried material, using the QIAquick Gel Extraction Kit using a microcentrifuge (Quiagen, Crawley, West Sussex, UK). *PhyC* was amplified with internal and external primers from Mathews and Donoghue (1999) and primers designed for Araceae (Cusimano *et al.* 2010, Fig. 2). Amplification reactions were performed with 10 μM of primers, 25 μM $MgCl_2$, 1.25 μM of each dNTP, 2.5 μl of 10x PCR-buffer, 0.5 U BioTherm DNA polymerase (Genecraft, Lüdinghausen, Germany), and 10-50 ng of template DNA per 25 μl reaction volume. For recalcitrant material, we used a more reactive polymerase (PhusionTM High Fidelity PCR Kit by Finnzymes). The PCR program for the BioTherm DNA polymerase was: 5 min. initial denaturation at 95 $^\circ$C; 39 cycles of 30 sec. at 95 $^\circ$C, 1 min. at 48 $^\circ$C, 1 min. at 72 $^\circ$C; 10 min. final extension at 72 $^\circ$C. The PCR program for the Phusion polymerase was: 30 sec. initial denaturation at 98 $^\circ$C; 39 cycles of 10 sec. at 98 $^\circ$C, 30 sec. at 48 $^\circ$C, 20 sec. at 72 $^\circ$C; 7 min. final extension at 72 $^\circ$C. PCR products were purified using 0.15 μL of SAP enzyme, 0.015 μL of Exo1 enzyme (Fermentas GmbH, St. Leon-Rot, Germany) and 5 μL of water for 5 μL of DNA. Products were heated at

35 ° C for 15 min., then at 80 ° C for 15 min.

Sequencing relied on Big Dye Terminator kits 441 (Applied Biosystems, Warrington, U.K.) and the amplification primers. The cycle-sequencing program was: 1 min. initial denaturation at 96 ° C, 34 cycles of 10 sec. at 96 ° C, 5 sec. at 54 ° C, 4 min. at 60 ° C. Cycle sequencing products were cleaned with Sephadex G-50 Superfine gel filtration (Amersham, Uppsala Sweden) on MultiScreen TM-HV membrane plates (Millipore, Bedford, U.S.A.) according to the manufacturers' protocols to remove unincorporated nucleotides. Fragments were separated in an ABI 3100 Avant capillary sequencer, assembled and edited using the software Sequencher (Gene Codes, Ann Arbor, Michigan, U.S.A.). Sequences were tentatively identified with BLAST procedure in GenBank (http://blast.ncbi.nlm.nih.gov/blas.cgi). Sequences were deposited in GenBank (for accession numbers see Table 1).

Phylogenetic analyses The alignment of the *Phytochrome C* sequences was generated using the ClustalW system implemented in Mega version 4 (Tamura *et al.* 2007). Phylogenetic inference of the matrix (60 taxa, 1091 aligned nucleotides reduced to 991 when removing the ambiguous beginning and end of the alignment) were estimated with maximum likelihood (ML) as implemented in the RAxML BlackBox (Stamatakis *et al.* 2008, http//phylobench.vital-it.ch/raxml-bb/) and with a Bayesian approach as implemented in MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Bootstrapping under ML used 1000 replicates performed in RAxML. RAxML used the GTRCAT approximation of the GTR + G +I model, with the gamma shape parameter having 25 rates categories. Bayesian analyses were performed with the GTR model of sequences evolution. Bayesian runs were started from independent random starting trees and repeated four times. Markov chain Monte Carlo runs extended for 2000000 generations, with trees sampled every 100th generation (resulting in 20000 trees for each run). Convergence of all runs was assessed when the average standard deviation of split frequency was stabilized under a value of 0.01, and using the convergence diagnostic in MrBayes. After eliminating the first 5000 trees as "burn-in," a 50% majority-rule consensus tree was constructed, with nodal values representing the probability ("posterior probability") that the recovered clades exist.

The 54 matching taxa of the *PhyC* alignment were then combined with the alignment of the six combined chloroplast markers (*rbcL*, *matK*, partial *trnK* intron, partial *tRNA-Leu* gene, *trnL – trnF* spacer, and partial *tRNA-Phe* gene) from Cusimano *et al.* (2011), resulting in a 5489 pb length matrix. For 31 of the genera, *PhyC* and the chloroplastic set of markers could be

concatenated for the same species. As the operational taxonomic unit was the genus, for the 21 remaining genera, the *PhyC* sequences were concatenated with the sequences for the chloroplast markers from different species (Table 1). For the 64 species for which the *PhyC* sequence was not available, the corresponding part of the alignment was filed with the appropriate character for missing data. As the topologies from nuclear and chloroplast markers did not show supported differences, phylogenetic analyses of the complete data set were performed following the ML and Bayesian methods as explained previously for the phylogenetic analysis of *PhyC*.

3.4 RESULTS

Phylogenetic reconstruction with PhyC The *PhyC* alignment matrix consisted of 61 taxa (see Appendix) and 991 aligned characters. Maximum Likelihood and Bayesian methods gave two trees with the same topology (Fig. 3). In the following text, statistical support values (Sv) are reported as follow: Sv = Maximum Likelihood bootstraps values/Bayesian posterior probabilities). Following Cusimano *et al.* (2011), and to allow comparison, nodes were considered as "strongly supported" when they received both bootstraps values of at least 85% and posterior probabilities equal or higher than 0.97. When only the posterior probabilities were at least 0.97, the corresponding nodes were considered as "well supported". As our results were mostly coherent with Cusimano *et al.* (2011), their clade terminology will be used in the following sections.

Both analyses strongly supported the Araceae family (Fig. 3, Sv = 100/1). None of the *PhyC* sequences obtained from the Proto-Araceae (*Orontium aquaticum, Lysichiton camtschatcensis* and *L. americanum*) could be aligned. The two *Lysichiton* sequences could be aligned together but neither with *Orontium* nor with the rest of the sequences. Thus, in this reconstruction, Lemnoideae was the first diverging clade of the family (Sv = 51/0.97).

The three subfamilies Monsteroideae, Pothoideae and Lasioideae as described by Mayo *et al.* (1997) were all strongly supported (Sv = 100/1, 100/1 and 99/1 respectively), but the *PhyC* marker by itself was not sufficient to resolve their inter-relationships.

Within the Monsteroideae, three subgroups were strongly supported: the Spathiphylleae (*Spathiphyllum-Holochlamys*, Sv = 100/1) as first diverging clade (Sv=100/0.99), and the sister clades *Heteropsis* (*Stenospermation-Rhodospatha*) and *Rhaphidophora* (*Monstera-Amydrium*)(both Sv = 100/1). Relationships within the Lasioideae were less supported, with only *Dracontioides* well supported as first diverging genus (Sv = 82/0.97), and the clade *Lasimorpha-Urospatha* strongly supported (Sv = 100/1).

The remaining Unisexual Flower clade (subfamily Aroideae sensu Mayo *et al.* 1997) was weakly supported (Sv = 36/0.92). With *PhyC*, the *Stylochaeton* clade (*Zamioculcas-Gonatopus-Styochaeton*) was not supported, with only *Zamioculcas-Gonatopus* grouped together (Sv = 100/1). The next diverging Aroideae clade (Aperigoniate Aroideae sensu Mayo *et al.* 1997) was not supported in this analysis. It was composed of *Callopsis*, a weak supported clade grouping *Calla* with *Montrichardia* and *Anubias* (Sv = 44/0.93), followed by the strongly supported Clade 1 (Fig. 1, Sv = 81/0.99). In the Clade 1, relationships are not resolved between *Nephthytis*, *Aglaonema*, the Spathicarpeae (*Dieffenbachia-Spathicarpa-Gorgonidium*), *Cercestis*, *Philodendron*

and the well supported Rheophyte clade (Sv = 100/1) (Fig. 3).

In the remaining strongly supported *Dracunculus* clade (Sv = 98/1), relationships are congruent with what was previously found by Cusimano *et al.* (2011), with the exception of *Typhonium* diverging before *Theriophonum* (Sv = 99/1) in the *Alocasia* clade.

In this reconstruction, the three genera represented by two or three species (*Arisaema, Biarum* and *Arum*) were strongly supported (respectively Sv = 100/1, 97/1, and 99/1). In addition, the two samples of *Calla palustris* were grouped together (Sv = 100/1).

Phylogenetic reconstruction with the combined alignment The addition of *PhyC* to the six combined chloroplast markers aligned by Cusimano *et al.* (2011) led to no dramatic change in the main topology of the tree (Fig. 4), with the exception of *Calla*, which was formerly embedded in the *Philonotion* clade, and appeared newly placed at the base of the Aroideae, grouped together with *Anubias* and *Montrichardia* (Sv = 46/0.98).

In the remaining clades, 14 nodes had higher support values, and 8 had lower support values (highlighted in the fig. 4). In the deeper nodes of the phylogeny, two clades came out less supported: the True Araceae clade (Sv = 84/1) and the *Podolasia* clade (Sv = 62/1). The two next diverging clades were on the contrary well supported with Sv = 59/1 for the Unisexual flowers clade and Sv = 73/1 for the Aroideae. Within the Aroideae, a new clade, which we propose to name "*Cercestis* clade" came out well supported (Sv = 64/1).

The remaining changes occurred in the relationships within the subfamilies. In the Lemnoideae, *Landoltia*, *Wollfia* and *Wolffiella* came out as a strongly supported group (Sv = 86/0.99). In the Monsteroideae, the *Heteropsis* clade and the *Rhaphidophora* clade formed a new strongly supported group (Sv = 95/0.97). In the *Heteropsis* clade, the support values grouping *Rhodospatha* and *Alloschemone* (Sv = 91/1) with *Heteropsis* (Sv = 88/1) were both improved, whereas in the *Rhaphidophora* clade, the group *Rhaphidophora-Anadendrum* was no more significantly supported. In the Lasioideae, the group *Anaphyllopsis-Dracontium-Dracontioides* changed from "well" to "strongly" supported (Sv = 88/1). The remaining Lasioideae were well supported together (Sv = 69/0.99), as was the group *Urospatha-Lasimorpha-Amydrium* (Sv = 63/0.98). In the *Zantedeschia* clade (Aroideae), the Culcasieae and the *Philodendron* clade were no longer significantly grouped together. In the Rheophyte clade, *Philonotion* was no longer significantly supported as first diverging genus, but the grouping of *Schismatoglottis* and *Phymatarum* changed from well to strongly supported (Sv = 86/1). In the Caladieae, the first diverging clade changed from well to strongly supported (Sv = 85/1) but the groupings of *Xanthosoma-Chlorospatha* and

Scaphispatha-Zomicarpella-Zomicarpa were no longer supported. The *Alocasia* clade changed from well to strongly supported (Sv = 88/1) with *Alocasia* strongly supported as a basal genus (Sv = 89/1). In the Areae, the relationships between *Typhonium, Lazarum, Theriophonum* and the terminal clade are no more resolved. Finally, the divergence of *Eminium* after *Sauromatum* is strongly supported (Sv = 99/1).

3.5 DISCUSSION

Up to now, no nuclear genetic information had been used to infer phylogenetic relationships within the Araceae family, and some major polytomies remained to be resolved, as well as the position of *Calla palustris* in the phylogenetic tree (Cabrera *et al.* 2008, Cusimano *et al.* 2011). Even if the addition of *PhyC* brought no major change to the phylogenetic inferences from the last published phylogeny, it enabled us anyway to resolve two new "deep" nodes in the phylogeny, and to place for the first time *Calla* with two genera (*Montrichardia* and *Anubias*) in a well supported clade (Sv = 46/0.98). The major change allowing the resolution of the deep nodes may be the removing of *Calla palutris* from the middle of the Aroideae clade to its base. Finding the position of *Calla* in the tree has been challenging for a long time, either in phylogenies inferred from morphological (Engler 1876, Grayum 1990, Bogner and Nicolson 1991, Mayo *et al.* 1997) or from molecular data (Barabé *et al.* 2004, Cabrera *et al.* 2008, Cusimano *et al.* 2011) since this monospecific genus appears in different positions according to the study. Our analysis suggests thus that *Calla*, a genus with aperigoniate bisexual flowers, belongs to the first aperigonate group with unisexual flowers at the base of the Aroideae subfamily (Fig. 4). This phylogenetic position indicates that there may have been one unique reversion to bisexual flowers in this genus maybe in relation with the loss of the floral perigone.

With the new position of *Calla*, our phylogeny gave more significant support values for the Unisexual Flowers Clade (Aroideae sensus Mayo *et al.* 1997). This clade was composed of the *Stylochaeton* clade as sister group to the Aroideae (Aperigoniate Aroideae sensu Mayo *et al.* 1997), as proposed by Cusimano *et al.* (2011). In the Aroideae, the genus *Callopsis* remained isolated, but the two other formerly isolated genera *Montrichardia* and *Anubias* formed a newly well supported clade with *Calla*. Then, the *Zantedeschia* clade and the *Philonotion* clade came out grouped together in a new well supported clade, which we propose to name *Cercestis* clade. Based on the character data matrix from Cusimano *et al.* 2011 (Mayo *et al.* 1997), there is no autapomorphy for this clade, but all genera present unisexual flowers without perigone and inaperturate pollen, the major internode in the inflorescence is the peduncle, the flowering sequence of the axis is basipetal, their continuation shoot is in axil of penultimate leaf before spathe, their leaves presents a distinct and differentiated blade, and they present no trichosclereids.

The Aroideae is a clade comprising 74 genera and 1960 species out of 3373. The major anatomical changes in this clade are considered to be the apparition of aperigoniate unisexual

flowers arranged in a male and a female part, biforines and laticifers, inaperturate pollen and the absence of sporopollenin in the exine (Mayo *et al.* 1997, Hesse *et al.* 2006). In possessing sporopollenin, two pollen apertures, and lacking biforines and spadix zonation, the position of *Calla* embedded in the Aroideae (sensu Cusimano *et al.* 2011) is uncertain, but its position within the Unisexual Flowers clade is well supported. According to Grayum (1990), the two genera sharing most morphological characteristics with *Calla* are *Callopsis*, at the base of the Aroideae (sensus Cusimano *et al.* 2011) in our phylogeny, and *Nephtytis*, in the *Zantedeschia* clade in our phylogeny. The position of *Calla* grouped with *Anubias* and *Montrichardia* is anyway not fully satisfactory. Some key morphological characteristics are not shared with *Anubias* and *Montrichardia*, for instance the spiral phylotaxy in *Calla*, which is a basal trait in Araceae, its diaperturate pollen (only found in the outgroup *Tofieldia*) and its base number of chromosomes, which is x = 18, as for *Callopsis*, *Philodendron*, *Cryptocoryne* and *Lagenandra*, but which is x = 12 for *Anubias* and *Montrichardia* (Cusimano *et al.* 2011). In addition, even if the three genera share the same aquatic habitat, *Montrichardia* is a South American species and *Anubias* grows in Africa whereas *Calla* is mainly distributed in Europe. In conclusion, even if there are cues for an even more basal position of *Calla*, from a morphological point of view, its position as inferred with the addition of *PhyC* is the more supported found so far with molecular data.

The resolution of the basal Unisexual Flowers clade has always been problematic, either when working with molecular or morphological data (Mayo *et al.* 1997, Cabrera *et al.* 2008, Cusimano *et al.* 2011). This lack of resolution may be due to numerous extinct species leading to missing molecular information. These species losses may have accompanied the dramatic changes arising in the clade Aroideae when flowers went unisexual and the spadix organization changed. The apparition of unisexual flowers and differentiation of their inflorescence structure in the Aroideae clade might also be the result of an ancient adaptive radiation and incomplete lineage sorting (Rokas and Caroll 2006, Wiens and Moen 2008). Up to now, six chloroplast markers combined with a nuclear marker have allowed to obtain a quite well supported phylogeny (Fig. 4), that we suppose strong enough to be used in the future for evolutionary ecology studies. Anyway, the addition of more numerous neutral nuclear markers may improve this result, but it seems to be challenging. For this study, we try to add ITS and ETS sequences (Gautier *et al.* 1995), which were too much variable to be aligned, and made preliminary work on GAI1 (Wen *et al.* 2007), PhyA (Mathew and Donoghue 1999) and PhyB (Mathews *et al.* 2000, Ito *et al.* 2010) which could not be amplified or sequenced. These markers may be interesting to develop, but they would need to be adapted for Araceae or other basal Angiosperms.

3.6 ACKNOWLEDGMENTS

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3.8 FIGURE LEGENDS

Figure 1. Inflorescence of *Calla palustris* from Gerardmer (Vosges, France).

Figure 2. Relative positions of the primers used to amplify *PhyC*. Primer sequences are: 748R-Ara: 5'ACA AGA TCC ATG ACA TTA GGT GAT T3' (present study), 750R: 5'AAG ATC CAT AAC ATT TGG TGA T3', 430F: 5'TCG TGA TGT CTG TCA CAA TAA3' (Mathews and Donoghue 1999), AF: 5'ATA GAC CTG GAA CCA GTG AAT3', A20F: 5'CAC TCA ATC CTA CAA ACT GGC3' and AR: 5'GAA TAG CAT CCA TTT CAA CAT3' (Cusimano *et al.* 2010).

Figure 3. Phylogenetic reconstruction of the Araceae family inferred from ML and Bayesian analyses of *PhyC* sequences (991 bp). PP = Posterior probabilities, BP = Bootstrap values. Clades in capital letters and in black indicate subfamilies and clades as recognized by Mayo *et al.* 1997, clades in grey color indicate those discussed in the text following the terminology proposed by Cusimano *et al.* 2011. H = *Heteropsis* clade, S = *Stenospermation* clade, R = *Rhaphidophora* clade.

Figure 4. Phylogenetic reconstruction of the Araceae family inferred from ML and Bayesian analyses of *PhyC* sequences (991 bp) together with the complete Cusimano *et al.* 2011 alignment of six combined chloroplast markers (4494 bp). PP = Posterior probabilities, BP=Bootstrap values. Clades in capital letters and in black indicate subfamilies and clades as recognized by Mayo *et al.* 1997, clades in grey indicate those discussed in the text following the terminology proposed by Cusimano *et al.* (2011). H = *Heteropsis* clade.

Table 1. Provenance, voucher information and GenBank accession numbers of the Phytochrome C sequences for the taxa used in this study. Species added to the alignment from Cusimano et al. 2010 for phylogenetic analyses: • same species than in Cusimano *et al.* 2010, ∗ same genus than in Cusimano *et al.* 2010. Symboles in gray indicate that we could amplify only half of the PhyC sequence. Herbarium acronyms of botanical gardens: LY = Lyon Botanical Garden (France), M = Munich Botanical Gardens (Germany), N = Botanical Garden of the Montet (Nancy, France).

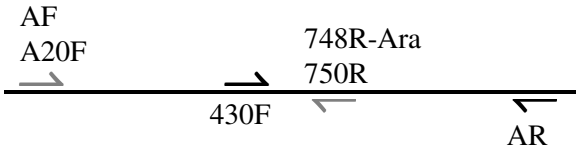
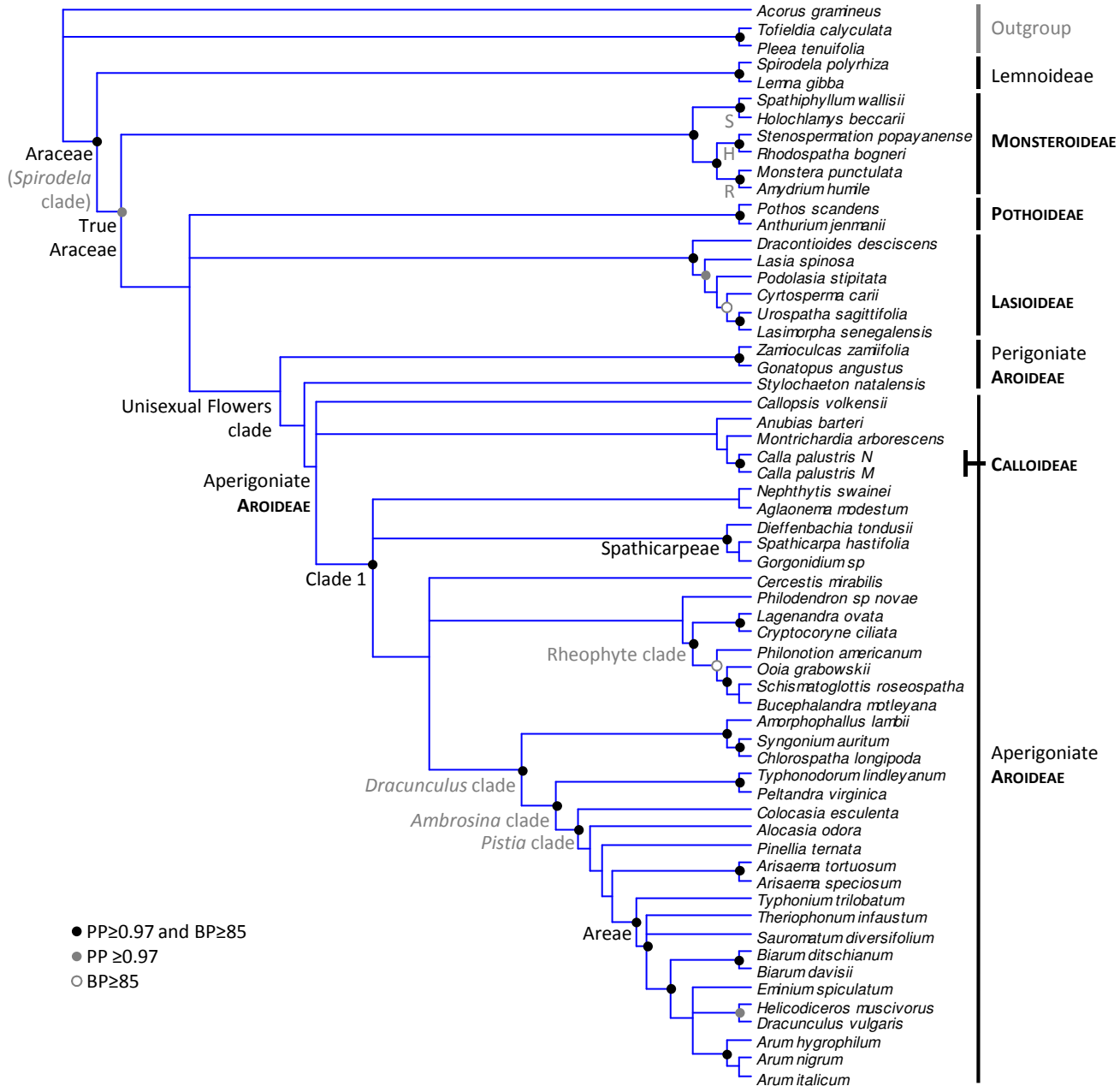


Figure 1 Figure 2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Pleea tenuifolia
Spirodela polyrhiza
Lemna gibba
Spathiphyllum wallisii
Holochlamys beccarii
Stenospermation popayanense
Rhodospatha bogneri
Monstera punctulata
Amydrium humile
Pothos scandens
Anthurium jenmanii
Dracontioides desciscens
Lasia spinosa
Podolasia stipitata
Cyrtosperma carii
Urospatha sagittifolia
Lasimorpha senegalensis
Zamioculcas zamiifolia
Gonatopus angustus
Stylochaeton natalensis
Callopsis volkensii
Anubias barteri
Montrichardia arborescens
Calla palustris N
Calla palustris M
Nephthytis swainei
Aglaonema modestum
Dieffenbachia tondusii
Spathicarpa hastifolia
Gorgonidium sp
Cercestis mirabilis
Philodendron sp novae
Lagenandra ovata
Cryptocoryne ciliata
Philonotion americanum
Ooia grabowskii
Schismatoglottis roseospatha
Bucephalandra motleyana
Amorphophallus lambii
Syngonium auritum
Chlorospatha longipoda
Typhonodorum lindleyanum
Peltandra virginica
Colocasia esculenta
Alocasia odora
Pinellia ternata
Arisaema tortuosum
Arisaema speciosum
Typhonium trilobatum
Theriophonum infaustum
Sauromatum diversifolium
Biarum ditschianum
Biarum davisii
Eminium spiculatum
Helicodiceros muscivorus
Dracunculus vulgaris
Arum hygrophilum
Arum nigrum
Arum italicum
S
H
R
Araceae (Spirodela clade)
True Araceae
Unisexual Flowers clade
Aperigoniate AROIDEAE
Clade 1
Spathicarpeae
Rheophyte clade
Dracunculus clade
Ambrosina clade
Pistia clade
Areae
Outgroup
Lemnoideae
MONSTEROIDEAE
POTHOIDEAE
LASIOIDEAE
Perigoniate AROIDEAE
CALLOIDEAE
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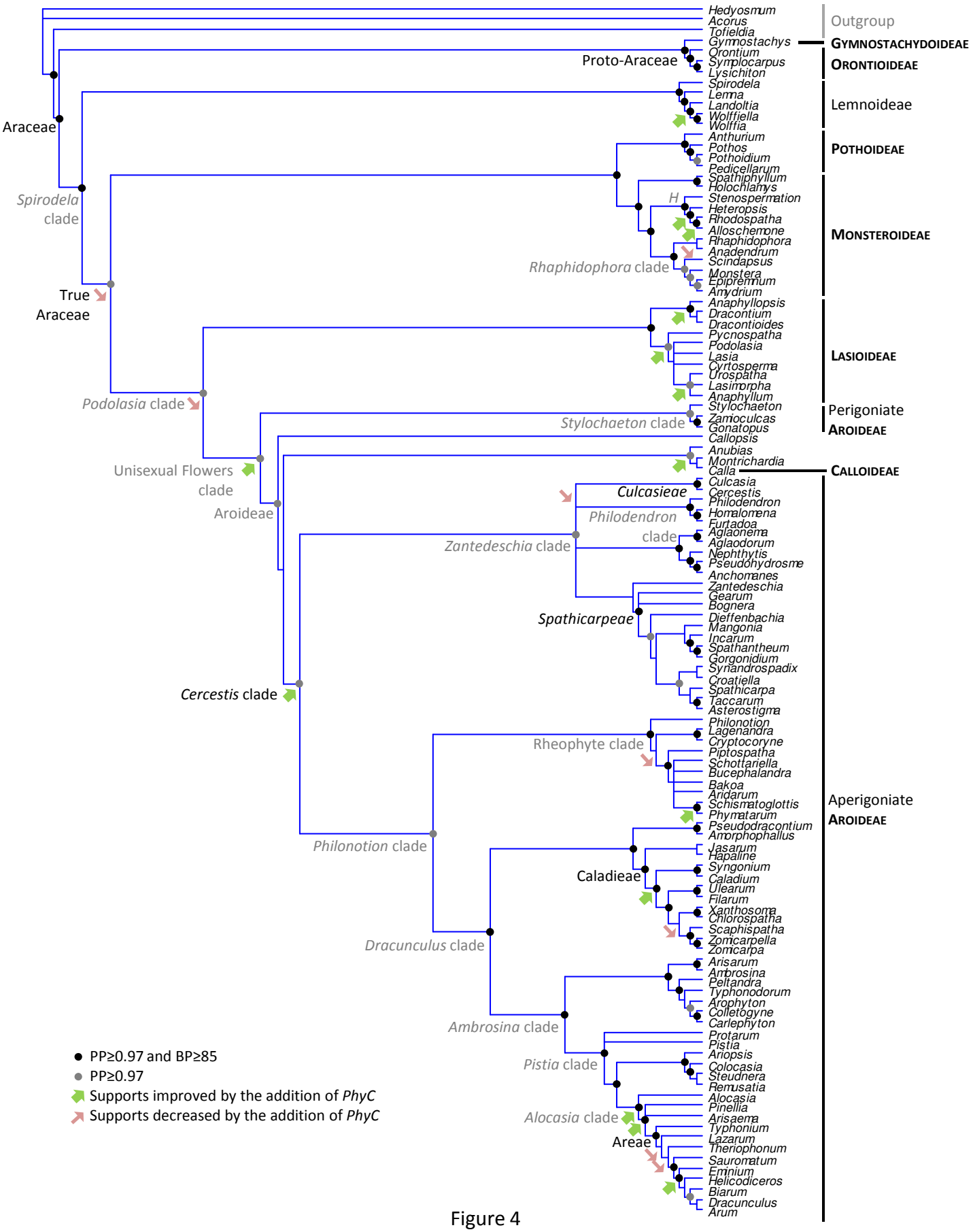
PP≥0.97 and BP≥85
PP ≥0.97
BP≥85

Figure 3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Acorus
Tofieldia
Gymnostachys
Orontium
Symplocarpus
Lysichiton
Spirodela
Lemna
Landoltia
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Wolffia
Anthurium
Pothos
Pothoidium
Pedicellarum
Spathiphyllum
Holochlamys
Stenospermation
Heteropsis
Rhodospatha
Alloschemone
Rhaphidophora
Anadendrum
Scindapsus
Monstera
Epipremnum
Amydrium
Anaphyllopsis
Dracontium
Dracontioides
Pycnospatha
Podolasia
Lasia
Cyrtosperma
Urospatha
Lasimorpha
Anaphyllum
Stylochaeton
Zamioculcas
Gonatopus
Callopsis
Anubias
Montrichardia
Calla
Culcasia
Cercestis
Philodendron
Homalomena
Furtadoa
Aglaonema
Aglaodorum
Nephthytis
Pseudohydrosme
Anchomanes
Zantedeschia
Gearum
Bognera
Dieffenbachia
Mangonia
Incarum
Spathantheum
Gorgonidium
Synandrospadix
Croatiella
Spathicarpa
Taccarum
Asterostigma
Philonotion
Lagenandra
Cryptocoryne
Piptospatha
Schottariella
Bucephalandra
Bakoa
Aridarum
Schismatoglottis
Phymatarum
Pseudodracontium
Amorphophallus
Jasarum
Hapaline
Syngonium
Caladium
Ulearum
Filarum
Xanthosoma
Chlorospatha
Scaphispatha
Zomicarpella
Zomicarpa
Arisarum
Ambrosina
Peltandra
Typhonodorum
Arophyton
Colletogyne
Carlephyton
Protarum
Pistia
Ariopsis
Colocasia
Steudnera
Remusatia
Alocasia
Pinellia
Arisaema
Typhonium
Lazarum
Theriophonum
Sauromatum
Eminium
Helicodiceros
Biarum
Dracunculus
Arum
Proto-Araceae
Araceae
Spirodela clade
True Araceae
Podolasia clade
Unisexual Flowers clade
Aroideae
Cercestis clade
Philonotion clade
Dracunculus clade
Ambrosina clade
Pistia clade
Alocasia clade
Areae
H
Rhaphidophora clade
Stylochaeton clade
Culcasieae
Philodendron clade
Zantedeschia clade
Spathicarpeae
Rheophyte clade
Caladieae
Outgroup
GYMNOSTACHYDOIDEAE
ORONTIOIDEAE
Lemnoideae
POTHOIDEAE
MONSTEROIDEAE
LASIOIDEAE
Perigoniate AROIDEAE
CALLOIDEAE
Aperigoniate AROIDEAE
PP≥0.97 and BP≥85
PP≥0.97
Supports improved by the addition of PhyC
Supports decreased by the addition of PhyC

Figure 4

Sequence.source		Species	Voucher.number	GenBankAcc
		Acoraceae		
Mathews & Donoghue 1999	*	*Acorus gramineus* Solander	NA	AF190061
		Araceae		
present paper	•	*Aglaonema modestum* Schott ex Engl.	NCY013334 (N)	JF776574
present paper	•	*Alocasia odora* (Lindl.) K. Koch	J. Bogner 2959 (M)	
present paper	*	*Amorphophallus lambii* Mayo & Widjaja	NCY004756 (N)	JF776575
present paper	•	*Amydrium humile* Schott	J. Bogner 2446 (M)	JF776576
present paper	•	*Anthurium jenmanii E* ngl.	NCY002096 (N)	JF776577
present paper	•	*Anubias barteri* Schott	BotGardNb 2001.3.139 (N)	JF776578
Cusimano *et al.* 2010		*Arisaema speciosum* (Wall.) Mart.	Hetterscheid H.AR.294 (L, spirit coll)	EU886470
Cusimano *et al.* 2010	*	*Arisaema tortuosum* (Wall.) Schott	Anaimudi 20/5	EU886469
Cusimano *et al.* 2010	•	*Arum hygrophilum* Boiss.	CY-0-BONN-6427 (BONN)	EU886471
Cusimano *et al.* 2010		*Arum italicum* Mill.	BG Mainz, cult. 20 Jul 2001	EU886472
Cusimano *et al.* 2010		*Arum nigrum* Schott	Cusimano06-1 (M)	EU886473
Cusimano *et al.* 2010	*	*Biarum davisii* Turiill	MO living acc. 78231 (MO)	EU886479
Cusimano *et al.* 2010		*Biarum ditschianum* Bogner & Boyce	BG Bonn 4695 (BONN)	EU886477
present paper	•	*Bucephalendra motleyana* Schott	J. Bogner 2902 (M)	JF776579
present paper	•	*Calla palustris* L.	J. Bogner 2968 (M)	JF776580
present paper		*Calla palustris* L.	BotGardNb 1970.8.001 (N)	JF776581
present paper	•	*Callopsis volkensii* Engl.	NCY002305 (N)	JF776582
present paper	•	*Cercestis mirabilis* (N. E. Br.) Bogner	NCY002307 (N)	JF776583
present paper	*	*Chlorospatha longipoda* (K.Krause) Madison	NCY009369 (N)	JF776584
present paper	•	*Colocasia esculenta* (L.) Schott	NA (N)	JF776585
present paper	*	*Cryptocoryne ciliata* (Roxb.) Fisch. ex Wydler	BotGardNb 020151 (LY)	JF776586
present paper	*	*Cyrtosperma carrii* A. Hay	J. Bogner 2451 (M)	JF776587
present paper	*	*Dieffenbachia tondusii* Croat & Grayum	NA (LY)	JF776588
present paper	•	*Dracontioides desciscens* Engl.	NA (M)	JF776589
Cusimano *et al.* 2010	•	*Dracunculus vulgaris* Schott	T. Croat 78286 (MO)	EU886476
Cusimano *et al.* 2010	•	*Eminium spiculatum* (Blume) Schott	M. Neumann 27/96 (BONN)	EU886474
present paper	•	*Gonatopus angustus* N. E.Br.	NCY002370 (N)	JF776590
present paper	*	*Gorgonidium sp*	Weigend&Förther97/910 (M)	JF776591
Cusimano *et al.* 2010	•	*Helicodiceros muscivorus* (L. f.) Engl.	MO living acc. 71821 (MO)	EU886480
present paper	•	*Holochlamys beccarii* (Engl.) Engl.	J. Bogner 1269 (M)	JF776592
present paper	•	*Lagenandra ovata* (L.) Thwaites	BotGardNb 020200 (LY)	JF776593
present paper	•	*Lasia spinosa* (L.) Thwaites	BotGardNb 383792 (LY)	JF776594
present paper	•	*Lasimorpha senegalensis* Schott	(M)	JF776595
Mathews & Donoghue 1999	*	*Lemna gibba* L.	NA	AF190093
present paper	*	*Monstera punctulata* (Schott) Schott ex Engl.	J. Bogner 2448 (M)	JF776596
present paper	•	*Montrichardia arborescens* (L.) Schott	NCY010134 (N)	JF776597
present paper	*	*Nephthytis swainei* Bogner	Swain&HallGC44621 (M)	JF776599
present paper		*Ooia grabowskii* (Engl.) Engl.	J. Bogner 2977 (M)	JF776602
Cusimano, unpubl. data	•	*Peltandra virginica* Raf.	J. Bogner 2119 (M)	
present paper	*	*Philodendron sp novae Scherberich*	BotGardNb 080206 (LY)	JF776600
present paper	•	*Philonotion americanum* (Jonku & Jonku) Wong & Boyce	J. Bogner 2911 (M)	JF776601
Cusimano, unpubl. data	*	*Pinellia ternata* Thunb.	J. McClements s. n., 30 Jul. 2001	
present paper	•	*Podolasia stipitata* N. E. Br.	(M)	JF776603
present paper	•	*Pothos scandens* L.	NCY002646 (N)	JF776604
present paper	*	*Rhodospatha bogneri*	NCY013046 (N)	JF776605
Cusimano *et al.* 2010	*	*Sauromatum diversifolium* (Wall.) Cusimano & Hett.	Hetterscheid H.AR.484 (L, spirit coll)	EU886482
present paper	*	*Schismatoglottis roseospatha* Bogner	Knüppel & Linke s. n. (M)	JF776606
present paper	•	*Spathicarpa hastifolia* Plook	NCY013581 (N)	JF776607
present paper	•	*Spathiphyllum wallisii* Regel	BotGardNb 013182 (LY)	JF776608
present paper	•	*Spirodela polyrhiza* (L.) Schleid	J. Bogner 2976 (M)	JF776609
present paper	•	*Stenospermation popayanense* Schott	NCY001911 (N)	JF776610
present paper	*	*Stylochaeton natalensis* Schott	J. Bogner 2979 (M)	JF776611
present paper	•	*Syngonium auritum* (L.) Schott	BotGardNb 013185 (LY)	JF776612
Cusimano *et al.* 2010	*	*Theriophonum infaustum* N.E.Br.	P. Bruggemann PB 099	EU886485
Cusimano *et al.* 2010	*	*Typhonium trilobatum* (L.) Schott	J. Murata 5	EU886496
present paper	*	*Typhonodorum lindleyanum* Schott	NCY013595 (N)	JF776613
present paper	•	*Urospatha sagittifolia* (Rudge) Schott	BotGardNb 060077 (LY)	JF776614
present paper	•	*Zamioculcas zamiifolia* (Lodd.) Engl.	NCY004630 (N)	JF776615
		Tofieldiaceae		
Mathews & Donoghue 1999		*Pleea tenuifolia* Michx.	M. W. Chase 152 (GH)	AF276736
Duvall *et al.* 2003	*	*Tofieldia calyculata* Wahlemb	NA	AY396715

Table 1

Chapitre 4

Preuves phylogénétiques de l'évolution et de l'histoire des traits liés à la pollinisation chez les Aracées

Soumission prévue dans ***Molecular Ecology*** .

Auteurs : Marion CHARTIER, Marc GIBERNAU.

Evolutionary history of pollination syndromes: a large scale phylogenetic approach in Araceae

4.1 ABSTRACT

As pollination efficiency directly affects plant reproductive success, floral traits linked to pollination efficiency are under strong selective pressures by pollinators. Thus, a change in pollinator among species is likely to lead to a different set of floral traits in certain plant lineages. Pollinator shifts and the correlated changes in floral morphologies can be nowadays analyzed from an evolutionary point of view using phylogenetic inferences in association with recent statistical methods, like the Bayesian stochastic mapping.

In this study, pollinator driven selection on floral traits was tested in the Araceae family (Monocotyledons) at a large phylogenetic scale. The most recent molecular phylogeny was used to reconstruct the ancestral states of pollination modalities, and of a large set of qualitative and quantitative floral traits, and finally to test for any correlated evolution of these traits using the software SIMMAP v. 1.5.

According to the results, the Araceae ancestor was probably involved in a rewarding pollination mutualism with a generalist interaction, which is also the state of the most basal subfamilies. Pollination interactions then specialized toward rewarding pollination mutualism involving Hymenoptera in the two next diverging subfamilies. Then, mating pollination mutualism involving Coleoptera probably appeared twice and evolved toward nursery pollination mutualism involving Diptera in two clades, or nursery pollination evolved once with a reversion toward mating pollination mutualism in one clade. Deception appeared four times, mostly from Diptera pollination mutualism. Phylogenetic analyses of correlated evolution showed several significant changes in both the type of pollinators and several floral traits. Hence, correlations between floral traits evolution and pollination modalities implied that pollination syndromes do exist in Araceae. Finally, it appears that in this plant family, the floral evolutive unit is the inflorescence more than the flower.

4.2 INTRODUCTION

Animal pollinated plants have developed an amazing diversity of floral features to attract pollinators and ensure their reproduction (Faegri and van der Pijl 1966, Proctor *et al.* 1996). These floral features (e.g. shape, colour, floral display, odour) often constitute adaptations to pollinators, and are associated with attractive rewards (nectar, pollen, floral tissues) or with pollination efficiency and specialization (stigmatic areas, pollen shape, chemical compounds, anthesis duration, breeding system etc., Schemske and Bradshaw 1999, Shuttleworth and Johnson 2010, Schlumpberger *et al.* 2009). The observation that phylogenetically distant plant species share similar floral features associated with the same pollination mode has lead to the concept of *pollination syndromes*. Pollination syndromes are the convergence of complexes of floral traits resulting from the same selective pressures exerted by the same pollinators on plants from different lineages (Stebbins 1970, Faegri and van der Pijl 1979, Fenster *et al.* 2004). The concept of pollination syndromes is consequently linked *per se* to the phylogenetic context of the studied taxa.

Phylogenetic data can be included in studies on pollination, which link pollinator shifts with the evolution of floral features or ecological conditions (Johnson *et al.* 1998, Patterson and Givnish 2004, Duchen and Renner 2010). The addition of phylogenetic data to the former comparative studies is a powerful tool to understand the direction of pollinator shifts, retrace their history, and to detect the correlated evolution of some floral traits with pollination modalities, as it allows to take into account the morphological or phylogenetic constraints (Pérez *et al.* 2006, 2007, Blomberg and Garland 2002, DeWitt Smith 2010). Statistics are nowadays available to test for correlations between the evolutions of quantitative (Felsenstein 1985, Grafen 1989, Maddison 1990) but also qualitative characters (Huelsenbeck *et al.* 2003, Pagel and Meade 2006). These analytical methods constitute efficient tools to infer the evolutionary history of pollination modes, as well as the potential links between these ecological shifts and changes in floral traits. At present time, such studies are limited by the availability of large data sets in taxonomic groups showing important pollination/floral feature shifts, and from which phylogenetic relationships are well resolved (Glor 2010). So far, such phylogenetic studies have been mainly conducted on taxa at the tribe or genus level, and mainly on species pollinated by bees, hummingbirds, bats and hawkmoths (reviewed by Trip and Manos 2008, see also Knapp 2010, Marten-Rodriguez *et al.* 2010). In addition, these studies have rarely investigated relationships between a high number of floral traits and documented pollinators at a high phylogenetic level (but see Ackermann and

Weigend 2006, Friedman and Barett 2008 for wind pollination, Alcantara and Lohmann 2010).

Here, the evolutionary history of qualitative, but also quantitative floral traits was studied in relation to pollinators and the evolution of pollination modes at the family level in Araceae. Araceae is a widespread family from the monocotyledons, containing 3373 described species distributed in 117 genera (CATE Araceae 2011). Its pollination has been well documented and involves striking floral features making this family an excellent model for testing the concept of floral syndromes (Grayum 1992, Mayo *et al.* 1997, Chouteau *et al.* 2008, Gibernau 2003, 2011, Gibernau *et al.* 2010). In this family, species are mainly pollinated by insects belonging to three different orders: Diptera, Coleoptera and Hymenoptera, and are involved in four different types of pollination. The first type is a *rewarding pollination mutualism*. In this case, pollinators visit inflorescences looking for a reward which can be nutritive (Tanaka 2004) or an odorant wax (e.g. Hentrich *et al.* 2010a). This type of pollination has been recorded in association with the three orders of pollinators, and some generalist systems (inflorescences attracting pollinators from different orders at the same time). The second pollination type is a *mating pollination mutualism* and is associated with Coleoptera and Diptera. In this case, pollinators meet in the inflorescence to mate and eat floral parts (e.g. Maia and Schlindwein 2006, Seymour *et al.* 2009, Maia *et al.* 2010). The third pollination type is a *nursery pollination mutualism* and is associated with Diptera. In this case, pollinators come into the inflorescences to mate and feed but also to oviposit, and the larvae develop later eating the decaying floral parts (e.g. Sultana *et al.* 2006). The last pollination type is *deceptive pollination*. In this case, insects looking for an oviposition site are attracted by inflorescences which mimic the odour, colour and shape of different decaying organic matter. Insects are usually trapped into a floral chamber where they spend several hours without being able to oviposit. Deception has been documented for plants pollinated by Diptera and Coleoptera (e.g. Angioy *et al.* 2004, Diaz and Kite 2006, Punekar and Kumaran 2010).

Among Araceae, inflorescences have developed a great variety of floral features (Fig. 1) which might be linked with pollination, including different shapes, colours, presence of sterile flowers, appendices (e.g. scented sterile organs) and trapping features (Mayo *et al.* 1997). Correlation between pollen ornamentation and pollinator orders have already been suggested by Grayum (1992) and phylogenetically demonstrated by Sannier *et al.* (2009), who found a correlation between the evolution of echinulate pollen and Diptera pollination, and between smooth pollen and Coleoptera pollination.

Recently, it has also been shown in two complementary comparative studies (Chouteau *et al.*

2008, Gibernau *et al.* 2010) that some quantitative traits, like flower number per inflorescence, pollen grain volume or pollen number per inflorescence, were linked with pollinator orders, and could be used to infer pollinator type of non documented species. If these results are in part interpretable from an ecological point of view, they might be better understood under the light of the phylogenetic relationships between taxa, and under the light of the evolution of pollination in Araceae.

The phylogeny of Araceae has been well resolved with morphological data and up to six chloroplast markers (Cabrera *et al.* 2008, Cusimano *et al.* 2011) to which a nuclear marker has recently been added (Chartier *et al.* unpublished data).

Here, we used the last inferred phylogeny of Araceae (Chartier *et al.* unpublished data) to reconstruct ancestral states of pollination and floral traits, and tested for their correlated evolution. Our work aimed to answer the following specific questions: (1) What are the ancestral states of pollinator order and pollination type in Araceae and how many times did they change along the phylogeny? (2) Is there a correlated evolution of the pollination type or pollinator order and some floral traits? (3) Is there a correlated evolution of some floral traits? (4) How important and frequent is the pollinator-mediated selection among floral traits in Araceae?

4.3 MATERIALS AND METHODS

Construction of the character matrix A qualitative character matrix was constructed with informations on the pollination type, the pollinator orders, and on a total of 20 floral traits potentially linked with pollination.

First, the matrix was filled with data from 109 species belonging to 64 genera from Chouteau *et al.* (2008) and Gibernau *et al.* (2010) with the following quantitative floral traits: male flower number per inflorescence, female flower number per inflorescence, mean number of ovules per flower, mean number of locules per flower, mean number of pollen grains per flower, mean volume of pollen grains, mean stigmatic area and pollen-ovule ratio per inflorescence. Measures and counts were added from five inflorescences of *Calla palustris* harvested in 2008 in three different sites around Gerardmer (Retournemer, Belbriette and Gerardmer, Vosges, France) following the protocol or Gibernau *et al.* (2010). The information was summarized at the genus level by averaging quantitative data for the species of the same genus. In order to map the characters and to perform the correlation analyses, quantitative data were log transformed and coded as qualitative data by the method of simple gap coding (Almeida and Bisby 1984, Penneys and Judd 2011). This method consists in plotting the frequency curve of a character, and visually determin discontinuities in the distribution by the presence of peaks, dips or gaps. This method was used to discriminate 5 ordered classes for each quantitative character.

The character matrix was completed by adding qualitative characters taken from Cusimano *et al.* (2011): floral sexuality, type of pollen exine surface, spathe structure, presence of pollen starch, spadix zonation, and from Mayo *et al.* (1997): life mode, stratus (level at which the inflorescences open), repartition, presence of pollen adherence, sterile flowers type (the matrix is available as supplementary data).

The three pollinator orders (Diptera, Hymenoptera or Coleoptera) and the four pollination types (reward mutualism, mating mutualism, nursery mutualism and deception) were filled in with the data from Chouteau *et al.* (2008), Gibernau *et al.* (2010) and the recent review on the subject from Gibernau (2011).

Ancestral characters reconstruction of pollination modalities Ancestral state reconstructions were performed in SIMMAP v.1.5 for pollination order and pollination types (Bollback 2006, http://www.simmap.com). For this analysis, the posterior distributions of the 10000 post burnin trees obtained from the four runs of the Bayesian analysis of Chartier *et*

al. (unpublished data) were used. The genera for which no morpho-ecological information was available were removed from these trees, leading to a 64-genera sampling. Before running simulations, posterior distributions of the rates and bias priors parameters for each character were estimated in SIMMAP 1.5 and R v2.12.2, following the MCMC approach described in the SIMMAP manual, and using the default settings (see Supplementary data for priors values). Result representations were obtained with the software R v.2.12.2. Scripts are available upon request from the first author.

Tests for correlated evolution Correlated character evolutions were identified with the Character association (correlation) test in SIMMAP 1.5. This test is based on a number of stochastic mapping reconstructions on a tree. In a mapping reconstruction, the amount of time spent in a character state is the probability of being in this state in the tree. Thus, the probability of being in a combination of two states from two different characters is the amount of time spent in the tree under the two character states. The strength of the association is determined by comparing the observed associations of the two character states to the expected associations obtained under the hypothesis that the characters are independent, which is here the probability of being in the first state multiplied by the probability of being in the second state. This difference is quantified using a statistic (D). The significance of the differences between the observed and expected associations is then assessed by comparison with data randomly simulated so that there is genuinely no correlation between the traits (predictive sampling, Huelsenbeck *et al.* 2003, Bollback 2006). For the analysis, the Bayesian consensus tree from Chartier *et al.* (unpublished data) was used. A thousand samples were performed to obtain the statistic D, and 500 predictive samples to assess its significance.

4.4 RESULTS

Correlated evolution of pollination modalities The evolution of pollinator order was significantly correlated with the evolution of pollination type (p = 0.002, Table 1). The evolution of generalist pollination was positively correlated to the evolution of rewarding (p = 0.01) mutualism, and negatively correlated with the evolution of mating mutualism (p = 0.03). The evolution of Hymenoptera pollination was strongly associated with rewarding mutualism (p = 0.018).The evolution of Coleoptera pollination was strongly correlated to the evolution of mating mutualism (p < 1.10-6) and negatively correlated to rewarding mutualism (p = 0.012). Finally, the evolution of Diptera pollination was significantly correlated with the evolution of nursery mutualism (p = 0.020), and deception (p = 0.022), and was negatively correlated with the evolution of rewarding (p = 0.006) or mating mutualisms (p = 0.032).

Correlated evolution of pollination and floral traits Four of the 20 tested floral characters evolved in correlation with pollinator orders. The evolution of the same characters was also correlated with pollination types, in addition to five other characters (Table 2 and 3). Generalist and Hymenoptera pollination modes evolved in correlation with bisexual flowers (p = 0.002, resp. $p < 1.10^{-6}$) with no spadix zonation (p = 0.03, resp. p = 0.008). In addition, the evolution of generalist pollination was also associated with the absence of pollen starch (p = 0.018) and a distribution in cold and temperate zones (p = 0.004). On the contrary, Diptera and Coleoptera pollinations evolved in correlation with unisexual flowers (p = 0.002 resp. p = 0.008). The evolution of Diptera pollination was also correlated with the presence of pollen starch (p = 0.032), and a spadix zonation with two zones of sterile flowers (p = 0.006), whereas Coleoptera pollination was correlated with a spadix zonation consisting of a female and a male part (p = 0.02), sometimes with sterile flowers in between (p = 0.02), and tended to be correlated with a distribution in inter-tropical zones (p = 0.054).

The traits evolving in correlation with rewarding mutualism were bisexual flowers ($p < 1.10^{-6}$), no sterile flowers (p = 0.004), no spadix zonation ($p < 1.10^{-6}$), a high number of female flowers (p = 0.014), a low total stigma area (p = 0.018), reticulate pollen exine surface (p = 0.002), no pollen starch (p = 0.004), a spathe absent or inconspicuous (p = 0.03) and a distribution in cold or temperate zones (p = 0.018). On the contrary, mating mutualism evolved in correlation with unisexual flowers (p = 0.002), common sterile flowers (p = 0.002), a male and a female zone (p = 0.002) sometimes with sterile flowers in between (p = 0.016), a low flower

number ($p < 1.10^{-6}$), a high total stigmatic area ($p = 0.012$), smooth pollen exine surface ($p = 0.006$), presence of pollen starch ($p = 0.022$) and a repartition in inter-tropical zones ($p = 0.004$). Nursery mutualism and deceptive pollination evolution were both associated with unisexual flowers ($p = 0.026$, resp. $p = 0.048$), a spadix zonation consisting in a female, a male and two zones of sterile flowers ($p = 0.012$, resp. $p = 0.046$), and medium stigma areas ($p = 0.034$, resp. $p = 0.036$). Deception was in addition associated with the presence of a floral chamber ($p = 0.048$) and spinose pollen exine surface ($p = 0.034$). The correlated evolution of nursery mutualism with spinose exine surface was just a tendency ($p = 0.062$).

Correlated evolution of floral traits Most of the floral traits correlated with pollinator order of pollination type also evolved in a correlated way (in the following section, overall statistic values are reported in the text, and individual statistical values are available in tables or as supplementary material).

Thus, high stigmatic areas evolved in correlation with distribution in tropical zones whereas low stigmatic areas evolved in correlation with distribution in cold/temperate zones ($p = 0.032$). Pollen exine surface and spathe shape evolutions were also correlated ($p = 0.036$) with the evolution of reticulate exine significantly correlated with the evolution of boat shaped spathes, and the evolution spinose exine significantly correlated with the evolution of floral chambers.

Pollen starch evolved together with spinose pollen ($p = 0.048$), floral chambers ($p = 0.04$), a low number of female flowers ($p = 0.05$), repartition in inter-tropical zones ($p = 0.024$) and nutritive sterile flower type ($p < 1.10^{-6}$). The evolution of spadix zonation was significantly correlated with the evolution of pollen exine surface ($p < 1.10^{-6}$), spathe structure ($p = 0.03$), female flower number ($p = 0.01$), pollen starch ($p = 0.014$) and sterile flowers type ($p = 0.01$): spadices with no zonation evolved together with reticulate pollen exine, absent or inconspicuous spathes, a high number of female flowers, no pollen starch and no sterile flowers. Spadix zonation consisting in a female and a male part evolved together with smooth pollen ornamentation, a low number of female flowers and common sterile flowers. Spadix zonation with a male and a female flower zones with sterile flowers in between evolved together with common sterile flower type. Finally, Spadix zonation with a male and a female zone, and two sterile flower zones evolved together with spinose pollen ornamentation, spathes with floral chambers and production of pollen starch.

Even if the pollen-ovule ratio did not evolve in correlation with pollinator order ($p = 0.166$) or pollination type ($p = 0.084$), it evolved in correlation with pollen ornamentation ($p = 0.026$),

spathe structure (p = 0.012), spadix zonation (p = 0.024) and the number of ovules per flower (p = 0.024), but not with the number of pollen grains per flower (p = 0.06). Thus, species presenting the highest pollen-ovule ratios also evolved toward reticulate pollen ornamentation, boat shaped spathe and low ovule number per flower. On the contrary, species presenting the lowest pollen-ovule ratios also evolved toward spinose pollen ornamentation, spathes forming a floral chamber, spadix with one or two sterile flower zones, and a high ovule number per flower (Table 4). In addition, the evolution of the number of female flowers was positively correlated to the evolution of the number of pollen grains per flower (p = 0.034).

Finally, 14 of the studied floral traits were correlated with flower sexuality (see supplementary material and discussion).

Pollination ancestral states reconstruction The reconstruction of pollinator order, pollination type, and correlated floral traits allowed to infer the evolutive history of pollination in Araceae. In the following paragraph, nodes representative of the ancestors of the main Araceae clades are numbered according to figure 2.

The ancestor of the Araceae family was probably generalist (p = 0.72), and pollinated by rewarding mutualism (p = 0.96, Fig. 2). It grew in cold/temperate climatic zones, its spathe was likely to be inconspicuous (p = 0.41) or boat shaped (p = 0.25, Fig. 3), its flowers were bisexual (p = 1), arranged in a spadix with no zonation (p = 0.96). Its number of female flowers was high (probability of the states increased with flower quantity) and its pollen starchless (p = 0.76).

Ancestors of the two early-diverging clades Proto-Araceae (node 1) and Pothoideae-Monsteroideae (node 2) were also probably pollinated by a rewarding mutualism (p = 0.99 for both clades ancestors). The ancestor of Proto-Araceae was generalist (p = 0.94) and was likely to have evolved toward loss of the spathe (p = 0.62), which became boat-shaped in *Lysichiton* and *Symplocarpus*. *Lysichiton* also lost the production of starch in pollen. Pollinators switched from rewarding generalist to rewarding (p = 0.99) Hymenoptera pollination (p = 0.9) in the next two diverging subfamilies Pothoideae and Monsteroideae (node 2). At this stage, Araceae switched from cold/temperate zones to inter-tropical zones (p = 1). The ancestor of this clade evolved likely toward a boat shaped spathe (p = 0.66), with the exception of *Pothos* (unconspicuous spathe) and the evolution toward a fully expanded spathe in *Anthurium*, *Spathiphyllum* and *Holochlamys*. Pollen starch appeared in the ancestor of these two subfamilies (p = 0.6), but was lost in the ancestor of the clade Pothoideae (p = 0.74), *Spathiphyllum*, and *Holochlamys*. Diptera pollination was likely to appear for the first time in the next diverging clade Lasioideae

(node 3, p = 0.89), probably in association with deception (p = 0.70) or rewarding mutualism (p = 0.21). Spathe was still boat-shaped (p = 0.99), with the apparition of a floral chamber in *Dracontioides*, and hair like sterile flowers in *Dracontium*. In this clade, there was a reversion toward starchless pollen (p = 0.97). Coleoptera (p = 0.93) associated with a nursery pollination mutualism (p = 0.97) probably appeared in the ancestor of the next Unisexual Flowers clade (node 4). This changing in pollination type and order was associated with the most dramatic change in Araceae which is the apparition of unisexual flowers (p = 1), associated with a spathe forming a floral chamber (p = 0.79), a spadix zonation consisting in a male and a female zone (p = 1), and when they exist, nutritive sterile flowers (p = 0.70). The number of female flowers decreased (probability of the states decreased with flower quantity) and stigma area increased (probability of the states increased with flower quantity). Pollen exine was still reticulate (p = 0.95) and presented starch (p = 0.97).

At the base of this clade, the *Stylochaeton* clade (node 5) and *Callopsis* are nevertheless pollinated by Diptera (Fig. 2). In the *Stylochaeton* clade, *Zamioculcas* and *Gonatopus* are Diptera pollinated, and little is known about their pollination mode, but their most recent common ancestor (node 5) was likely to be pollinated by Coleoptera (p = 0.78) and mating mutualism (p = 0.83). The three genera forming the *Stylochaeton* clade present spathes forming a floral chamber, unisexual flowers arranged in a male and a female zone, nutritive sterile flowers, rather high stigma areas, presence of pollen starch and reticulate exine surface. *Callopsis* is also pollinated by Diptera, by rewarding mutualism, and share the same traits that in the *Stylochaeton* clade, but with a fully expended spathe, no sterile flowers and a low stigma area.

Relationships between *Callopsis*, the clade *Montrichardia*, *Anubias*, *Calla*, and the rest of the Aroideae (*Cercestis* clade) were poorly resolved. Differences of floral features of *Calla* and the genera at the base of the Aroideae clade has been already discussed (Cusimano *et al.* 2011, Chartier *et al.* unpublished). Note anyway that whereas *Montrichardia* and *Anubias* are Coleoptera pollinated by mating mutualism, *Calla* is probably generalist and pollinated by rewarding mutualism (Chartier, unpublished data). Within Aroideae, the ancestor of the first diverging *Zantedeschia* clade (node 6) was also pollinated by Coleoptera (p = 0.99) in a mating mutualism (p = 1), and probably presented a boat shaped spathe (p = 0.70), a male and a female flowers zone (p = 0.99), even if in this clade five genera possess a sterile zone with nutritive sterile flowers (*Philodendron*, *Homalomena*, *Dieffenbachia*, *Bognera* and *Synandrospadix*). The number of female flowers was rather low and the stigmatic area large. Pollen possessed starch (p = 1), and its exine was likely to be smooth (p = 0.92) but some genera in this clade also present

reticulate pollen, and *Synandrospadix*, a fly pollinated genus, presents spinose pollen. In this clade, Diptera pollination appeared in the genera *Aglaonema*, *Spathantheum*, *Synandrospadix* and *Spathicarpa*.

The ancestor of the *Philonotion* clade (node 7) was probably Diptera (p = 0.60), and/or Coleoptera (p = 0.39) pollinated, by mating (p = 0.68) or breeding mutualism (p = 0.30). Then, Diptera and nursery pollination mutualism evolved twice in the Rheophyte clade (node 8) and in the *Ambrosina* clade (node 10), or Coleoptera pollination by mating mutualism evolved a second time in the Caladiae clade (node 9).

The ancestor of the Rheophyte clade (8) was Diptera pollinated by nursery mutualism (p = 0.97 and p = 0.90). The exceptions in this clade are *Aridarum* pollinated by mating mutualism, and *Cryptocoryne* by deception (Fig. 2). The ancestor of this clade probably bared a boat-shaped spathe (p = 0.63) or a floral chamber (p = 0.25). Spadix zonation comprises a female and a male zone with sterile flowers in between (p = 0.99 for the ancestor) and sterile flowers are nutritive (p = 0.89). Stigma area were pretty large, all species produce starchy (p = 0.99 in the ancestor) and smooth pollen (p = 0.93 in the ancestor).

Coleoptera pollination associated with a mating mutualism appeared a second time or was inherited in the Caladiae clade (9, p = 0.74 for both characters) with notable exceptions of deceptive pollination in the genus *Pseudodracontium*, and Diptera pollination in the genus *Ulearum* (Fig. 2). In this clade, inflorescences possess a floral chamber, a spadix zonation consisting of a male and a female zone separated by a zone with sterile flowers, few female flowers with large stigmatic areas. All kind of pollen exine can be found and starchy pollen in all genera (except *Chlorospatha*). *Pseudodracontium* and *Ulearum* differ in having boat shaped spathes and a sterile zone at the apex for *Pseudodracontium*, and two sterile flower zones for *Ulearum*.

The ancestor of the *Ambrosina* clade (10) was pollinated by Diptera (p = 0.98) in a nursery mutualism (p = 0.98). In this clade, all documented species are Diptera pollinated, and pollination by deception probably evolved a fourth time in the terminal clade (11, p = 1) comprising the deceptive genera *Pinellia*, *Arisaema*, *Typhonium*, *Helicodiceros*, *Dracunculus* and *Arum*. Breeding mutualist species from this clade are inter-tropical distributed, whereas deceptive species, except *Typhonium* and some *Arisaema* species, are distributed in cold/temperate areas. All genera present spathes with a floral chamber, and a spadix zonation presenting two sterile flower zones, with the exception of *Colletogyne* and *Carlephyton*, in which there has been a reversion to boat-shaped spathes. The pollination mode of these two genera and *Arophyton*, all from Madagascar, remains unknown, and the three genera lack a sterile flower zone. In addition, the

evolution toward deception in this clade was probably accompanied by a change from nutritive sterile flowers to hair-like sterile flowers, as it was the case for *Dracontium* in the Lasioideae (3) but with the exception of *Dracunculus*. Finally, all species in this clade except *Arum* present starchy pollen, and pollen exine is mainly spinose except for *Pistia*, *Colocasia*, and the deceptive genus *Dracunculus*.

4.5 DISCUSSION

Evolution of pollination in Araceae Contrary to the previous study of Sannier *et al.* (2009), whose results inferred that the ancestor of Araceae was beetle pollinated, our results predicted that ancestral pollination in Araceae was a generalist pollination system by reward mutualism, actually observed in several early-diverging Proto-Araceae (Gibernau 2011). Evolution of pollination and floral traits in Araceae showed an original and striking case of successively derived pollination syndromes detectable at the family level, and evolving from generalized systems to more specialized interactions, a broadly accepted direction of change in pollination (Ollerton *et al.* 2007 but see Marten-Rodriguez *et al.* 2010). The ancestral Araceae rewarding system specialized to Hymenoptera in South American Pothoideae and Monsteroideae, even if some species are beetle-pollinated (Chouteau *et al.* 2007). The Lasioideae clade is poorly known in term of pollination, and is suspected to be an intermediate stage between Coleoptera and Diptera pollination, and between generalist and specialized systems (Gibernau 2003). The major changes in floral characters (mostly the apparition of unisexual flowers) lead to the apparition of mating mutualism with Coleoptera pollination in the Unisexual Flowers clade, evolving twice toward Diptera nursery pollination in the Rheophyte clade and *Ambrosina* clade, or evolving toward Diptera pollination and reversing toward Coleoptera pollination in the Caladieae. The phylogenetic reconstruction could not distinguish among these two scenarios even if the second hypothesis is more probable. The apparition of Diptera pollination and deception had already been proposed by Stebbins (1970) to be derived from Coleoptera pollination, in which the floral chamber were likely to have evolved toward constricted spathes forming keetle traps. Deceptive pollination appeared four times, three times from Diptera pollination in the clades Lasioideae (*Dracontium*), Rheophyte (*Cryptocoryne*) and *Ambrosina* (Alocasia clade), and once from Coleoptera pollination in the Caladieae (*Pseudodracontium*).

Pollination syndromes Our study allowed to describe four floral syndromes associated with pollination modes in Araceae, understandable in the light of Araceae biology and ecology.

Genera involved in rewarding pollination mutualisms attract insects looking for a resource which is not directly related to reproduction, following two differentes modalities. Generalist rewarding (plants pollinated by insects from different orders) is the case of Proto-Araceae, *Urospatha* (Lasioideae) and *Calla* in cold/temperate zones. These species reward pollinators looking for a nutritive resource, mainly pollen, as Araceae do not produce nectar (Mayo *et al.*

1997, Schwerdtfeger *et al.* 2002). Hymenoptera-pollinated rewarding species belong to Pothoideae and Monsteroideae in inter-tropical zones (Schwerdtfeger *et al.* 2002, Gibernau 2011). This pollination type has also been described in two Cretean *Arum* species attracting food collecting bees (Diaz and Kite 2006, Urru *et al.* 2010). Only known from South America tropical species, this is also the case of euglossine bees harvesting an odorant wax on the spadix in order to attract females (Hentrich *et al.* 2007, 2010). In a previous comparative analysis, generalists and Hymenoptera-pollinated species had already been classified in two close groups based on their floral traits (Gibernau *et al.* 2010). These modes of pollination are associated with inconspicuous or boat-shaped spathes, no spadix zonation and no sterile flowers, representative of the most "simple" inflorescence types in Araceae (Gibernau *et al.* 2010). A high number of female flowers (and thus of total flower number) in comparison to other Araceae genera and small stigmatic areas are likely to be related to the efficiency of these pollination modes, as it is related to the capacity of pollen deposition on stigmas by pollinators (Ne'eman *et al.* 2010). Pollinators may need to visit inflorescences several times to effectively pollinate flowers: as Araceae are protogynous and sequentially flower, pollen must be imported once at stigma receptivity, and then exported when it is released from anthers several days after. A high number of flowers thus increases the probability of pollinator visits and consequently pollination efficiency. A higher number of flowers, and bisexual flowers had already been reported in Hymenoptera-pollinated species (Gibernau *et al.* 2010).

Note that pollination by euglossine bees, as it is the case in *Anthurium* and *Spathiphyllum*, is mainly based on an olfactory signal as it has been described in other euglossine pollinated Angiosperms: Orchidaceae, Gesneriaceae, Annonaceae, Gentianaceae (Dressler 1986, Teichert *et al.* 2008, Hentrich *et al.* 2010a, Hentrich *et al.* 2010b). All these species are characterized by the production of this attractive odour, and a specific compound, carveol, has been described in the odour of most of them (Whitten *et al.* 1986).

The second pollination syndrome is associated with mating mutualism, in association with Coleoptera pollination. This pollination type is likely to have appeared in the Unisexual Flowers Clade, and is a major pollination system in South America in the genus *Philodendron* and in the clades Caladieae and Spathicarpeae, but is also known from *Homalomena* in Tropical Asia (Gibernau 2003, 2011, Maia *et al.* 2010). Coleoptera, mostly from the Scarabaeideae family, mate and eat in the inflorescences, protected by the spathe surrounding the floral axis. This mode of pollination was associated in our study with plants growing in inter-tropical zones, presenting unisexual flowers arranged in a male and a female zone, sometimes with sterile flowers in between.

The floral cycle of the Coleoptera-pollinated Araceae is very short, lasting around 48h (Gibernau *et al.* 1999, 2003), and could not have evolved without the major changes appearing in the Unisexual Flowers Clade. First, the stay of pollinators into the inflorescences may be favored by the boat-shaped or closed spathe forming a floral chamber. The zonation of the spadix results in the location of female flowers at the bottom of the floral chamber, where the insects copulate and eat (Gibernau 2003). The male flowers are above the floral chamber, so that pollen falls on the insects or the insects will have to walk onto the male flowers to leave the inflorescences favoring pollen deposition on their body before departure. This type of pollination also evolved with the production of nutritive sterile flowers and starchy pollen, which may constitute food rewards for the Coleoptera, which are known to eat food bodies, pollen or stigma (Bernhardt 2000). This type of pollination also evolved with the production of smooth pollen, having lost the sporo-pollenin. The correlation of smooth pollen with Coleoptera pollination had already been predicted by Grayum (1992) and demonstrated by Sannier *et al.* 2009. Smooth pollen is in general associated with wind (Osborn *et al.* 1991, Hesse 2000) or water dispersion (Hesse 2000). In Coleoptera-pollinated Araceae, pollen is released in sticky strands of pollenkitt, and in some species, stigmatic fluids or resin secreted by their male flowers or the spathes have been described to stick the pollen grains to the relatively smooth insect body (Grayum 1992, Mayo *et al.* 1997). The lost of costly sporo-pollenin in the Unisexual Flowers Clade is also believed to be associated with the short time flowering cycles, in which pollen does not need to remain viable more than several hours (Hesse 2006). Cantharophily has also been described in other angiosperms, as Arecaceae, Annonaceae, Magnoliaceae, Winteraceae or Nympheaceae (Gottsberger 1986). These species share with Araceae the flower protogyny, a short floral cycle, but also other floral traits likely to be linked with cantharophily which are production of heat, heavy spicy or fruity attractive odours, or stark floral features believed to protect ovaries from the damaging Coleoptera (Gottsberger 1999). Finally, correlations were found between the evolution of Coleoptera pollination and a decrease of the number of female flowers and an increase of stigma area. First, the decrease of the number of female flowers may be linked to the increase of pollination efficiency as in this obligate mutualism, pollinator visits are far more efficient and specificity ensures the deposit of "good" pollen (Cruden 1977, 2000). Secondly, the increase of stigmatic areas might be due to the large size of beetles and their non-precise behavior of pollen deposit.

Nursery pollination mutualism involving Diptera is likely to have evolved from mating mutualism in Araceae species (Fig. 2). This type of pollination could have arisen from an

ancestor (node 7 on the Fig. 2) for which Diptera were involved in a mating mutualism, evolving toward insect mating plus eggs ovipositing onto the inflorescences. This type of mutualism has also been thought to be derived from a parasitism of Diptera laying eggs in the inflorescences (Sakai 2002). In Araceae, it has been shown to be principally associated with *Colocasiomyia* flies (Drosophilidae) in very specialized interactions, and distributed in Asia (Miyake and Yafuso 2005, Sultana *et al.* 2006). These flies mate, eat and deposit eggs in the inflorescences during a short flowering cycle (one or two days). This mode of pollination evolved with unisexual flowers, the apparition of spinose pollen, which had already been associated with entomophilous pollination (Faegri and Van de Pijl 1966, Osborn *et al.* 1991, Proctor *et al.* 1996, Hesse 2000) and particulary pollination by Diptera in Araceae, as it is believed to help pollen aggregation and hanging on the hairy insects bodies (Grayum 1992, Sannier *et al.* 2009). This pollination syndrome is also associated with a medium stigma area, the presence of two sterile zones in the spadix, the apparition of appendix specialized in heat production and odour release instead of the male flowers like it is the case in cantharophilous species. Finally, Gibernau *et al.* (2010) found an association between Diptera pollination and low flower number and low pollen number. In our study, the correlation with a low female flower number was just a tendency, and may be the result of a more efficient pollination system, like in mating rewarding systems. Nursery pollination mutualisms in which insects oviposit in plants and in which larvae later develop in decaying floral parts have been described in various other Angiosperms Zamiaceae, Aupomatiaceae, Arecaceae, Cyclanthaceae, Aristolochiaceae, Siparounaceae and Moraceae, and are often associated with heat production and strong odour emission (Sakai 2002). In Aristolochiaceae, this kind of pollination is believed to have lead to the apparition of deceptive plant species (Sakai 2002).

Our results go toward the same conclusion in Araceae, where deceptive pollination is likely to have evolved four times, once from Coleoptera (or Diptera) breeding species (*Pseudodracontium*) and three times from Diptera pollinated breeding species (*Dracontium*, *Cryptocoryne*, the Areae clade Fig 2). These species mimic the foul Diptera breeding site odours, but the insects cannot breed in the inflorescences, and are sometimes sequestrated several days in the floral keetle traps (Gibernau *et al.* 2004). Such traps evolved for instance in *Cryptocoryne*, *Helicodiceros* or *Arum* (Yadav 1998, Angioy et al 2004, Gibernau *et al.* 2004). Deception also evolved in correlation with the apparition of two sterile flower zones, like the Diptera-pollinated species by nursery mutualism. In some species, like *Arum*, the sterile flower zones have a hair shape preventing the insects from escaping the inflorescences. This may be the reason why hair-like sterile flowers are correlated (but not significantly) with deception (Urru *et al.* 2010, Table 3). Deception is

correlated to unisexual flowers, and mainly appeared in the Unisexual Flowers clade. Finally, like for breeding-mutualist systems, the correlation between deception and the apparition of spinose pollen is likely to be due to the correlation between deception and Diptera-pollination (see above). Gibernau *et al.* (2010) already found that the set of floral traits of fly deceptive pollination system was embedded within the Diptera pollination syndrome.

This type of deception, with mimicry of an ovipositing site also evolved in numerous Angiosperms lineages like Aristolochiaceae, Asclepiadaceae, Burmanniaceae, Hydnoraceae or Rafflesiaceae, in species sharing with Araceae their foul odours of carrion, dung or decaying matter, their hairy surfaces, heating floral features, light windows and trapping flowers or inflorescences (Renner 2006).

Correlated evolution of floral traits The correlated evolution of floral traits related to pollination may result from several processes linked to pollinator selective pressures and to other factors, like for instance developmental or functionnal morphological constraints (Galen 1999, Murren 2002, Pigliucci 2003, Fenster *et al.* 2004).

For instance, in Araceae, the correlated evolution of smooth pollen with a spadix with two flower zones is more likely to be due to the fact that both smooth pollen and spadix with two flower zones are correlated with the evolution of Coleoptera mating mutualism. On the contrary, the fact that the evolution of a spadix with two flower zones was correlated to the evolution of a low number of female flowers may result from an unknown indirect effect, or to an absence of pollinator selective pressures: decrease of flower number may be associated with an increase of pollinator efficiency (Cruden 1977, Ne'eman *et al.* 2009), which may be the case in the more specialized pollination systems involving unisexual flowers arranged in a two zones spadix. However, in these inflorescences, a low number of female flowers may also result from differences in resources allocations (Klinkhamer *et al.* 1997) or to morphological constraints which may be due to the important change in Aroideae flower disposition in the spadix.

Neither the number of ovules or locules per flower was correlated to any other trait, contrary to the pollen/ovule ratio, calculated per inflorescences (Table 4). These results confirm that in Araceae, pollination selective pressures are exerted on the whole inflorescence, in which floral traits have evolved together. That the inflorescence, and not the flower, constitutes the pollination unit, has been suggested by Chouteau *et al.* (2006) and by Gibernau *et al.* (2010).

The fact that the two third of floral traits evolutions were correlated to the evolution of flower sexuality is not surprising, as the major shift in pollination types occurred on the ancestor

of the Unisexual Flowers Clade, and was associated with major changes in the inflorescence structure (spadix zonation, spathe shape, number of flowers).

Conclusion and perspectives In conclusion, we found here many phylogenetic evidences for the apparition of floral features in correlation with pollinators in Araceae, which suggest that shifts in pollination lead to changes in floral morphology, like pollination by night-active beetles must have favored the apparition of a protecting floral chambers, but also that the apparition of some types of pollination, like deception by brood site mimicking, may have been allowed to evolved from inflorescences already possessing the potential to form trapping floral chambers, which had already been suggested by Stebbins (1970) : *"the relatively broad chambers characteristic of flytrap flowers are more likely to evolve from colepteran-pollinated flowers, which have a similar shape"*.

Adding phylogenetic inferences to the former correlative analysis of Gibernau *et al.* (2010) allowed to confirm the existence of pollination syndromes in Araceae and to highlight broad tendencies of floral traits convergences and evolution.

In the future, more fine analyses of these syndromes at lower taxonomic levels, or on a particular pollination type, like deception, might help understanding the origin and evolution of the different pollination systems in Araceae. Our study for example lacked the inclusion of some important floral traits linked to pollination in Araceae, and difficult to code in a family tree. One example is the attractive odour of Araceae, also found in other cantharophilous, bee pollinated or deceptive angiosperms, and released by flowers, spathes or appendices. Odours have been chemically described in a few species in Monsteroideae (Hentrich *et al.* 2010a), Pothoideae (Hentrich 2007, 2010a), Aroideae (Kite and Hetterschieid 1997, Diaz and Kite 2002), and have been shown to be the major attractant of pollinator in some species (Maia *et al.* 2010, Kite 1992). This trait may be labile between species, and its coding is fussy, as different compounds combination may lead to the same type of odour (Raguso 2008). The evolution of floral odours in *Arum* (Aroideae) has been investigated by Diaz and Kite 2002, Gibernau *et al.* (2004) and Urru *et al.* (2011), who described different substrates mimicking species associated with different pollinators, and different odours in the deceptive or rewarding species of the genus. The second lacking trait is the production of heat by some Araceae. This trait is also delicate to use to infer pollination type as it has been shown to play several roles in Araceae. These roles have been shown to be attraction of pollinators in *Helicodiceros* (Angioy *et al.* 2003), energetic reward for Coleoptera in *Philodendron* (Seymour *et al.* 2003, 2009), and have been suggested to be be climatic stress avoidance in *Symplocarpus* (Ito *et al.* 2004) and odour release in *Arum*

(Bermadinger-Stabentheiner and Stabentheiner 1995, Kite 1995).

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4.8 FIGURE LEGENDS

Figure 1. Araceae inflorescences and their associate pollination modalities (pollinator order-pollination type). (a) *Lysichiton camtschacensis* generalist-rewarding (b) *Anthurium sp. Guyane* bee-rewarding (c) *Monstera adansonii* bee-rewarding (d) *Dracontium polyphyllum* fly-deception (e) *Stylochaeton hypogaeus* ?-? (f) *Calla palustris* generalist-rewarding(g) *Montrichardia linifera* beetle-mating (h) *Philodendron melinonii* beetle-mating (i) *Dieffenbachia amoena* beetle-mating (j) *Pseudodracontium lacouri* generalist-deception (k) *Colocasia gigantea* fly-breeding (l) *Arum maculatum* fly-deception. Picture (a) is from Frederic Muller, picture (c), (d) and (e) are from David Scherberich.

Figure 2 Evolution of pollinator orders and pollination types in Araceae inferred from the Bayesian ancestral state reconstruction on 10000 post-burnin Bayesian trees (Chartier *et al.* unpublished data). Pie diagrams reflect the marginal posterior probability of each state at the ancestral node for the clade. Correspondence between colours and character states is given in the legend. Numbered clades are those discussed in the text.

Figure 3. Evolution of pollination modalities and spathe shape in Araceae inferred from the Bayesian ancestral state reconstruction on 10000 post-burnin Bayesian trees (Chartier *et al.* unpublished data). Pie diagrams reflect the marginal posterior probability of each state at the ancestral node for the clade. Correspondence between colours and character states is given in the legend. Numbered clades are those discussed in the text.

Table 1. Correlation between pollinator order and pollination type evolutions in Araceae: d (p-value). Negative values of d means a negative correlation between the states, positive values mean a positive correlation. ns = non significant correlations.

Table 2. Correlation between pollinator order and floral trait evolution in Araceae: d (p-value). Negative values of d means a negative correlation between the states, positive values mean a positive correlation. ns = non significant correlations, S = sterile.

Table 3. Correlation between pollination type and floral trait evolution in Araceae: d (p-value). Negative values of d means a negative correlation between the states, positive values

mean a positive correlation. ns = non significant correlation. S = sterile.

Table 4. Correlation between floral trait and pollen-ovule ratio evolution: d (p-value). Negative values of d means a negative correlation between the states, positive values mean a positive correlation. ns = non significant correlation, S = sterile.

Figure 1

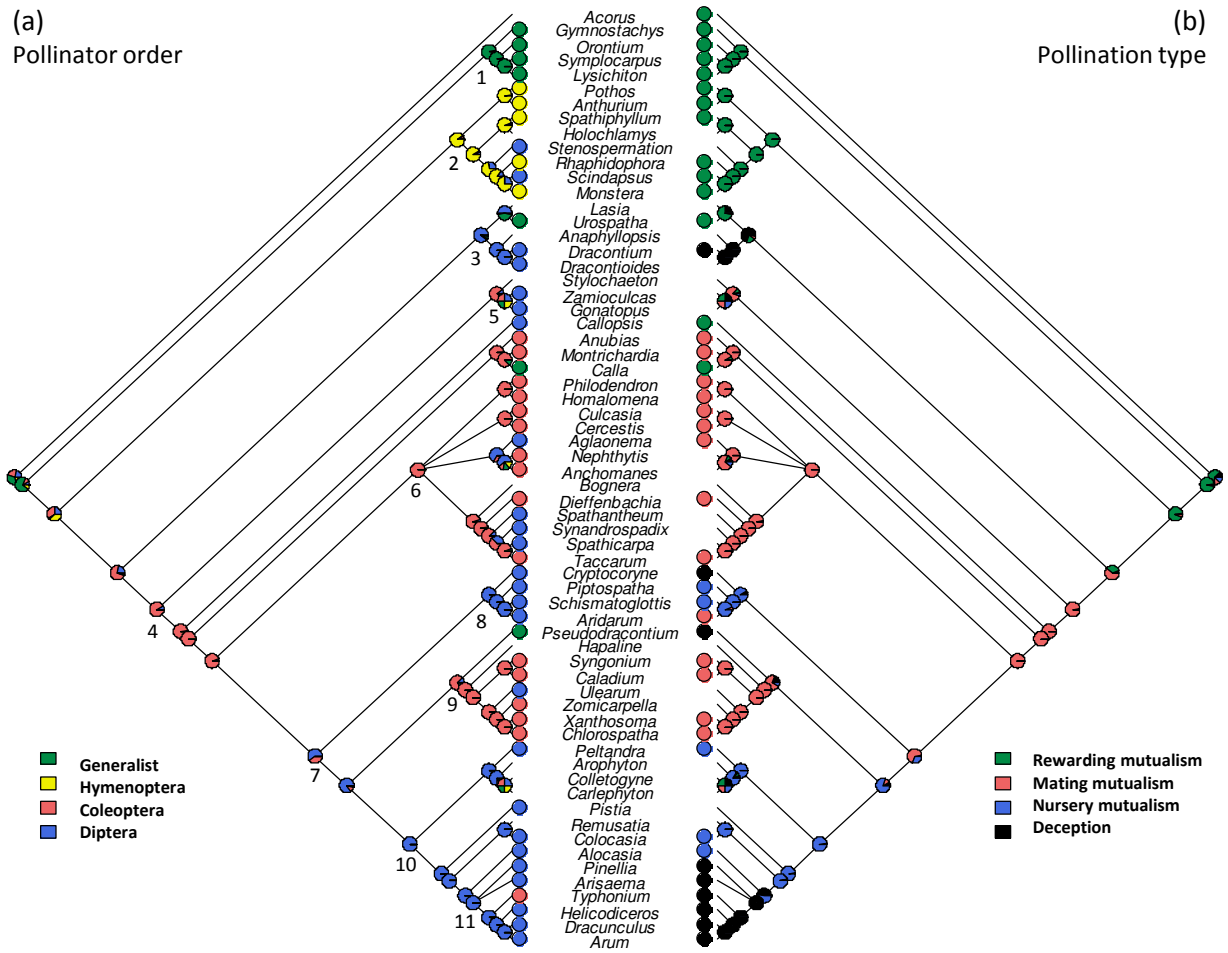
(a)
Pollinator order
(b)
Pollination type
Acorus
Gymnostachys
Orontium
Symplocarpus
Lysichiton
Pothos
Anthurium
Spathiphyllum
Holochlamys
Stenospermation
Rhaphidophora
Scindapsus
Monstera
Lasia
Urospatha
Anaphyllopsis
Dracontium
Dracontioides
Stylochaeton
Zamioculcas
Gonatopus
Callopsis
Anubias
Montrichardia
Calla
Philodendron
Homalomena
Culcasia
Cercestis
Aglaonema
Nephthytis
Anchomanes
Bognera
Dieffenbachia
Spathantheum
Synandrospadix
Spathicarpa
Taccarum
Cryptocoryne
Piptospatha
Schismatoglottis
Aridarum
Pseudodracontium
Hapaline
Syngonium
Caladium
Ulearum
Zomicarpella
Xanthosoma
Chlorospatha
Peltandra
Arophyton
Colletogyne
Carlephyton
Pistia
Remusatia
Colocasia
Alocasia
Pinellia
Arisaema
Typhonium
Helicodiceros
Dracunculus
Arum
1
2
3
4
5
6
7
8
9
10
11
Generalist
Hymenoptera
Coleoptera
Diptera
Rewarding mutualism
Mating mutualism
Nursery mutualism
Deception

Figure 2

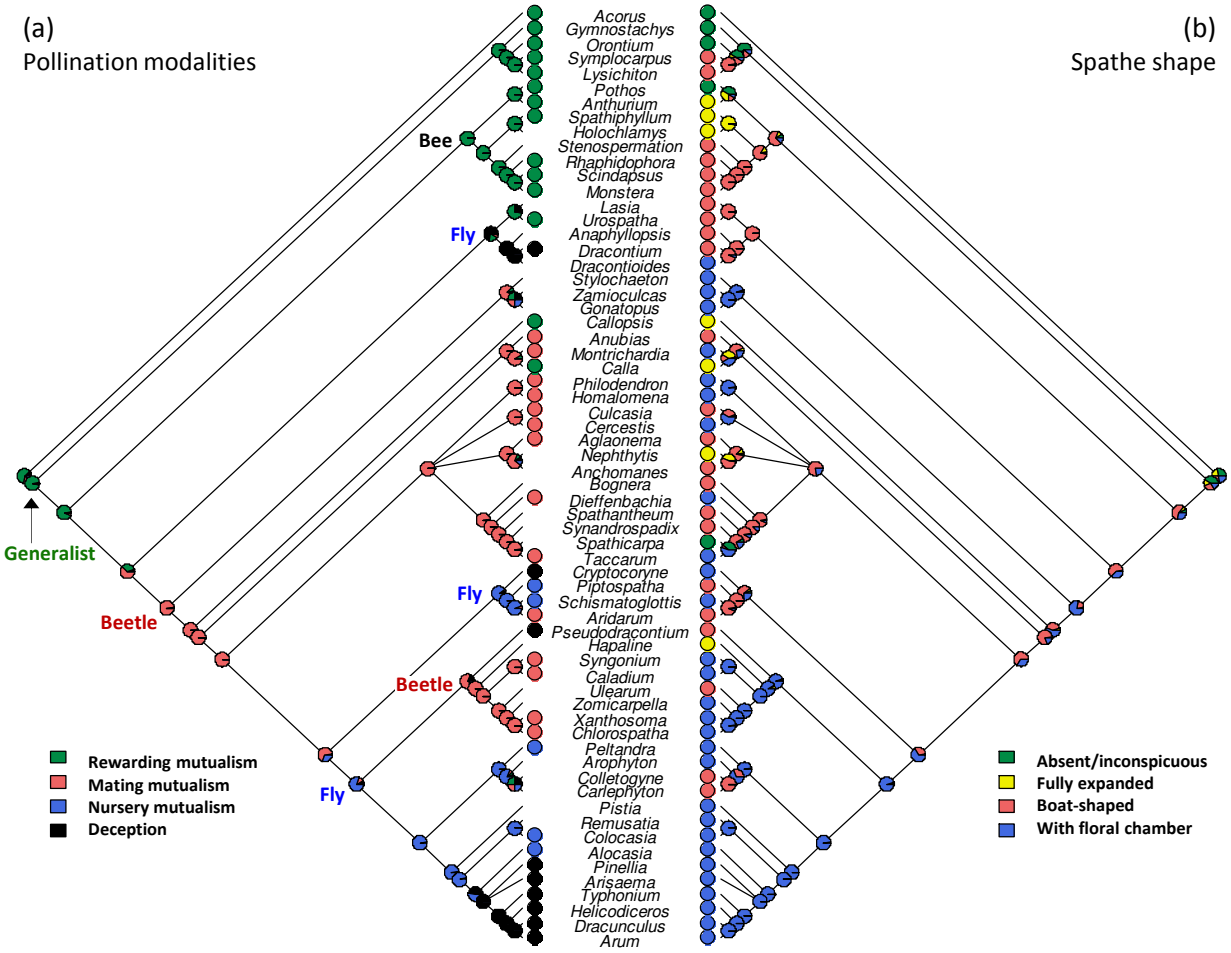
(a)
Pollination modalities
(b)
Spathe shape
Acorus
Gymnostachys
Orontium
Symplocarpus
Lysichiton
Pothos
Anthurium
Spathiphyllum
Holochlamys
Stenospermation
Rhaphidophora
Scindapsus
Monstera
Lasia
Urospatha
Anaphyllopsis
Dracontium
Dracontioides
Stylochaeton
Zamioculcas
Gonatopus
Callopsis
Anubias
Montrichardia
Calla
Philodendron
Homalomena
Culcasia
Cercestis
Aglaonema
Nephthytis
Anchomanes
Bognera
Dieffenbachia
Spathantheum
Synandrospadix
Spathicarpa
Taccarum
Cryptocoryne
Piptospatha
Schismatoglottis
Aridarum
Pseudodracontium
Hapaline
Syngonium
Caladium
Ulearum
Zomicarpella
Xanthosoma
Chlorospatha
Peltandra
Arophyton
Colletogyne
Carlephyton
Pistia
Remusatia
Colocasia
Alocasia
Pinellia
Arisaema
Typhonium
Helicodiceros
Dracunculus
Arum
Bee
Fly
Generalist
Beetle
Fly
Beetle
Fly
Rewarding mutualism
Mating mutualism
Nursery mutualism
Deception
Absent/inconspicuous
Fully expanded
Boat-shaped
With floral chamber

Figure 3

		Pollinator order			
Character	States	Generalist	Hymenoptera	Coleoptera	Diptera
Pollination type	Rew. mutualism	0.059 (0.01)	0.061 (0.018)	-0.072 (0.012)	-0.049 (0.006)
p=0.002	Mating mutualism	-0.040 (0.03)	ns	0.113 (<1.10-6)	-0.035 (0.032)
	Nursery mutualism	ns	ns	ns	0.045 (0.02)
	Deception	ns	ns	ns	0.039 (0.022)

Table 1

		Pollinator order			
Character	States	Generalist	Hymenoptera	Coleoptera	Diptera
Flower sexe	Bisexual	0.072 (0.002)	0.069 ($<1.10^{-6}$)	-0.079 (0.002)	-0.062 (0.008)
$p<1.10^{-6}$	Unisexual	-0.072 (0.002)	-0.069 ($<1.10^{-6}$)	0.079 (0.002)	0.062 (0.008)
Distribution	Cold/Temperate zones	0.076 (0.004)	ns	ns	ns
p=0.038	Inter-tropical zones	-0.076 (0.004)	ns	ns	ns
Pollen starch	Absent	0.056 (0.018)	ns	ns	-0.046 (0.032)
p=0.04	Present	-0.056 (0.018)	ns	ns	0.046 (0.032)
Spadix zonation	No zonation	0.065 (0.030)	0.064 (0.008)	-0.074 (0.004)	-0.055 (0.014)
$p<1.10^{-6}$	Female/male	ns	-0.028 (0.032)	0.055 (0.02)	ns
	Female/S/male	ns	ns	0.050 (0.02)	ns
	Female/male/S	ns	ns	ns	ns
	Female/S/male/S	ns	ns	-0.032 (0.018)	0.078 (0.006)

Table 2

		Pollination type			
	States	Rewarding mutualism	Mating mutualism	Nursery mutualism	Deception
Sexe	Bisexual flowers	0.175 (<1.10^{-6})	-0.115 (0.002)	-0.037 (0.026)	-0.023 (0.048)
p<1.10^{-6}	Unisexual flowers	-0.175 (<1.10^{-6})	0.115 (0.002)	0.037 (0.026)	0.023 (0.048)
Sterile flower type	No sterile flowers	0.105 (0.004)	-0.07 (0.014)	ns	ns
p=0.004	Nutritive sterile flowers	-0.104 (<1.10^{-6})	0.088 (0.002)	ns	ns
	Hair like sterile flowers	ns	ns	ns	ns
Spadix zonation	No zonation	0.162 (<1.10^{-6})	-0.104 (0.004)	ns	ns
p<1.10^{-6}	Female/male	-0.05 (0.016)	0.08 (0.02)	ns	ns
	Female/S/male	ns	0.058 (0.016)	ns	ns
	Female/male/S	ns	ns	ns	ns
	Female/S/male/S	-0.067 (0.006)	-0.032 (0.038)	0.053 (0.012)	0.047 (0.046)
Spathe structure	Absent/inconspicuous	0.043 (0.03)	ns	ns	ns
p=0.048	Fully expanded	ns	ns	ns	ns
	Boat-shaped	ns	ns	ns	ns
	With floral chamber	-0.087 (<1.10^{-6})	ns	ns	0.03 (0.048)
Female flower number	1-15,7	-0.023 (0.05)	0.021 (0.056)	ns	ns
p=0.012	22-37,4	-0.026 (0.008)	0.033 (<1.10^{-6})	ns	ns
	43,5-98	-0.012 (0.04)	ns	ns	ns
	119,5-211	0.019 (0.034)	-0.028 (0.002)	ns	ns
	238,5-1442,4	0.042 (0.014)	-0.030 (0.01)	ns	ns
Stigma area (mm2)	0,002-0,04	0.013 (0.018)	-0.013 (0.016)	ns	ns
p=0.034	0,07-0,2	0.007 (0.016)	-0.010 (0.01)	ns	ns
	0,29-0,66	-0.004 (0.018)	ns	0.003 (0.034)	0.003 (0.036)
	0,79-2,31	-0.008 (0.01)	0.01 (0.01)	ns	ns
	2,61-13,08	ns	0.015 (0.012)	ns	ns
Pollen exine surface	Reticulate/other	0.132 (0.002)	-0.061 (0.016)	-0.043 (0.034)	-0.028 (0.044)
p=0.004	Smooth	-0.071 (0.002)	0.076 (0.006)	ns	ns
	Spinose	-0.067 (0.018)	ns	ns	0.033 (0.034)
	Striate	ns	ns	ns	ns
Pollen starch	Absent	0.091 (0.004)	-0.054 (0.022)	ns	ns
p=0.026	Present	-0.091 (0.004)	0.054 (0.022)	ns	ns
Distribution	Cold/Temperate zones	0.062 (0.018)	-0.068 (0.004)	ns	ns
p=0.046	Inter-tropical zones	-0.062 (0.018)	0.068 (0.004)	ns	ns

Table 3

		Pollen-ovule ratio				
	States	5-812	1286-4913	6205-11350	15222-40430	51611-122055
Pollen exine surface	Reticulate/other	-0.035 (<1.10^{-6})	-0.016 (0.008)	ns	0.016 (0.008)	0.030 (0.006)
p=0.026	Spinose	0.026 (0.008)	0.015 (0.008)	-0.001 (0.01)	ns	-0.024 (0.012)
Spathe structure	Boat-shaped	-0.026 (0.004)	-0.02 (0.002)	ns	0.016 (0.006)	0.028 (0.006)
p=0.012	With floral chamber	0.032 (0.004)	0.018 (0.004)	-0.005 (0.06)	-0.02 (0.002)	-0.025 (0.006)
Spadix zonation	No zonation	-0.029 (0.004)	-0.013 (0.014)	0.006 (0.03)	0.013 (0.016)	0.02 (0.012)
p=0.024	Female/S/male	0.019 (0.03)	ns	-0.005 (0.032)	ns	ns
	Female/S/male/S	0.026 (0.006)	0.015 (0.014)	ns	-0.015 (0.012)	-0.022 (0.006)
Ovule number	1	-0.025 (0.002)	-0.018 (0.002)	ns	0.014 (0.004)	0.025 (0.006)
per flower	1.5-6.1	ns	0.005 (0.024)	ns	ns	ns
p=0.024	8-11	0.008 (0.026)	ns	ns	-0.005 (0.032)	-0.01 (0.018)
	13.9-22.3	0.014 (0.008)	ns	-0.004 (0.014)	-0.007 (0.012)	-0.008 (0.05)

Table 4

Troisième partie

ÉTUDE À L'ÉCHELLE DE LA POPULATION DE L'INTÉRACTION *ARUM*-POLLINISATEURS

Chapitre 5

Résumé des articles

La PARTIE 3 est constituée de trois articles dont le but est d'étudier les variations géographiques et temporelles des odeurs attractives chez deux espèces d'*Arum*, *Arum italicum* et *Arum maculatum*, en relation avec les variations locales de leurs modalités de pollinisation. *A. italicum* et *A. maculatum* sont deux espèces sapromyophiles, c'est à dire pollinisées par des diptères dans une interaction de duperie. Les insectes sont en effets attirés par une odeur de bouse ou d'urine émise par la plante, et caractéristique de leur site d'oviposition, et glissent dans une chambre floral formant un piège dans lequel ils sont séquestrés pendant environs vingt-quatre heures. Ils sont attirés dans les inflorescences le premier soir de la floraison, au moment de la réceptivité des stigmates, et peuvent donc polliniser les inflorescences s'ils emmènent du pollen. Ils ne sont libérés que lendemain en fin de matinée, au moment où le pollen est libéré. Ils se font alors saupoudrer de pollen qu'ils exportent en sortant des inflorescences (Figure 1).

Le CHAPITRE 6 de cette partie, publié en 2011, est une étude des variations inter-population des guildes de pollinisateurs et des odeurs attractives d'*Arum italicum* et d'*A. maculatum*. Cette étude avait pour premier but de décrire la diversité des pollinisateurs des deux espèces dans différentes populations. *A. italicum* et *A. maculatum* étant soupçonnés d'être respectivement opportuniste et spécialiste du point de vue de leur pollinisation (Kite 1995, Diaz et Kite 2002, Albre et Gibernau 2008), il fallait en effet savoir si ces tendances se confirmaient lors de l'étude de plus de populations, et ensuite si cette différence écologique de pollinisation était corrélée à des schémas différents de variations des odeurs attractives pour les pollinisateurs entre les populations des deux espèces. L'hypothèse de départ étaient que les profils d'odeurs de l'espèce généraliste *A. italicum* soient plus structurés entre les populations, car adaptées à des faunes

locales différentes, tandis que les profils d'odeur de l'espèce spécialiste *A. maculatum* soient plus homogènes entre les différentes populations, dans lesquelles il attireraient toujours le même pollinisateur.

Pour tester ces hypothèses, les pollinisateurs des deux espèces ont été collectés dans quatre populations différentes d'*A. italicum* et deux populations différentes d'*A. maculatum* en 2008. La récolte des insectes dans les inflorescences d'*Arum* est très simple, puisqu'au stade de réceptivité femelle les insectes sont captifs dans les inflorescences. Les insectes de 232 inflorescences ont ainsi pu être déterminés au niveau de la famille dans les différentes populations.

En parallèle, des plants d'*Arum* des différentes populations ont été récoltés et cultivés sous serre dans les mêmes conditions. Lors de l'ouverture des inflorescences, les odeurs ont été collectées et analysées par chromatographie en phase gazeuse. Les profils d'odeurs obtenus ont ensuite été analysés pour obtenir les pourcentages relatifs de chaque composé dans chaque prélèvement, qui ont servi à comparer les individus entre eux.

L'identification à la famille des insectes a montré que dans les populations étudiées, *A. italicum* était principalement pollinisé par des diptères appartenant aux familles des Psychodidae, Chironomidae, et en moindre proportions Ceratopogonidae, Sciaridae, et certaines espèces de Brachycères, ainsi que des coléoptères de la famille des Staphylinidae. Ces proportions variaient selon les populations, et le pollinisateur principal n'était donc pas toujours le même. À l'inverse, *A. maculatum* s'est avéré être pollinisé par plus de 90% de Psychodidae dans les deux populations étudiées. Ces deux résultats confirmaient bien les statuts respectifs de pollinisation opportuniste et spécialiste d'*A. italicum* et *A. maculatum*. De plus, *A. maculatum* s'est avéré être jusqu'à cent fois plus attractif qu'*A. italicum* dans certaines populations. Par contre, contrairement à l'hypothèse de départ, les profils de l'odeur attractive des inflorescences d'*A. italicum* se sont avérés peu structurés entre les populations étudiées, contrairement aux profils d'odeurs d'*A. maculatum*, qui étaient significativement différents entre les deux populations étudiées.

Les différences de structuration des pollinisateurs attrapés et des odeurs attractives entre les populations de ces deux espèces peuvent avoir plusieurs causes. Tout d'abord, les pollinisateurs disponibles sont susceptibles de varier dans le temps et l'espace, ce qui pourrait expliquer le fait qu'*A. italicum* n'attrape pas toujours la même diversité d'insectes. Ensuite, ces variations pourraient être causées par la variation de certains composés dans l'odeur d'un site, à l'autre, non visibles lors de l'étude des profils d'odeurs complets. Par ailleurs, la structuration étonnante des odeurs entre les populations d'*A. maculatum* pourrait être due à des flux de gènes moins importants entre les populations d'*A. maculatum* qu'entre celles d'*A. italicum*.

L'étude présentée dans le CHAPITRE 7 (article en préparation) avait pour but de poursuivre l'étude de 2008 sur *A. italicum* et *A. maculatum* et de répondre à certaines des questions alors posées. Pour cela, les odeurs et pollinisateurs d'*A. italicum* ont été échantillonnés dans 6 populations différentes en 2009 (dont les populations échantillonnées en 2008), et quatre d'entre elles ont été ré-échantillonnées en 2010. Les populations d'*A. maculatum* étudiées ont été échantillonnées dans les mêmes populations qu'en 2008 en 2009 et 2010, ainsi que dans une nouvelle population en 2009. Les insectes appartenant à la famille des Psychodidae ont été cette fois identifiés au niveau de l'espèce, pour déterminer précisément le degré de spécialisation d'*A. maculatum* pour ses pollinisateurs, et comparer la diversité de Psychodidae attrapés par les inflorescences des deux espèces d'*Arum* dans les deux sites étudiés où elles poussent en sympatrie (Smarves, Poitou, France, et Bagnères-de-Bigorre, Hautes Pyrénées, France).

Les odeurs ont également été collectées sur ces sites, et analysées par chromatographie en phase gazeuse et spectrométrie de masse, ce qui a permis d'identifier la majorité des composés olfactifs. Les pourcentages relatifs des composés émis par les inflorescences de chaque individu ont ensuite été comparés.

Enfin, des tests de transplantations de plants d'*A. italicum* et d'*A. maculatum* ont été effectués en 2008 et 2009 entre deux sites séparés d'une centaine de kilomètres, l'un à Toulouse (Midi-Pyrénées, France), où pousse une population d'*A. italicum*, et l'autre à Bagnères-de-Bigorre, où les deux espèces poussent en sympatrie.

Comme en 2008, les pollinisateurs attirés par les deux espèces se sont avérés varier entre les populations et d'une année sur l'autre pour *A. italicum* et être beaucoup plus stables pour *A. maculatum*. Les proportions de Psychodidae attrapées par les inflorescences des deux espèces ont varié d'un site à l'autre, mais pour chaque site, sont restées stables d'une année à l'autre. Ainsi, *A. italicum* a attiré en tout cinq espèces de Psychodidae différentes parmi les populations étudiées, tandis qu'*A. maculatum* a majoritairement attiré deux espèces, *Psychoda phalaenoides* et *Psycha grisescens* selon la population. Une étude récente (Espindola *et al.* 2011) a également montré qu'*A. maculatum* était pollinisé par ces deux même espèces de Psychodidae à l'échelle de l'Europe, dont la répartition dépendrait probablement des conditions climatiques. Il est intéressant de noter que dans les sites où les deux espèces d'*Arum* poussent en sympatrie, les espèces de Psychodidae qu'elles attirent ne sont pas les mêmes, et qu'il est possible qu'un isolement reproducteur ait été mis en place dans ces populations là.

Lors des tests de transplantation, à Bagnères-de-Bigorre ou à Toulouse, les inflorescences d'*A. italicum* transplantées ont attiré la même diversité et les mêmes quantités d'insectes que les inflorescences natives du site de transplantation. Ceci suggère que la guilde de pollinisateurs attrapée par cette espèce dépend fortement de la disponibilité en insectes du milieu, et non d'une adaptation locale de l'attractivité de la plante ou des préférences des pollinisateurs. Le fait que les odeurs des inflorescences d'*A. italicum* étudiées en 2009 montrent peu de structuration entre les populations corrobore le résultat identique trouvé en 2008 et le caractère "opportuniste" d'*A. italicum* du point de vue de la pollinisation. Notons qu'à Smarves en 2009, un des sites de sympatrie des deux espèces, l'odeur des inflorescences d'*A. italicum* différait faiblement des autres populations d'*A. italicum* étudiées (mais pas significativement) et était significativement différente en 2010 de l'odeur des inflorescences d'*A. italicum* de Bagnères-de-Bigorre (l'autre site de sympatrie des deux espèces).

Transplantées de Bagnères-de-Bigorre à Toulouse, les inflorescences d'*A. maculatum* ont toutes attrapé une majorité de Psychodidae, qui se sont avérés appartenir à la même espèce, *Psychoda phalaenoides*, quel que soit le site. Les tests de transplantation réciproque n'ayant pas pu être effectués sur cette espèce (qui ne pousse pas naturellement à Toulouse), il n'est pas possible d'affirmer si ce résultat est dû à une spécificité particulière des inflorescences de Bagnères-de-Bigorre pour *Psychoda phalaenoides*. Néanmoins, les différences entre les odeurs des populations d'*A. maculatum* semblaient être corrélées aux différences de Psychodideae capturés entre les populations en 2009 et 2010, et une adaptation plus importante aux pollinisateurs de l'odeur d'*A. maculatum*, l'espèce spécialiste, que de l'odeur d'*A. italicum*, l'espèce opportuniste, semble possible.

Pour aller plus loin, l'étude du CHAPITRE 8 (article soumis à *Annals of Botany*) a consisté à étudier plus précisément le rôle de l'odeur attractive d'*A. italicum* et d'*A. maculatum* dans l'isolement reproducteur des deux espèces. En effet, à Bagnères-de-Bigorre, où les deux espèces poussent en sympatrie, il a été montré qu'*A. italicum* pouvait attirer jusqu'à 40% de Psychodidae, dont certains appartenaient à l'espèce qui pollinisait *A. maculatum*. Sur ce site, les deux espèces sont donc potentiellement en compétition pour cette espèce de pollinisateur. Par ailleurs, *A. italicum* est une espèce hexaploïde, tandis qu'*A. maculatum* est tetraploïde (Bedalov et Küpfer 2005). Leur hybridation devrait donc donner des hybrides pentaploïdes, peu ou pas fertiles, à cause d'anomalies chromosomiques (Rieseberg et Willis 2007). Dans ce cas, un isolement reproducteur pourrait avoir été sélectionné entre les deux espèces sur ce site.

L'isolement reproducteur entre deux espèces entomophiles de plantes phylogénétiquement proches peut se faire de différentes manières. Tout d'abord, il peut résulter d'un décalage dans la période de floraison ou d'attraction des pollinisateurs dans le temps. Ensuite, il peut être dû à une attraction différentielle des pollinisateurs, évitant ainsi les flux inter-spécifiques de pollen. Enfin, il peut être dû à des mécanismes physiologiques apparaissant après l'évènement de pollinisation, comme l'incompatibilité pollinique ou l'avortement des fruits formés. Pour tester ces hypothèses, la phénologie des deux espèces a été suivie en 2010 pendant la quasi-totalité de leur période de floraison. Les hybrides potentiels ont été recensés sur le site, ainsi que les pollinisateurs et les odeurs des deux espèces et de leurs hybrides potentiels, reconnus à leurs couleurs florales intermédiaires. Les traits floraux des trois sortes d'*Arum* ont été comparés, et des tests de fécondation croisés opérés entre *A. italicum* et *A. maculatum*. La période de la journée pendant laquelle les inflorescences d'*A. italicum* et d'*A. maculatum* attirent leurs pollinisateurs a également été déterminée pour chaque espèce par un suivi de trois inflorescences par espèce pendant toute une soirée. Enfin, des tests de choix ont été opérés en plaçant des inflorescences d'*A. italicum* et d'*A. maculatum* au stade attractif dans une cage de toile opaque, et en y relâchant des insectes prélevé le matin même dans des inflorescences de l'une ou l'autre des deux espèces.

Les barrières de reproduction pré-pollinisation se sont avérées faibles entre les deux espèces d'*Arum*. En effet, les périodes de floraisons et d'attraction des pollinisateurs se recouvraient largement, et les deux espèces attiraient le même pollinisateur, *Psychoda phalaenoides*, majoritairement pour *A. maculatum* et de façon minoritaire pour *A. italicum*. Les barrières de reproduction post-pollinisation se sont également avérées faibles, car la majorité des inflorescences pollinisées à la main ont produit des fruits quelques soit le traitement. L'analyse des odeurs a cependant permis d'apporter une réponse à la coexistance apparemment stable des deux espèces sur ce site. Des tests de corrélations ont en effet montré que la différence de spécificité des deux espèces d'*Arum* pour leurs pollinisateurs était surement due à une différence dans les composés attractifs émis, expliquant qu'*A. italicum* n'attire qu'une moindre proportion de *Psychoda phalaenoides*. Par ailleurs, *A. maculatum* s'est avéré particulièrement attractif sur ce site, attirant plus de deux fois le nombre d'insectes attirés par *A. italicum* sur deux années consécutives, et lors des tests de choix. Ainsi, le comportement opportuniste d'*A. italicum* et la forte attractivité pour son pollinisateur spécifique d'*A. maculatum* sont probablement les deux facteurs expliquant l'évitement d'une exclusion compétitive entre les deux espèces à Bagnères-de-Bigorre.

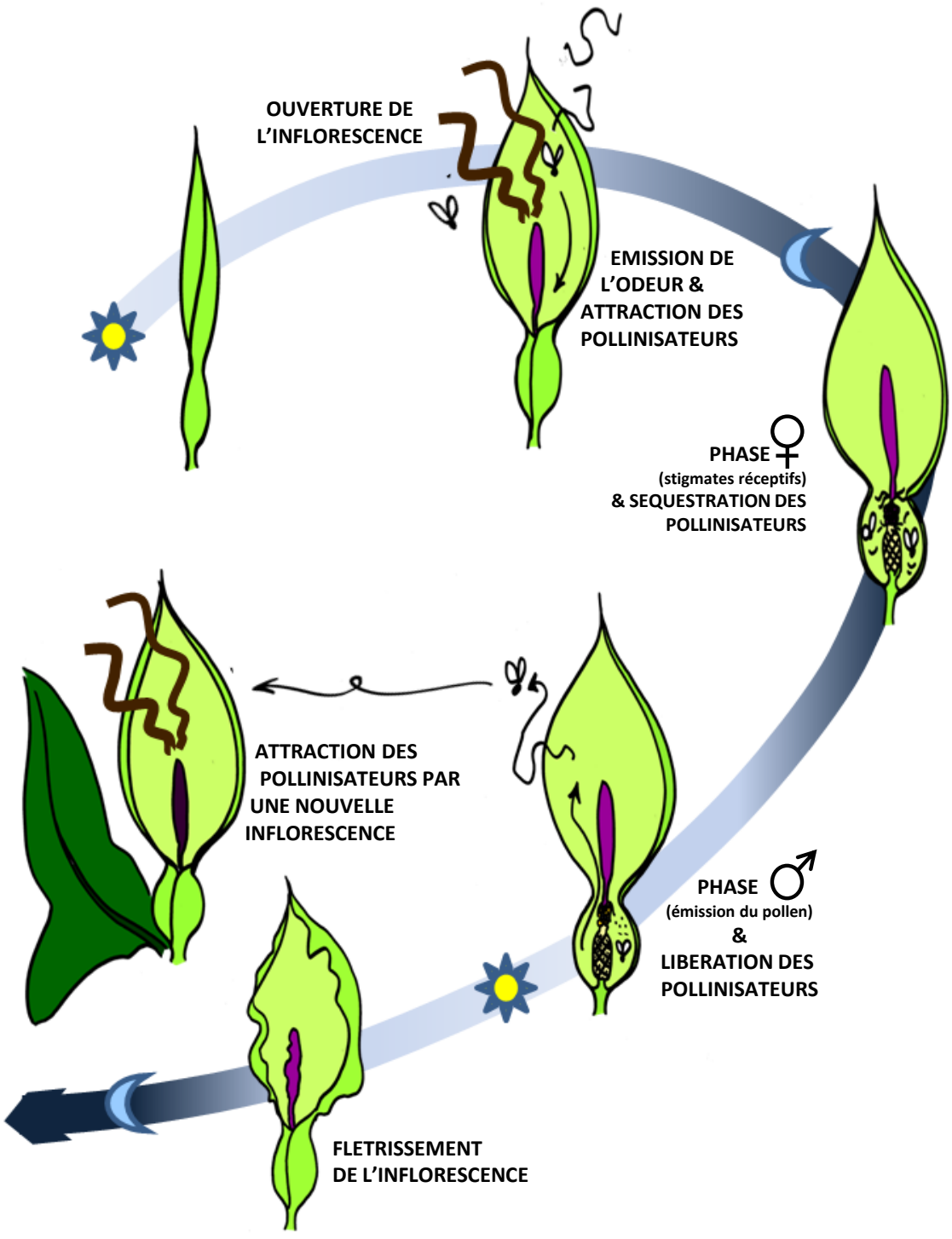

Figure 1. Schéma des différentes étapes du cycle floral d'*Arum italicum* et d'*A. maculatum.*

Chapitre 6

Variations géographiques des profils d'odeurs d'*A. italicum* et *A. maculatum* et spécificité pour leurs pollinisateurs

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Do floral odor profiles geographically vary with the degree of specificity for pollinators? Investigation in two sapromyophilous *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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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 *Psychodida*e species were found in Spain and in the South of France (Mendez & Obseo 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 "opportunist" *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 mimicks the oviposting 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?

(ii) 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 were *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 Chantonnay, 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 a 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	***A. italicum***								***A. maculatum***			
Population	Chantonnay		Igeldo		Toulouse		Uzer		Uzer		Smarves	
N	12		35		135		25		22		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 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 Chantonnay, 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%).

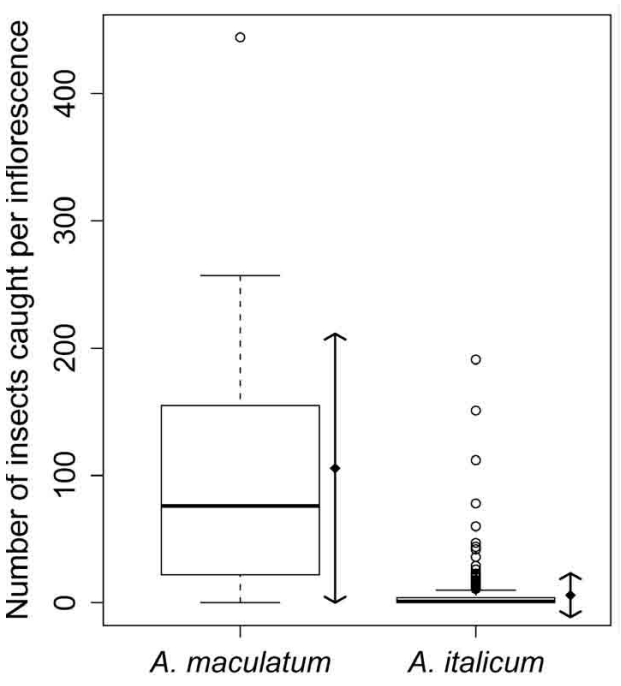

Figure 1
Boxplot of the number of insects caught in the inflorescences of *Arum italicum* and *A. maculatum* in the five studied populations. Quartiles = 0, 22, 76, 155, 257 for *A. maculatum* (N = 25) and 0, 0, 1, 4, 10 for *A. italicum* (N = 207). Arrows indicate means and standard deviations.

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).

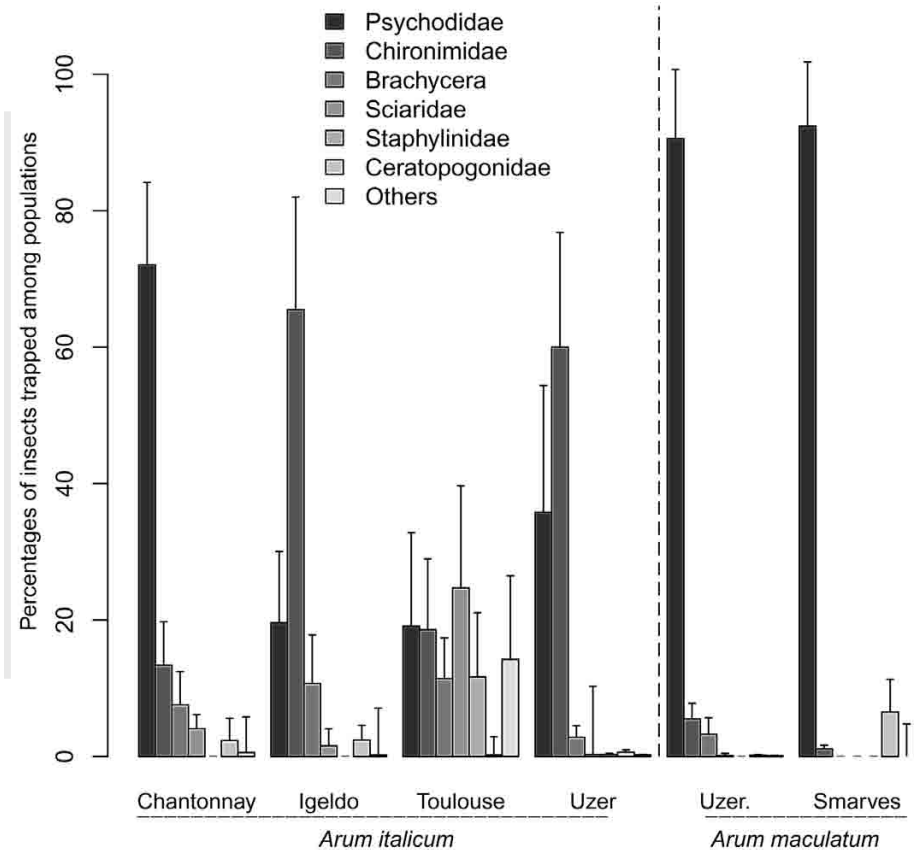

Figure 2
Diversity of the inflorescence visitors of *Arum italicum* and *A. maculatum*. Percentages and standard deviations of the different insect groups caught in the inflorescences from the five studied populations are given. See tab. 1 for the sample size and total number of insects.

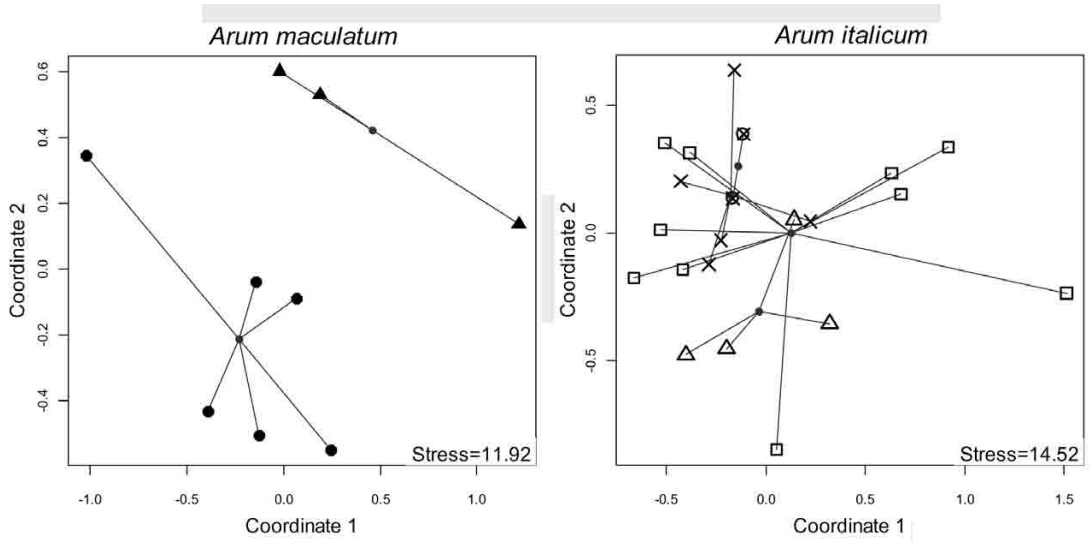

Figure 3
Non-metric MDS representation of the inflorescence odour profiles obtained from *Arum italicum* from Chantonnay (crosses), Igeldo (empty triangles), Toulouse (empty squares), Pierrelatte (empty circles with crosses) and *A. maculatum* from Smarves (full triangles) and Uzer (full circles). The distance between symbols represents the difference between 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 Chantonnay), Chironomidae (respectively 66 and 60% in Igeldo and Uzer) and Sciaridae (25% in Toulouse). Interestingly, in the northernmost population in Chantonnay, 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 Chantonnay, 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 consistant 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 Chantonnay 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 Chantonnay 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 wich 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.

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Chapitre 7

Variations géographiques des odeurs et des pollinisateurs de deux espèces d'*Arum*, et recherche des adaptations locales aux pollinisateurs par des tests de transplantation

Soumission prévue dans ***Functional Evology*** .

Auteurs : Marion CHARTIER, Laurent PÉLOZUELO, Bruno BUATOIS, Jean-Marie BESSIÈRE, Marc GIBERNAU

Geographic variations of odour and pollinators in a deceptive pollination system: a test for local adaptation by reciprocal transplants of two European *Arum* species (Araceae)

7.1 ABSTRACT

Interactions between entomophilous plants and their pollinators are one of the major factors shaping the evolution of floral features. As species are distributed in more or less connected populations, they evolved in a geographic mosaic of co-evolution, were the outcome of the plant-pollinator interaction is likely to vary from site to site as a result from local adaptations.

Arum italicum and *A. maculatum* are two Araceae species deceiving their Diptera pollinators by mimicking the dung odour of their oviposition sites. Whereas *A. italicum* is known to be pollinated by insects belonging to different Diptera families (i.e. opportunist), *A. maculatum* has been shown to be specialized to two pollinator species over its European repartition area, but only one pollinator species in a given population. To test for local adaptation for pollinators through variations of the pollinator-attractive odour, the temporal and geographical variations of pollinators and pollinator-attractive odour were described in several populations of the two species over two consecutive years. To assess whether pollinator geographical variations were due to differences in local insect availability or to local adaptation of the *Arum* species, transplants of inflorescences of the two species were performed in two consecutive years between two sites from South of France.

Pollinators of the opportunist species *A. italicum* varied in time and space, but the floral odour was not structured between populations, indicating potentially high gene flows or selection for a variable odour due to fluctuations of pollinator availability, or in relation to the deceptive pollination system of the plant species. When transplanted, inflorescences of *A. italicum* trapped the same diversity of insects than in native inflorescences in the site, indicating that pollinator diversity highly depends on insect availability in the site for this species. On the contrary, the specialist species *A. maculatum* only trapped in high quantities one of its two known main pollinators *Psychoda phalaenoides* or *Psycha grisescens* (Diptera, Psychodidae), but the species varies according to the population. The floral odour variations tended to be more structured for this species, indicating a potential adaptation of the plant to the pollinator species in each population.

The comparison of these two related deceptive pollination systems suggests that different degrees of specificity may have consequences in terms of odour and population structure. But a striking result is that local pollination conditions (i.e. pollinator diversity and availability) strongly affect the degree of geographical variations of this interaction.

7.2 INTRODUCTION

Angiosperm diversification is believed to have been shaped by the interactions which evolved between many plants species and their insect pollinators (reviewed by Johnson 2006, Herrera *et al.* 2006). Floral traits directly dedicated to pollinators' attraction and rewarding (which are called "pollination syndromes") have been strongly selected in plants such as flower colour, display, odour, size and/or shape, but also rewards like edible floral tissues, nectar or secretions (Stebbins, 1970, Proctor *et al.* 1996, Faegri and van der Pijl 1979, Fenster *et al.* 2004). Pollinators and pollination syndromes can vary among populations of the same species, leading to plant and pollinator species interacting in populations under different selective pressures, resulting in a geographic mosaic of co-evolution (Thompson 2005, Gomez *et al.* 2009). These populations are more or less inter-connected by gene flux, which control the degree of differentiation among populations. In the case of sufficient divergence of one of these populations, speciation is likely to occur (Levin 2000, Thompson 2005). This process may for instance occur through pollinator shifts: pollinators, through visitation preference, can isolate a genotype from another (Gould and Johnston 1972, Kiester *et al.* 1984, Bradshaw and Schemske 2003). As pollinators are likely to vary from a site to another, they may be one important factor shaping geographical variations of floral traits. Thus, studies on geographical variations of floral traits linked to pollinator variations among populations might help understanding the mechanism leading to species formation and evolution (Herrera *et al.* 2006). Recently, some of these studies have demonstrated striking correlations between pollinator shift or pollinator traits changes and floral features variations (e.g. Anderson and Johnson 2007, Gomez *et al.* 2008, Cosacov *et al.* 2008, Schlumpberger *et al.* 2009, Brown *et al.* 2011, but see Ellis and Johnson 2009, Nattero *et al.* 2010) or between climatic variations and floral traits variations (Hodgins and Barrett 2008) or pollinator variations (Espindola *et al.* 2011).

Here, we investigated the geographical variations of a plant-pollinator interaction, focusing on its main pollinator-attractive signal: the floral odour. The emission of a floral odour is a widely spread trait in flowers, and its major function is recognition between plants and their pollinators (Pichersky and Gershenzon 2002, Knudsen 2006, Schaeffer and Ruxton 2011). In some plant species, floral odour has been shown to be the major or unique attractive cue, for example in the very specialized interactions between fig and fig wasps (Gibernau *et al.* 1998), orchids and male bees (Schiestl and Schlüter 2009), yucca and yucca moth (Svensson *et al.* 2006), or Araceae and euglossin bees (Hentrich et al, 2007, 2010). This is also the case for flowers

attracting pollinators at dusk or during the night, when visual cues are poorly informative, like plants species from the families Annonaceae (Gottsberger 1999), Cactaceae (Schlumpberger *et al.* 2009), or Araceae (Gibernau *et al.* 2004). Floral odours are labile, and can vary in chemical compounds composition, in the relative amount of compounds, or in their overall quantities (e.g. Dötterl *et al.* 2005, Raguso 2008).

In the context of the geographic mosaic of co-evolution as described by Thompson (2005), local adaptation to pollinators may cause floral scent variations. Floral scent variations have been tested in a few studies, in which gene flow and geographic distance have been more often proposed to explain the observed variations or stability, more than adaptation to pollinator local preferences (Knudsen 2002, Ackerman *et al.* 1997, Solers *et al.* 2010, but see Svenssen *et al.* 2005, Schlumpberger and Raguso 2008, Ibanez *et al.* 2010). Local adaptation to biotic and abiotic conditions can be easily tested through reciprocal transplant tests (Volis 2000, Campbell and Waser 2007, Angert *et al.* 2008, Leger *et al.* 2009), and they have been sometimes used in studies on pollination (Geber and Eckhart 2005, Campbell 2003, Watermann *et al.* 2011). So far, few studies investigated geographical variations of both pollinators and floral scent (Svensson *et al.* 2005, 2006, Pettersson and Knudsen 2001, Ibanez *et al.* 2010).

Floral odour profiles have also been shown to vary according to the specificity degree for their pollinators of two European *Arum* species, but the cause of pollinators and odour variations remained to be demonstrated (Chartier *et al.* 2011).

A. italicum and *A. maculatum* are two species from the Araceae family growing in temperate woodlands, on the forest floor (Mayo *et al.* 1997). The two species are sapromyophilous, as their inflorescences attract mainly Diptera insects and sequester them almost a day in a trap to ensure pollination (Lack and Diaz 1991, Albre *et al.* 2003, Gibernau *et al.* 2004). The classical floral cycle lasts about 24 hours over two days. On the first day afternoon, 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 up (Bermadinger and Bermadinger-Stabentheiner 1995, Albre *et al.* 2003) and emits the attractive odour (Kite 1995). Attracted insects land on the spathe and slide into the floral chamber. At this stage, if the insects carry fresh pollen, they can pollinate the receptive female flowers while moving onto the floral chamber. The next morning, the stigmas are no more receptive, and pollen is released on the insects. The hair corona blocking the exit of the floral chamber dries, and the insects leave the inflorescence carrying fresh pollen. If they are trapped again, they can cross pollinate a new inflorescence. Up to now, *A. maculatum* has been shown to be mainly pollinated by two

species from the Psychodidae family, *Psychoda phalaenoides* or *Psycha grisescens* according to the population (Prime 1960, Rohacek *et al.* 1990, Lack and Diaz 1991, Diaz and Kite 2002, Chartier *et al.* 2011, Espindola *et al.* 2011). On the contrary, the insect diversity found in the inflorescences of *A. italicum* fluctuates greatly between sites (Gibernau *et al.* 2004a, Chartier *et al.* 2011): different Psychodidae species were found in Spain and in the South of France, as well as diverse Diptera species from the families Ceratopogonidae, Sciaridae and Chironomidae (Mendez and Obeso 1992, Diaz and Kite 2002, Albre *et al.* 2003, Chartier *et al.* 2011). Therefore, pollinators of the "opportunist" *A. italicum* have been shown to vary among sites, whereas the main pollinator of the "specialist" *A. maculatum* has been shown to be always *P. phalaenoides* or *P. grisescens*.

In *Arum*, the principal pollinator-attractive feature is the floral odour mimicking the odour of the ovipositing site 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 and Kite 2002). Recently, the geographical variations of their profiles have been proposed to be linked to their different degree of specificity in several populations in France (Chartier *et al.* 2011). Floral odour profiles of *A. italicum* were not geographically structured among populations, suggesting a high level of 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 level of gene flow or adaptation to different local pollinator preferences, but these hypotheses remained to be tested, and the chemical composition of the floral blends remained to be identified (Chartier *et al.* 2011).

In this paper, odours and pollinators were studied in six populations of *A. italicum* and three populations of *A. maculatum* on a wider geographical scale. Furthermore, reciprocal transplantation tests were performed to assess whether pollinator variations were due to local variation of pollinator availability, or to difference in the plant attractiveness/polllinator preferences among sites. The specific questions are: (1) How do pollinators and odours composition vary in time and space in *Arum italicum* and *A. maculatum*? (2) Is there a link between odour and pollinator variations in the two species? (3) Are pollinator variations among sites due to differences in plant attractiveness or in pollinator availability?

7.3 MATERIALS AND METHODS

Pollinators capture and determination Insects caught in the floral chamber of *Arum italicum* and *A. maculatum* were collected in 7 locations. Inflorescences of *A. italicum* were sampled in 2009 and 2010 in Chantonnay (Vendée, France, 46 ° 40'N 1 ° 06'O), Smarves (Vienne, France, 46 ° 30'N 0 ° 22'E), Toulouse (Haute-Garonne, France, 43 ° 33'N 1 ° 28'E) and Bagnères-de-Bigorre (Midi-Pyrénées, France, 43 ° 04'N 0 ° 09'E) and only in 2009 in Pierrelatte (Rhône-Alpes, France, 44 ° 22'N 4 ° 14'E) and Igeldo (Gipuzkoa, Spain, 43 ° 18'N 2 ° 04'O). Three populations of *A. maculatum* were sampled in La Loubatière (2009, Languedoc-Roussillon, France, 43 ° 24'N 2 ° 15'E), Bagnères-de-Bigorre and Smarves (2009 and 2010). Note that Bagnères-de-Bigorre and Smarves are sites where *A. maculatum* and *A. italicum* grow nearby in sympatry.

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 as the sterile hair corona blocks the exit of the floral chamber. 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 precious help of a Diptera taxonomist (Prof. Alain Thomas).

As Psychodideae were the most numerous insects trapped, and the main pollinator of *A. maculatum*, we estimated their specific diversity in each population. Females were identified at the species level under a microscope based on their genitalia and antenna shapes (Vaillant 1988, Withers 1989, Ježek 1990) for 4 Psychodidae per inflorescence on 5 inflorescences per population.

Floral scent collection Odours of *Arum maculatum* and *A. italicum* were collected in the field in 2009 and 2010. Inflorescence odours were collected for both species in the evening, between 8 pm and 11 pm. At this stage, the spathe is widely open, the appendix is warm and the odour is strong. Each Inflorescence (spathe and spadix) was wrapped in a plastic inert bag (Nalophan NA colorless, caliber 90, available from ETS Charles-Frères, France) in order to create an "open static headspace": the bottom of the bag was kept close under the floral chamber with a bond, isolating the inflorescence from the leaves 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 are absorbed and desorbed from a fiber attached within the needle of a modified syringe. We used StableFlexTM 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 distant from the appendix for 20 min. Closed empty bags containing ambient air from 3-4 m away the inflorescence were used as controls to discard putative VOCs not originating from inflorescence. Fibers were stored in a freezer (-20 ° C) until analyses by Gas Chromatography-Mass Spectometry (GC-MS).

Floral scent analyses GC-MS analyses were performed on the Platform for Chemical Analyses in Ecology of the "SFR 119 Montpellier Environnement Biodiversité", at the "Centre d'Écologie Fonctionnelle et Évolutive (Montpellier, France)". SPME Fibers were desorbed 5 minutes at 250 ° C into the 1177 Split/Splitless injector of a CP-3800 gas chromatograph (Varian Inc., Palo Alto, CA, USA) coupled with a Saturn 2000 ion trap spectrometer (Varian Inc.). The carrier gas was helium with a constant flow rate set close to 1.0 mL/min. A split ratio of 1:4 was used. The temperature of the column (fused silica capillary column, 30 m x 0.25 mm x 0.25 μm, CP-Sil 8 CB lowbleed MS, Varian Inc. in 2009; Optima 5 Accent, Macherey-Nagel, Düren, Deutschland in 2010) was maintained at 50 ° C for 2 min after injection, linearly increased to 200 ° C at a rate of 5 ° C/min, and then increased to 250 ° C at a rate of 10 ° C/min and maintained at 250 ° C for 1 min. Mass spectra were recorded in scan mode from 38 to 300 m/z with an electronic impact (EI) at 70 eV. The chemical compounds were identified by comparison with the mass spectral library NIST98 MS and Adams 2007, and retention indices founds in libraries and published data (Adams 2007). All chromatogram peaks were manually integrated, the relative percentage area of each peak was calculated for each chromatogram (i.e. each sampled inflorescence). Only peaks representing more than 1% or the total peaks area per chromatogram were kept for the analyses.

Reciprocal tansplant experiment Plants were transplanted between the sites Bagnères-de-Bigorre and Toulouse in 2008 and 2009. Plants from one site were put in pot in the morning, before the opening of the spathe, and transported in the second site in the early afternoon. Pots were randomly disposed in the new site to avoid any environmental bias. Inflorescences opened in the evening, and the trapped insects were collected in the next morning. Insects were conserved in 70% ethanol until determination at the family level. In 2008, inflorescences of *A. italicum* were transplanted from Toulouse to Bagnères-de-Bigorre and inflorescences of *A. maculatum* from Bagnères-de-Bigorre to Toulouse. The diversity and number of the insects trapped in these transplanted inflorescences was compared to the data from Chartier *et al.* (2011). In

2009, inflorescences of *A. italicum* were transplanted from Toulouse to Bagnères-de-Bigorre and inflorescences of the two species from Bagnères-de-Bigorre to Toulouse.

The diversity of Psychodidae trapped in the transplanted inflorescences was estimated in 2009 by identifying females at the species level under a microscope based on their genitalia and antenna shapes (Vaillant 1988, Withers 1989, Ježek 1990) for 4 Psychodidae per inflorescence on 5 inflorescences per treatment.

Note that all trapped insects were harvested in the transplanted inflorescence, thus, no pollen from these inflorescences could be exported to pollinate native inflorescences.

Statistical analyses The quantity and diversity of insects trapped were compared between the two species, and between all populations for each species and each year. The number and the diversity of insects trapped in the transplanted inflorescences were compared to the native inflorescences from the sites of transplantations and from the site of provenance. Insect proportions were compared between groups by non parametric multivariate analyses of variance (npMANOVA) with the function adonis() from *vegan* package in R (Anderson 2001). The same test was used as post-hoc, with a Bonferroni correction. In the results part, only the statistic values for overall tests are given for all multi-comparison tests.

Floral scents were compared between species and between populations for each species and years. The significance of the differences between the different groups was assessed with a npMANOVA using the funtion adonis() in R. The same test was used as post-hoc, with a Bonferroni correction. The odour variability was compared within the two species by comparing the mean Jaccard distances among individuals per species (Ackerman *et al.* 2011). In addition, as the prelevment and analyses conditions were the same, the relative total amount of emitted VOCs per species was estimated by the mean total peak areas per chromatogram and compared among species.

To visualize individual variations of odours, VOCs were grouped by metabolic pathways into four classes: benzenoids, monoterpenoids, sesquiterpenoids and lipids. As indole, a nitrogen containing compound, was one of the main compounds emitted by *A. maculatum*, it was added as a fifth compound class. The relative amounts of each compound class for all individuals were compared between each group with a npMANOVA using the funtion adonis() in R.

7.4 RESULTS

Pollinator variations In all studied populations in 2009 and 2010, *Arum italicum* and *A. maculatum* trapped mainly insects from the families Psychodidae (10964 insects trapped), Chironomidae (2007), Ceratopogonidae (107), Sciaridae (34; Nematocera, Diptera), some Brachycera (209, Diptera) and Staphilinidae (62, Coleoptera). 51 other insects were not identified. *A. italicum* was significantly less attractive than *A. maculatum* (Mann-Whitney (MW): $W = 9811, p < 1.10^{-3}$) with an overall mean per inflorescence of 18.11 ± 2.87 trapped insects (including 8.70 ± 1.27 Psychodidae and 8.01 ± 2.09 Chironomidae) whereas *A. maculatum* trapped a mean of 84.93 ± 17.07 insects per inflorescence (including 82.94 ± 17.02 Psychodidae).

Geographical variations of pollinators The number of insects trapped by the inflorescences varied between populations and years for *A. italicum* (Table 1) and *A. maculatum* (Table 2).

In 2009, inflorescences of *A. italicum* trapped more insects in Smarves, Bagnères-de-Bigorre and Igeldo (respectively: $49.5 \pm 13.16, 23.22 \pm 4.84$ and 13.12 ± 3.22; Kruskal-Wallis (KW): $\chi^2_5 = 65.86, p < 1.10^{-12}$) than in Chantonnay, Pierrelatte and Toulouse (respectively: $6.16 \pm 1.39, 5.59 \pm 1.04$ and 1.13 ± 0.62). In 2010, the inflorescences of Bagnères-de-Bigorre trapped a significantly higher number of insects ($75.86 \pm 18.14; KW : \chi^2_3 = 50.85, p < 1.10^{-10}$) than in other studied populations of Toulouse, Chantonnay and Smarves (respectively: $1.13 \pm 0.62, 1.38 \pm 0.46, 1.48 \pm 0.36$).

Inflorescences of *A. maculatum* trapped significantly more insects in Bagnères-de-Bigorre (respectively 147.75 ± 52.88 in 2009 and 216.12 ± 39.89 in 2010; KW: $\chi^2_2 = 13.24, p < 1.10^{-3}$ in 2009; MW: $W = 788, p < 1.10^{-8}$ in 2010) than in Smarves (respectively 19.36 ± 7.56 in 2009 and 1.47 ± 0.35 in 2010) and la Loubatière (6.50 ± 1.44 in 2009).

In 2009, populations of *A. italicum* showed three different types of pollinator diversity (npMANOVA: $F = 15.43, r^2 = 0.378, p < 1.10^{-4}$, Fig. 1a). Inflorescences trapped mainly Psychodidae in Smarves (95.46%), Chantonnay (80.13%), Pierrelatte (72.23%) and Bagnères-de-Bigorre (64.34%). Insect diversity was significantly different from all populations in Igeldo and Toulouse. Inflorescences trapped mainly Chironomidae in Igeldo (61.75%), whereas in Toulouse they trapped few Psychodidae and Chironomidae (27.22% and 7.98%) but a higher diversity of insects than in the other populations (64.8% of all other categories).

In 2010, populations of *A. italicum* attracted a lower proportion of Psychodidae, and showed

two different tendencies (npMANOVA:$F = 7.66, r^2 = 0.302, p < 1.10^{-4}$; Fig. 1b). In Smarves, Chantonnay and Bagnères-de-Bigorre, inflorescences attracted a majority of Psychodidae and Chironomidae, with a high proportion of Brachycera in Chantonnay (28.1%) and of unidentified insects in Smarves (20.0%). The insect diversity found in inflorescences from Toulouse was significantly different from all populations, with 66.7% of Sciaridae and 33.3% of Brachycera (Fig. 1b).

All populations of *A. maculatum* trapped mainly Psychodidae in 2009 (Fig. 2a). In Smarves and La Loubatière, they trapped significantly less Psychodidae (76.76% and 77.64%) than in Bagnères-de-Bigorre (94.26%; npMANOVA: $F = 3.75, r^2 = 0.149, p = 0.006$). The remaining insects belonged to other categories, but mainly of Brachycera with 13.3% of the total trapped insects in La Loubatière and 10.1% in Smarves. In 2010, inflorescences of *A. maculatum* attracted significantly different proportions of insects in Smarves and Bagnères-de-Bigorre (npMANOVA: $F = 13.37, r^2 = 0.237, p < 1.10^{-4}$; Fig. 2b). In Bagnères-de-Bigorre, they trapped 95.6% of Psychodidae, whereas in Smarves they trapped 42.0% of Psychodidae, 23.6% of Chironomidae and 34.5% of insects from the other categories.

Temporal variations of the overall pollinator diversity The four studied populations of *A. italicum* showed significant temporal variations of their pollinator diversities between 2009 and 2010 (statistics values are given in Table 3). Psychodidae proportions were higher in 2009 than in 2010 and ranged from 95.5% (Smarves) to 27.2% (Toulouse) in 2009 and from 51.1% (Smarves) to 0% (Toulouse) in 2010. The mean number of total insects trapped per inflorescence increased significantly from 2009 to 2010 in Bagnères-de-Bigorre (from 23.22 ± 4.84 to 75.86 ± 18.14, MW: $W = 209, p = 0.003$), and decreased significantly in Smarves (from 49.5 ± 13.16 to 1.48 ± 0.36; MW: $W = 343, p < 1.10^{-6}$) and Chantonnay (from 6.16 ± 1.39 to 1.38 ± 0.46, MW: $W = 252, p < 1.10^{-3}$).

Pollinator diversity did not vary significantly for *A. maculatum* in Bagnères-de-Bigorre, with more than 90% of Psychodidae trapped in both years (Table 3). As for *A. italicum* in this site, the mean number of insects trapped per inflorescence was higher in 2010 (216.12 ± 39.89) than in 2009 (147.75 ± 52.88; MW: $W = 183, p = 0.031$). In contrast, Psychodidae proportions decreased from 2009 to 2010 in Smarves (from 76.8% to 42.0%, Table 3) as well as the mean number of total insects trapped per inflorescence (from 19.36 ± 7.56 to 1.47 ± 0.35; MW: $W = 341, p < 1.10^{-4}$).

Psychodidae diversity All identified Psychodidae belonged to six species: *Psycha gris-*

escens (98 insects), *Psychoda phalaenoides* (81 insects), *P. crassipenis* (41 insects), *Apsycha pusilla* (18 insects), *Logima surcoufi* (4 insects) and *L. albipennis* (= *Pychoda parthenogenetica*) (1 insect) (Ježek 1990, Ježek and Hájek 2007). As there were few Psychodidae trapped in the inflorescences in some populations, we could only identify 17 Psychodidae in the inflorescences of *A. italicum* and 14 in the inflorescences of *A. maculatum* in Smarves in 2010, 9 in the inflorescences of *A. italicum* in Toulouse in 2009 and no Psychodidae in 2010, and 2 in the inflorescences of *A. italicum* in Chantonnay in 2010 (Fig. 3).

All Psychodidae species but *Logima albipennis* were found in the inflorescences of *A. italicum*. *Psycha grisescens* was found in the inflorescences of all populations. In Smarves in 2009 and 2010, the main trapped Psychodidae species was *Psychoda crassipenis* (respectively 90 and 94%), whereas in Bagnères-de-Bigorre in 2009 and 2010 it was *Psycha grisescens* (respectively 70 and 85%). In Pierrelatte, the main species was *Apsycha pusilla* (90%). In the other populations, there was no main species: inflorescences trapped more or less equivalent proportions of *Psycha grisescens* and *Psychoda phalaenoides*, with in addition *P. crassipenis* in Chantonnay and Toulouse in 2009, or *Logima surcoufi* in Igeldo and Toulouse in 2009 (Fig. 3).

Inflorescences of *A. maculatum* trapped only Psychodidae from the species *Psychoda phalaenoides* and *Psycha grisescens*, with one specimen of *Logima albipennis* found in one inflorescence from Smarves in 2009. Inflorescences trapped mainly *P. grisescens* in Smarves in 2009 and 2010 (respectively 60 and 100%), or *P. phalaenoides* in Bagnères-de-Bigorre in 2009 and 2010 and in La Loubatière in 2009 (respectively 85, 95 and 71%, Fig. 3).

Reciprocal transplant experiment: overall insect diversity In 2008 and 2009, *Arum italicum* inflorescences transplanted from Toulouse to Bagnères-de-Bigorre trapped the same diversity of insects than the native *A. italicum* inflorescences in Bagnères-de-Bigorre, and a significantly different diversity than in their native site (overall test npMANOVA: $F = 10.64, r^2 = 0.113, p < 1.10^{-4}$ in 2008, $F = 9.67, r^2 = 0.29, p < 1.10^{-4}$ in 2009; Fig. 6 a and b). In Bagnères-de-Bigorre, transplanted and native inflorescences trapped a high percentage of Psychodidae (62.6% and 48.71%), contrary to the native inflorescences in Toulouse (19.7%). In both years, the total number of insects trapped was significantly higher in Bagnères-de-Bigorre in the transplanted (11.59 ± 2.51) and native inflorescences (23.22 ± 4.84) than in the native inflorescences in Toulouse (1.67 ± 0.36; KW: $\chi^2_2 = 30.8, p < 1.10^{-6}$ in 2008, $\chi^2_2 = 58.0, p < 1.10^{-11}$ in 2009). Note that for both years the proportion of Chironomidae trapped in Bagnères-de-Bigorre in the transplanted inflorescences (23.5% in 2008, 6.4% in 2009) was slightly lower than in the native inflorescences

(41.7% in 2008, 24.5% in 2009), as they attracted a significantly lower number of Chironomideae (KW: $\chi^2_2 = 30.8, p < 1.10^{-6}$ in 2008, $\chi^2_2 = 58.0, p < 1.10^{-11}$ in 2009).

In the same way, *Arum italicum* inflorescences transplanted from Bagnères-de-Bigorre to Toulouse trapped the same diversity of insects than the native *A. italicum* inflorescences in Toulouse, and a significantly different diversity of insects than in their native site (overall test npMANOVA: $F = 9.67, r^2 = 0.29, p < 1.10{-}4$). They trapped a low proportion of Psychodidae (16.7%) and a significantly lower total number of insects than in their native site (0.78 ± 0.37; MW: $\chi^2_2 = 58.0, p < 1.10^{-11}$).

The insects diversity found in the *A. maculatum* inflorescences transplanted from Bagnères-de-Bigorre to Toulouse was not significantly different from the native *A. maculatum* inflorescences in Bagnères-de-Bigorre (Fig. 6c; npMANOVA: $F = 2.61, r^2 = 0.09, p = 0.063$ in 2008; $F = 3.35, r^2 = 0.11, p = 0.051$ in 2009). In all cases, inflorescences trapped more than 85% of Psychodidae. In both years, the number of insects trapped by the transplanted inflorescences in Toulouse was dramatically lower (2.7 ± 0.47 in 2008, 4.6 ± 0.81 in 2009) than in the native inflorescences in Bagnères-de-Bigorre (115.6 ± 23.22 in 2008, 147.8 ± 52.88 in 2009). Note that *A. maculatum* does not naturally occur in Toulouse.

Reciprocal transplant experiment: psychodid diversity Transplanted inflorescences of *A. italicum* from Toulouse to Bagnères-de-Bigorre trapped the same proportions of Psychodid species than the native *A. italicum* inflorescences in Bagnères-de-Bigorre (65% of *Psycha grisescens* and 35% of *Psychoda phalaenoides*, Fig. 7). As inflorescences of *A. italicum* trapped few psychodids in Toulouse, we were only able to determine three psychodids from the inflorescences of Bagnères-de-Bigorre transplanted in Toulouse: one *Logima surcoufi* and two *Psychoda crassipenis*, which was consistent with the diversity of psychodids found in the native inflorescences of *A. italicum* in Toulouse. The inflorescences of *A. maculatum* transplanted from Bagnères-de-Bigorre to Toulouse trapped 89% of *P. phalaenoides*, like in their native site, but the minor trapped species were *Apsycha pusilla* and an unknown psychodid species (*P. sp.*), whereas in their native sites they trapped *Psycha grisescens* as a minor species.

Floral blend composition A total of 44 different VOCs were found in the blends of the two *Arum* species; most of it were mono- and sesqui- terpenoids. Nineteen VOCs were common to the two species. The odour of *A. italicum* presented 10 specific compounds (Table 5), whereas the odour of *A. maculatum* presented 15 specific compounds (Table 6). In *A. italicum*, seven

dominant compounds represented 75% of the odour: 3,7-dimethylocta-1,6-diene (β-citronellene) ($31.81\% \pm 1.89$), β-caryophyllene ($12.55\% \pm 1.85$), 3,7-dimethyloctene ($12.51\% \pm 1.39$), p-cresol ($11.12\% \pm 1.53$), 2,6-dimethyl-3-octene ($5.61\% \pm 0.84$) and a not identified dihydrosesquiterpene ($IR = 1395, 9.27\% \pm 1.14$). The remaining 21 compounds represented each less than 5% of the average blend. *A. maculatum* differed mainly from *A. italicum* by the production of indole ($21.43\% \pm 3.67$). In *A. maculatum*, three dominant compounds represented 45% of the odour: indole, limonene ($16.68\% \pm 3.76$) and α-pinene ($7.33\% \pm 1.86$), the remaining 30 compounds representing each less than 5% of the averaged blend.

A. italicum produced approximately ten times more VOCs than *A. maculatum*, as estimated by the different mean total chromatograms areas per species (*A. italicum*: area = 13.66 ± 2.49 x 10^6; *A. maculatum*: area = 9.03 ± 2.29 x 10^5; MW: $W = 83, p < 1.10^{-14}$). In addition, the overall inter-individual variations were significantly higher in *A. maculatum* (Jaccard index = 0.79 ± 0.01) than *A. italicum* (Jaccard index = 0.59 ± 0.004; Fig. 5; MW: $W = 111626, p < 1.10^{-15}$).

Floral blend geographical variations According to the variations of the 5 classes of compounds among individuals, odour profiles differed significantly between populations for the two *Arum* species, but no population was significantly different from all the other according to the post-hoc tests. In *Arum italicum* (npMANOVA: $F = 4.61, r^2 = 0.40, p = 1.10^{-4}$), Bagnères-de-Bigorre 2009 significantly differed from Toulouse 2009 and Bagnères-de-Bigorre 2010. In *A. maculatum* (npMANOVA: $F = 5.28, r^2 = 0.43, p = 2.10^{-4}$), odours in Smarves 2010 significantly differed from La Loubatière 2009 and Smarves 2009, and odours in Bagnères-de-Bigorre 2010 from La Loubatière 2009.

More detailed analyses taking into account of the relative percentage of each compound gave similar results. In 2009, odours varied significantly between populations of *A. italicum* (npMANOVA: $F = 3.91, r^2 = 0.32, p < 1.10^{-4}$) but no population was significantly different from all the others when considering post-hoc tests. Toulouse and Smarves are the most divergent populations in term of odour, as they both are different from each other and from Igeldo and Bagnères-de-Bigorre. Indeed, inflorescences in Toulouse emitted less 3,7-dimethyloctene, β-citronellene, and more p-cresol and β-caryophyllene than the others populations, whereas in Smarves they produced more p-cresol and dihydrosesquiterpene ($IR = 1395$) and less β-cayophyllene (Table 5). In 2010, the *A. italicum* inflorescence odours in the two studied populations (Smarves and Bagnères-de-Bigorre) were significantly different (npMANOVA: $F = 5.67, r^2 = 0.45, p = 0.008$), as inflorescences produced less p-cresol and β-caryophyllene in

Smarves than in Bagnères-de-Bigorre (see Table 5).

Odours did not differ between populations of *A. maculatum* in 2009 (npMANOVA: $F = 2, r^2 = 0.18, p = 0.036$, post-hoc tests not significant). On the contrary, odours in Bagnères-de-Bigorre and Smarves differed in 2010 (npMANOVA: $F = 2.84, r^2 = 0.29, p = 0.015$), as inflorescences produced a lower amount of indole in Smarves (Table 5).

Floral blend temporal variations Two populations (Bagnères-de-Bigorre and Smarves) of both *A. italicum* and *A. maculatum* were sampled during two successive years. Odours varied between 2009 and 2010 in the two studied populations of *A. italicum* (npMANOVA: $F = 13.97, r^2 = 0.52, p = 2.10^{-4}$ in Bagnères-de-Bigorre; $F = 3.57, r^2 = 0.28, p = 0.009$ in Smarves). In both populations, the emission of limonene increased in 2010, whereas the emission of β-caryophyllene increased in Bagnères-de-Bigorre and the emission of p-cresol and dihydrosesquiterpene ($IR = 1395$) decreased in Smarves (Table 5).

Odours also varied between the two studied populations of *A. maculatum* between 2009 and 2010 (npMANOVA: $F = 3.68, r^2 = 0.24, p = 0.02$ in Bagnères-de-Bigorre; $F = 13.58, r^2 = 0.58, p = 0.02$ in Smarves). Limonene emission increased in both populations in 2010, and indole emission decreased in Smarves in 2010.

7.5 DISCUSSION

Pollinator variations Pollinator variations in time and among populations is a common phenomenon occurring between closely related (e.g. Kato *et al.* 2000; Cosacov *et al.* 2008) or distant pollinator species (e.g. Schlumpberger *et al.* 2009), and it has long been overestimated by studies based on observations on a single site (Herrera *et al.* 2006). From a pollination point of view *Arum italicum* was shown here to be an opportunist species, as its pollinators varied in time and space, whereas *A. maculatum* was strongly specialized to two psychodidae species over the studied populations.

Arum italicum and *A. maculatum* pollinators belonged to the same functional groups: they were all little midges from the families psychodidae, Chironomidae, Ceratopogonidae, or Sciarideae (Diptera, Nematocera), plus some Brachycera (Diptera) and Staphylinidae (Coleoptera), as already described in the these populations (Albre *et al.* 2003, Chartier *et al.* 2011), or in populations from the rest of Europe (e.g. Diaz and Kite 2002, Gibernau *et al.* 2004, Espindola *et al.* 2011). In *A. italicum*, relative proportions of insects varied greatly between populations: some populations were specialized to insects from one family, mainly psychodidae (e.g. Pierrelatte, Chantonnay and Smarves in 2009), or Chironomidae (Igeldo), or to both families (e.g. Smarves, and Bagnères-de-Bigorre in 2010, Fig. 1). In four samples (Toulouse in 2009 and 2010, Smarves and Chantonnay in 2010) insects from the others categories were trapped in high proportions.

In *A. maculatum*, the main pollinators always belonged to two species, *Psychoda phalaenoides* or *Psycha grisescens* (psychodidae), the almost exclusive pollinators found in a large scale study over its European repartition range (Espindola *et al.* 2011). In Smarves in 2010 anyway, Chironomidae, Brachycera and insects from the other categories were exceptionally important in proportions (Fig. 2).

Interestingly, for both species, the sites where inflorescences attracted high proportions of insects from the other categories were the sites where populations attracted less than 4 insects per inflorescence in average (Table 1 and 2, Fig.1 and 2). In these populations, the mean number of insects from the other categories rarely reaches more than 2 insects per inflorescence, whatever the total number of insects trapped, which is similar to the quantities caught in inflorescences from all populations. Therefore, there must be a stable number of insects different from the main pollinators trapped in each population, increasing proportionally when the total number of insects decreases, leading to more generalist inflorescences in some sites. The site in Toulouse is known to present few insects (Albre *et al.* 2003, Chartier *et al.* 2011). Furthermore, climatic

conditions in 2010 were bad and insects were collected during cold and rainy weeks (excepted in Bagnères-de-Bigorre) which can explain the low number of insects trapped in these inflorescences. A decrease in insect pollinators when climatic conditions are bad have also been reported by Kite (1995) and Chartier *et al.* (2011). Plants are believed to adapt to their most common or efficient pollinator, in order to increase their reproductive success (Fenster *et al.* 2004). Whatever the degree of specificity, a few and stable proportion of "error" in pollinator attraction may constitute an adaptive advantage in the case were environmental perturbations lead to a decrease of the main pollinator (Thompson 2005, Barriault 2010).

When adding data from Chartier *et al.* (2011), pollinator diversity and number are available from 2008 to 2010 in some sites for both species. In *Arum italicum*, relative proportions of insects varied according to year. For instance, in Chantonnay, *A. italicum* trapped a majority of psychodidae in 2008 and 2009, and psychodidae, Chironomidae, Brachycera and insects from the other categories in 2010, when the mean number of insects trapped per inflorescence (1.4) was the lowest, whereas in Bagnères-de-Bigorre, inflorescences trapped a majority of Chironomidae in 2008 and 2010, but more psychodidae in 2009 (Fig. 1, Chartier *et al.* 2011). On the other hand, inflorescences of *A. maculatum* trapped a majority of psychodidae in each population from 2008 to 2010, excepted in Smarves in 2010, where this proportion decreased compared to the other insect categories, which might be explain by the very low total number of insects trapped at this site that year (1.5 insect per inflorescence, see above), and the bad weather conditions.

Interestingly, whereas proportions of the different insect families varied greatly among populations and year in *Arum italicum*, proportions of psychodid species seemed to vary between populations, but not between years for both *Arum* species (Fig. 3). Indeed, in both years, *Arum italicum* attracted mainly *Pychoda crassipenis* in Smarves, whereas *A. maculatum* attracted *Psycha grisescens*. In Bagnères-de-Bigorre, *A. italicum* attracted mainly *P. grisescens*, whereas *A. maculatum* attracted mainly *Psychoda phalaenoides*. This result is all the more interesting that in these two populations, the two *Arum* species grow in sympatry, and both attracted *P. grisescens* in small or high quantities according to the site, but in a manner that *P. grisescens* is not the main pollinator of both species in any site. *Arum italicum* and *A. maculatum* are respectively hexaploid and tetraploid, and their hybrids must thus be pentaploid (Beuret 1977, Bedalov 1984, Bedalov and Küpfer 2005). Pentaploid hybrids are likely to have a low fertility owing to chromosome abnormalities, and thus may be counter-selected (Rieseberg and Willis 2007). The difference in pollinator attraction in these two sympatric sites may possibly result from a shift in pollinator selected to avoid hybridization. Such sites constitute good opportunities

for studying a case of selection for reproductive isolation (Chartier *et al.* unpubl. data).

Variations of trapped pollinators from site to site and from year to year may reflect variations in (1) insect availability, (2) insect odour preference, and/or (3) plant attraction. First, insects distribution are likely to vary according to climatic conditions. In a large scale study covering *A. maculatum* distribution in Europe, Espindola *et al.* (2011) found that *A. maculatum* was mainly pollinated by *Psychoda phalaenoides* in northern and central Europe, and that the proportions of *Psycha grisescens* caught increased in the Mediterranean area and in north west of France, which was correlated to variations in climatic conditions. Our results are coherent with Espindola *et al.* (2011), as *A. maculatum* caught a majority of *P. phalaenoides* in the two populations sampled in non-Mediterranean south of France (Bagnères-de-Bigorre and La Loubatière), like their population of Pyrénées Orientales, and in the present study, it caught a majority of *P. grisescens* in the northeast population in France (Smarves), like in their population of La Mignonais (Pays de la Loire, France). In *A. italicum*, not enough populations were sampled to test for a geographical gradient of pollinator diversity, but, as suggested by Chartier *et al.* (2011) the northern populations (Chantonnay, Smarves) tended to attract more psychodidae like in England (Diaz and Kite 2002) than southern populations (Igeldo, Toulouse and Bagnères-de-Bigorre).

Reciprocal transplant experiment Transplantations are a simple way to test whether variations in trapped pollinators between sites is owing to variations in insects availability or to variation in insects preferences/plant attractiveness, due to local adaptation. In 2008 and 2009, plants of both *Arum* species trapped the same number of insects and the same family diversity as native inflorescences of the sites where they were transplanted, but different from inflorescences of their native sites (Fig. 6), indicating that pollinator attraction highly depends on pollinator availability of the sites. The result was the same for psychodid diversity. Note anyway that when it was transplanted from Bagnères-de-Bigorre to Toulouse, *A. maculatum* trapped a majority of *P. phalaenoides*, like in its native site, but the minor trapped psychodid species were different from the species trapped by native and transplanted *A. italicum* plants in Toulouse, and were different from *P. grisescens*, the minor *Psychoda* species trapped by *A. maculatum* in Bagnères-de-Bigorre, even if *P. grisescens* was present in Toulouse (it was found in *A. italicum* inflorescences, Fig. 7). Thus, there might be differences in *P. grisescens* preference for *A. maculatum* between Bagnères-de-Bigorre and Toulouse. Anyway, this effect is largely minor compared to the probable effect of pollinator availability variation between sites, and as *A. maculatum* did not grow naturally in Toulouse, it was not possible to test for a reciprocal

change in pollinators.

Local adaptation for pollinators has been demonstrated through reciprocal transplant tests in a few studies. For instance, Waterman *et al.* 2011 showed that seed set decreased in three pairs of recently diverged orchids when reciprocally transplanted out of their native sites, which might be due to local adaptations to pollinators (see also Campbell 2003). Gomez *et al.* (2009) showed higher attractiveness for pollinator from transplanted *Erysimum* species (Brassicaceae) coming from evolutionary hotspots than coldspots. In our study, nor attractiveness nor pollinator diversity seemed to depend from plants genotypes, but rather from pollinator availability in the site, implying that at least *A. italicum* and maybe *A. maculatum* show high ecological adaptive flexibility. This may in part explain the widespread distribution of the two species (Linz *et al.* 2010) as adaptation to local pollinators is a factor decreasing fitness of species when colonizing or introduced in new habitats (Gebert and Ekhart 2005, Angert *et al.* 2008). In *Arum italicum* and *A. maculatum*, the major pollinator attractive feature is known to be the attractive dung/urine-like odour emitted by the spadix appendix (Lack and Diaz 1991, reviewed by Gibernau et al. 2004 and Urru *et al.* 2011), and its variations may explain the two species pollinator-attraction "strategies". For instance, in their study on *A. maculatum* pollinators, Espindola *et al.* (2011) proposed that its odour could be adapted to attract a majority of *P. grisescens* in some populations, and of *P. phalaenoides* in some others.

Variation of the attractive odours The odours of both *Arum* species were mainly composed of sesquiterpenoids and monoterpenoids, with some fatty acid derivative, in higher proportions in *A. maculatum*, and a nitrogen containing compound, indole, exclusively found in *A. maculatum*. Among these compounds, some are very common components of flower scent, e.g. α-pinene, limonene and β-caryophyllene (Knudsen *et al.* 2006), and most of them had already been identified in the odour of these two species (Kite 1995, Kite *et al.* 1998, Diaz and Kite 2002, Table 2). Some of these compounds, like 2-heptanone (a ketone), p-cresol (a benzenoid), or indole (a nitrogen containing compound exclusively found in *A. maculatum*) have been shown to be attractive to psychodids flies, all the more when they were mixed together (Kite *et al.* 1998). These compounds, as well as α-pinene, limonene or β-caryophyllene, where also found in sheep, cow, horse, or boar dung odours (Kite 1995, Dormont *et al.* 2010, Johnson and Jürgens 2010), as well as in the odour of deceptive asclepiads (Apocynaceae: Asclepiadoideae) (Jürgens *et al.* 2006) and are typical of the odours of sapromyophilous plants (Urru *et al.* 2011).

An important proportion of hydrogenated linear monoterpenoids (myrcene, ocimene) was

found in the odours of *Arum italicum* and *A. maculatum* (six compounds, Table 4 and 5). These compounds might have been synthesized from geranyl pyrophosphate (GPP), a key compound of the monoterpene biosynthesis, through successive reductions of the two double bonds of the molecule. A synthesis pathway for their formation is proposed in Figure 8.

A tetrahydrosesquiterpene and three dihydrosesquiterpenes (RI = 1366, 1392, 1395) were also found in the odours of the two species (Table 4 and 5). Although they could not be identified from the literature, analogies with the monoterpenoids molecules described above suggest that they might be hydrogenated derivatives of farnesene. These more or less reduced compounds have been found in organic matter, like mammal dejections (Dormont *et al.* 2010), but are not really common in floral odours (except citronellene, Knudsen *et al.* 2006).

Half of the compounds emitted were common to the odours of the two species, even if their main compounds were different. This may explain why they share at least their minor pollinators, and some psychodid species. For instance, p-cresol and 2-heptanone, two of the compounds positively tested for their attractiveness to psychodids (Kite *et al.* 1998), were emitted by both species. Our sampling was here two small to perform significant correlation tests and more behavioural tests on insects may in addition be a good way to isolate the specific attractive compounds. In addition, *A. italicum* produced around ten times more VOCs as estimated by the mean total area of their respective chromatograms, but attracted far less insects per inflorescences (mean over all populations: 18 insects per inflorescences for *A. italicum*, 85 for *A. maculatum*). Their difference in attractiveness is thus more likely to be due to their odour qualitative differences.

In both *Arum* species, odours varied between individuals. In *A. italicum*, there was no structuration between populations in 2008 and 2009, (Chartier *et al.* 2011, present study), but odours from inflorescences of Smarves were different from Bagnères-de-Bigorre in 2010. At contrary, odour profiles of *A. maculatum* differed between Smarves and Bagnères-de-Bigorre in 2008 and 2010 (Chartier *et al.* 2011, present study), but overlapped with La Loubatière in 2009.

Inter-population structuration of pollinator attractive odours may be due to several non exclusive factors including abiotic condition, adaptation to local pollinators (Schlumpberger and Raguso 2008) or low gene flux between populations (Solers *et al.* 2010). On the contrary, a stable odour among different populations might be explained by high gene flux (Knudsen 2002, Svensson *et al.* 2005) or similar selective pressures of different pollinators among different sites (Svensson *et al.* 2006). In addition, high variations of odour between individuals might result from few selective pressures from pollinators, for instance if other cues play an important

role in pollinator attraction (Ibanez *et al.* 2010), or may hide a stability of the physiologically active compounds, which may represent a more or less important proportion of the total variable odour (Ayasse *et al.* 2000, Ibanez *et al.* 2010). Finally, this high variation may be an adaptive response to high variations of pollinators from year to year (Geber and Moeller 2006), or in the case of deception, to pollinator selective response, as deceived pollinators might be selected to recognize and avoid deceptive inflorescences (Ackerman *et al.* 2011). In the context of the geographic mosaic of coevolution (Thompson 2005), all these explanatory factors may vary and be expressed differently from a site to another.

In *Arum*, as an estimation of inter-individual variations, the mean Jaccard index between individual odours of *A. italicum* (0.44) and *A. maculatum* (0.64) were both closer to the mean inter-individual Jaccard index of deceptives flowers (0.55) than to rewarding flowers (0.28) in a comparative study over 12 species or rewarding or deceptive flowers (Ackerman *et al.* 2011), indicating that deception might be a factor selecting for the inter-individual variations in the deceptive *Arum*.

In addition, the reciprocal transplant tests showed that pollinator variations in *A. italicum* were probably due to temporal variations and local availabilities in insects, as attractively depended on the site more than on the plant population (Fig. 7). This is coherent with the absence of structuration of the odour between the studied populations, already found by Chartier *et al.* (2011) in 2008. In Smarves, the odour slightly differed from the other populations in 2009, and significantly from Bagnères-de-Bigorre in 2010 in containing fewer β-caryophyllene in both years, higher amount of dihydrosesquiterpene in 2009 and fewer p-cresol in 2010. In this site, the two species grow in sympatry, and there might be selective pressures acting on the attractive odour to avoid hybridization, maybe acting on β-caryophylene proportions, as this compound is also emitted by *A. maculatum* (Table 2). This would be coherent in this population, as *A. italicum* attracted high proportions of psychodidae, mainly *P. crassipenis*, whereas *A. maculatum* mainly attracted *Psycha grisescens* and *Psychoda phalaenoides* (Fig. 7), but the attractiveness of β-caryophyllene for *P. phalaenoides* and *P. grisescens* remains to be tested.

In the samples of the present study, five compounds dominated the odour of *A. italicum*: p-cresol, 3,7-dimethyloctene, β-citronellene, an unidentified dihydrosesquiterpene (IR = 1395) and β-caryophyllene. In England, the main compounds only slightly differed, being β-caryophyllene, an unidentified sesquiterpene (IR=1404), β-citronellene and 3,7-dimethyl-1-octene (Diaz and Kite 2002). In England, *A. italicum*'s main pollinators also belonged to the families psychodidae (mainly *Psychoda phalaenoides*) and Chironomidae (*Smittia partorum*). This suggest there might

be few genetic differentiation between French and English populations, or that selective pressures for pollinator attraction are the same in these distant populations.

Pollinator variations are low in *A. maculatum*, and mainly consist in a shift from *Psycha grisescens* to *Psychoda phalaenoides*, or to the attraction of both species (Smarves, 2010). Transplanted inflorescences of *A. maculatum* from Bagnères-de-Bigorre to Toulouse mainly trapped *P. phalaenoides* in their native or transplanted sites, even if *Psycha grisescens* was present in both sites (as they were found in *A. italicum* inflorescences). In their study of *A. maculatum* pollinator variations, Espindola *et al.* (2011) suggested that some populations of *A. maculatum* were specialized to one or the other of the psychodids species, according to their distribution areas. In addition, if there is selection for a reproductive isolation of the two *Arum* species in Smarves and Bagnères-de-Bigorre, *A. maculatum* may be selected to attract mainly *Psychoda phalaenoides* in Bagnères-de-Bigore, and one of the two species in Smarves (Fig. 7). In 2008 and 2009, *A. maculatum* odour was different between both sites, and we know at least that in 2009, they attracted different pdychodids species. In 2009, odours of Smarves, La Loubatière and Bagnères-de-Bigorre overlapped, and inflorescences attracted more or less *P. phalaenoides* in all sites (Fig. 7). Thus, contrary to *A. italicum*, there might be selective pressures on the odours of *A. maculatum* for the attraction of one or the other psychodids, but more field biotest on the insects remain to be done to confirm this hypothesis.

In conclusion, *A. italicum* seems to be an opportunistic species for which the variable odour between years and sites allows to deceive and attract a varying guild of pollinators. This high variability and opportunism might be an advantage explaining its widespread distribution across Europe. *Arum maculatum*'s odour seems to be more structured between populations, and the species is highly specialized to two main psychodidae species, switching from one to the other according to their large distribution. In both cases, pollinator availability in the sites seems to be the preponderant factor explaining pollinator variations across sites. In the future, informations on the genetic structuration of populations quantifying gene flux might preciously highlight the processes shaping the different attractive odour structuration of the two *Arum* species.

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7.8 FIGURE LEGENDS

Figure 1. Diversity of the inflorescence visitors of *Arum italicum* identified at the family level (mean ± standard errors of the relative insects proportions per inflorescence) ***a.*** in 2009, ***b.*** in 2010. ns = non significant difference.

Figure 2. Diversity of the inflorescence visitors of *Arum maculatum* identified at the family level (mean ± standard errors of the relative insects proportions per inflorescence) ***a.*** in 2009, ***b.*** in 2010. ns = non significant difference.

Figure 3. *Psychoda* diversity found in the inflorescences of *Arum italicum* and *A. maculatum* in 2009 and 2010 in seven different sites. When possible, four insects from five different inflorescences were identified per site and species.

Figure 4. Odor profile of each sampled inflorescence of *A. italicum* and *A. maculatum*, ordered per populations and years. Stacked bars represents the relative amounts of each main compounds classes (benzenoids in blue, sesquiterpenoids in magenta, monoterpenoids in pink, lipids in yellow), and indole (in brown). Proportions of unidentified compounds are in grey.

Figure 5. Bray-Curtis distance between inflorescences odors of *A. italicum* and *A. maculatum*. Both species are significantly different.

Figure 6. Diversity of the visitors of the native and transplanted inflorescences of *Arum italicum* and *A. maculatum* in Toulouse and Bagnères-de-Bigorre (mean ± standard errors of the relative insects proportions per inflorescence). ***a.*** *A. italicum* in 2008, ***b.*** in 2009, ***c.*** *A. maculatum* in 2008 and 2009. Toulouse TR = inflorescences from Toulouse transplanted in Bagnères-de-Bigorre. Bagnères TR = inflorescences from Bagnères-de-Bigorre transplanted in Toulouse. ns = non significant difference.

Figure 7. *Psychoda* diversity found in the native and transplanted inflorescences of *Arum italicum* and *A. maculatum* in Toulouse and Bagnères-de-Bigorre in 2009. When possible, idendifications were done on 4 insects from 5 different inflorescences per site and species. Toulouse TR = inflorescences from Toulouse transplanted in Bagnères-de-Bigorre. Bagnères TR = inflorescences

from Bagnères-de-Bigorre transplanted in Toulouse.

Figure 8. Proposition of pathway for the biosynthesis of the monoterpenes identified in the odours of *Arum italicum* and *A. maculatum*.

Table 1. Mean number (mean ± se) of insects trapped per inflorescence of *A. italicum* in 2009 and 2010. N = number of sampled inflorescences. Total = mean of the total trapped insects number per inflorescence.

Table 2. Mean number (mean ± se) of insects trapped per inflorescence of *A. maculatum* in 2009 and 2010. N = number of sampled inflorescences. Total = mean of the total trapped insects number per inflorescence.

Table 3. Statistical values of the npMANOVAs statistics for the tests of temporal variations of insects diversity for *Arum italicum* and *A. maculatum* between 2009 and 2010. n.s. = non significant temporal variation.

Table 4 Mean relative amounts of VOCs produced by *Arum italicum* in studied populations in 2009 and 2010. RI = retention index, RT = retention time, N = number of sampled inflorescences, SE = standard error, O = number of chromatogramms where the VOC was recorded. * Compounds also described by Kite *et al.* (1998) or Diaz and Kite (2002)

Table 5. Mean relative amounts of VOCs produced by *Arum maculatum* in 2009 and 2010. RI = retention index, RT = retention time, N = number of sampled inflorescences, SE = standard error, O = number of chromatogramms where the VOC was recorded.* Compounds also described by Kite *et al.* (1998) or Diaz and Kite (2002)

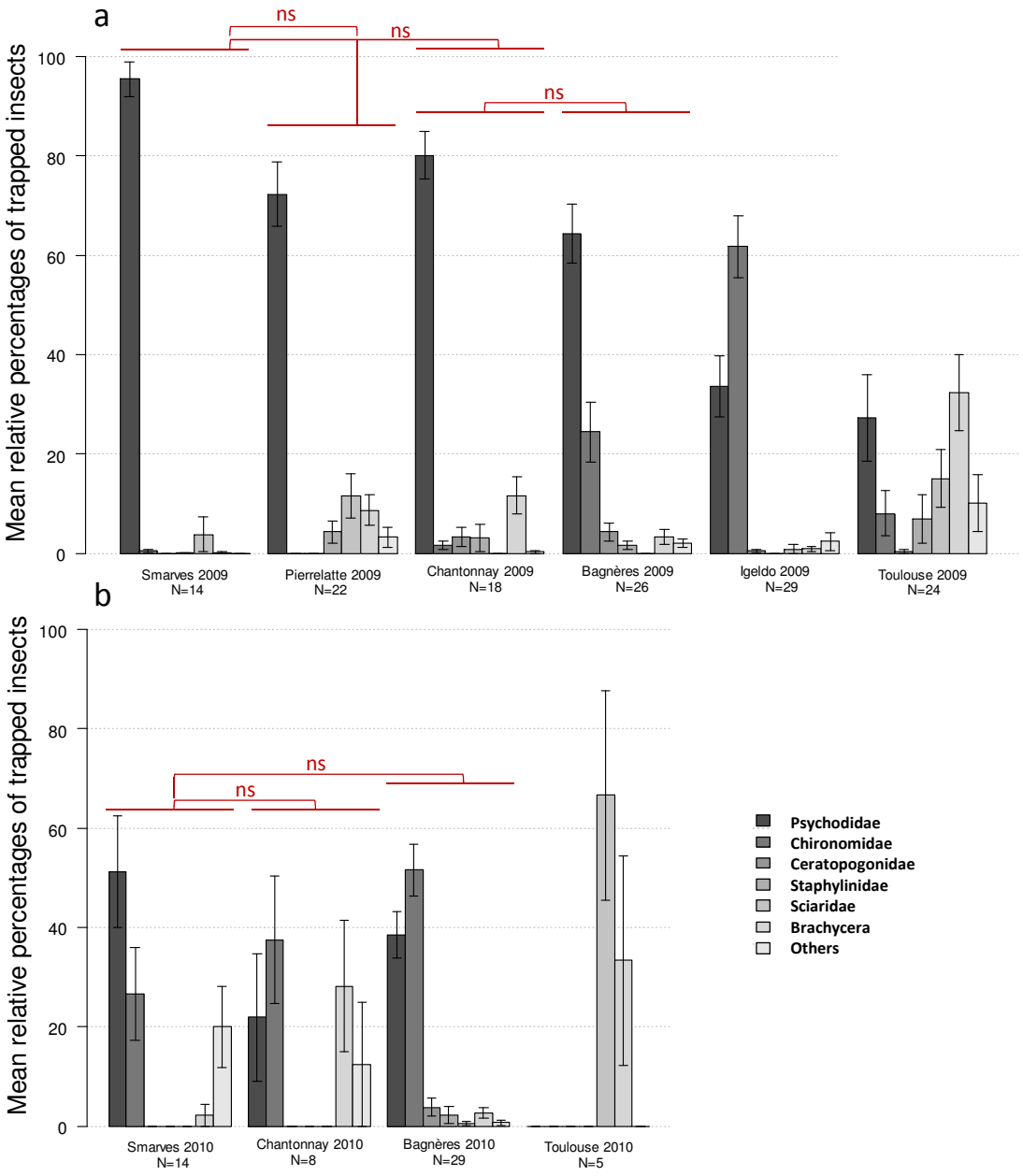

a
ns
ns
ns
Mean relative percentages of trapped insects
100
80
60
40
20
0
Smarves 2009
N=14
Pierrelatte 2009
N=22
Chantonnay 2009
N=18
Bagnères 2009
N=26
Igeldo 2009
N=29
Toulouse 2009
N=24
b
ns
ns
Mean relative percentages of trapped insects
100
80
60
40
20
0
Smarves 2010
N=14
Chantonnay 2010
N=8
Bagnères 2010
N=29
Toulouse 2010
N=5
Psychodidae
Chironomidae
Ceratopogonidae
Staphylinidae
Sciaridae
Brachycera
Others

Figure 1

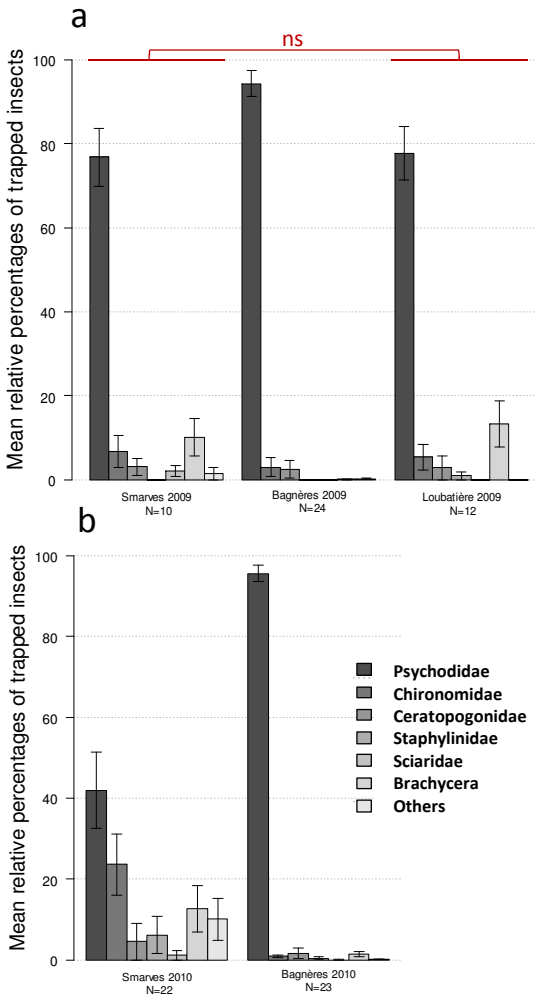


Figure 2

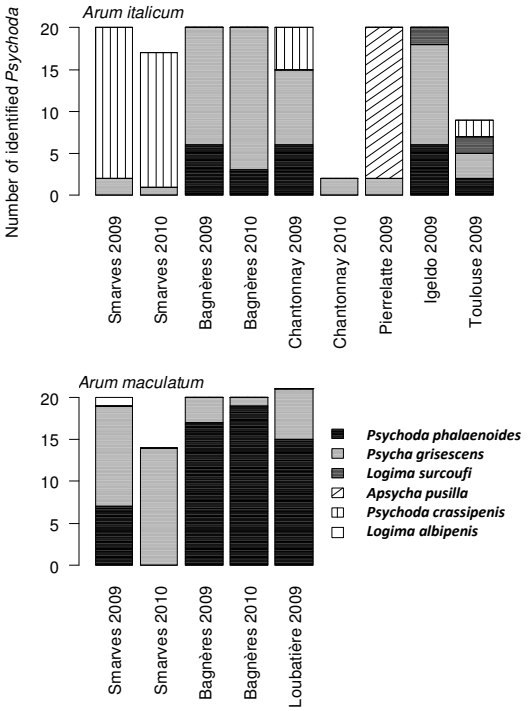
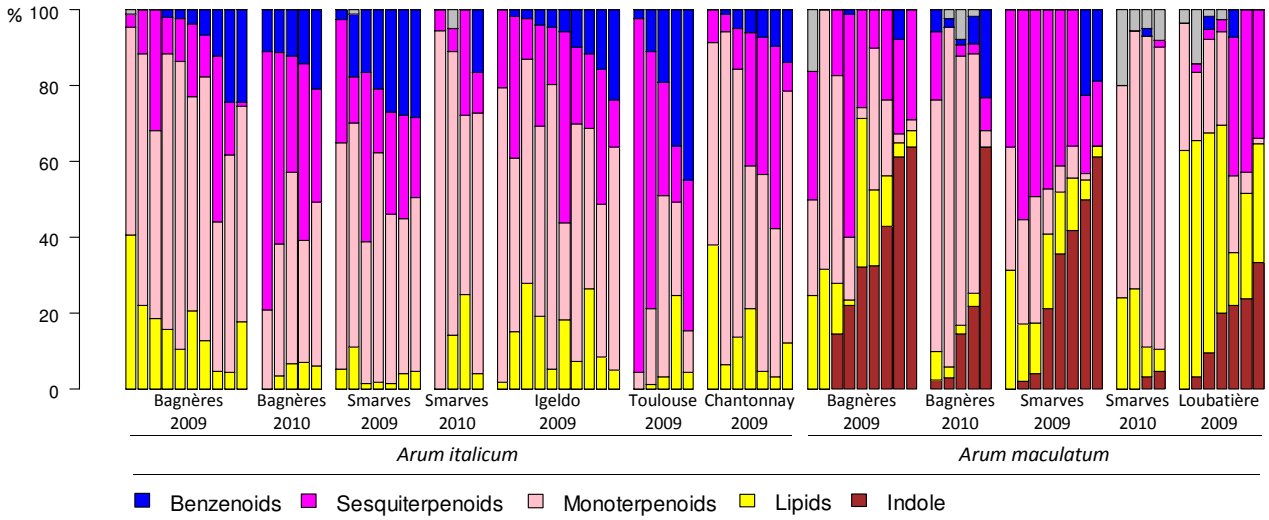


Figure 3



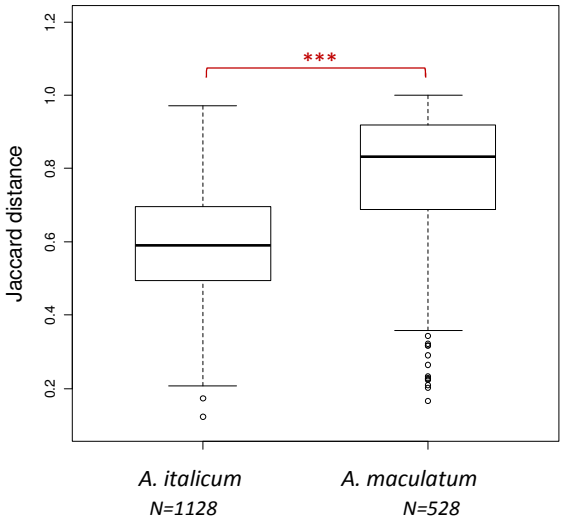
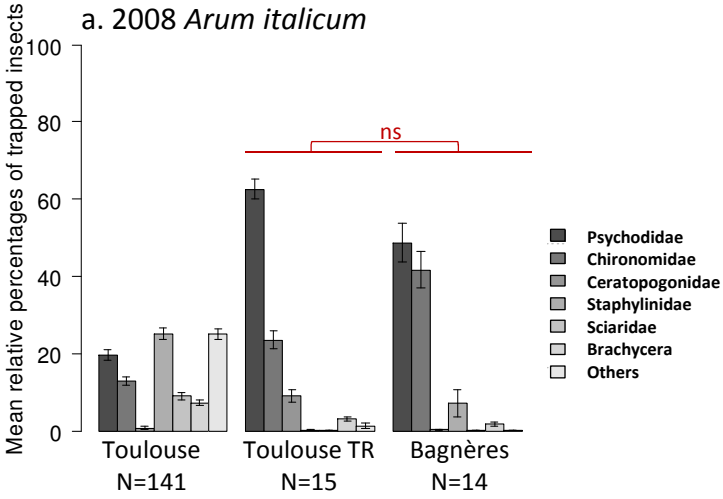
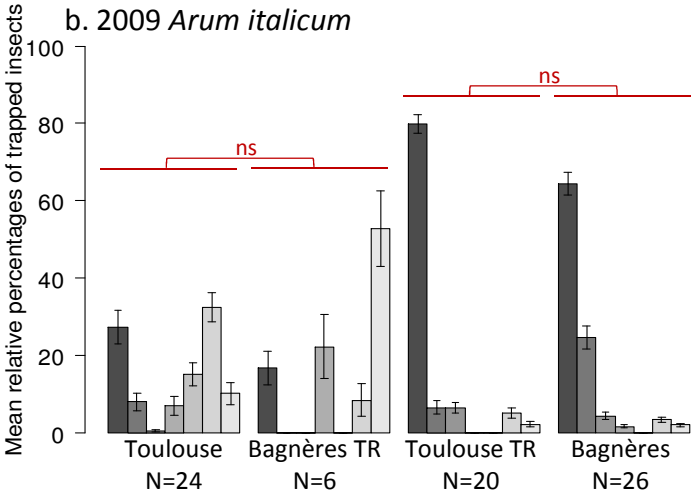
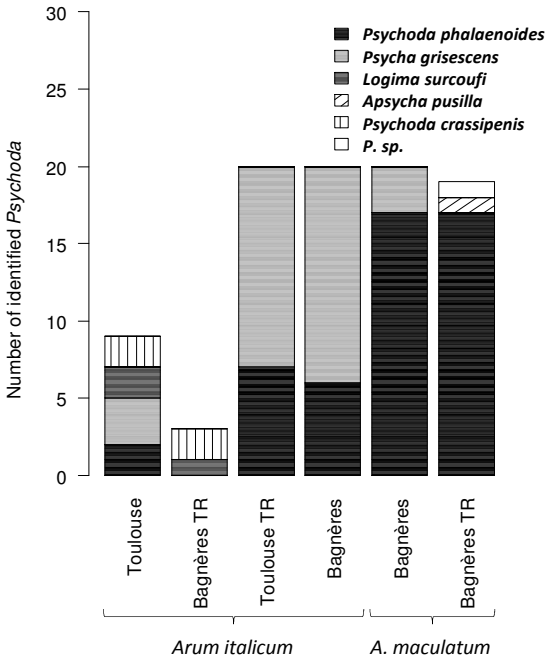


Figure 4

Figure 5







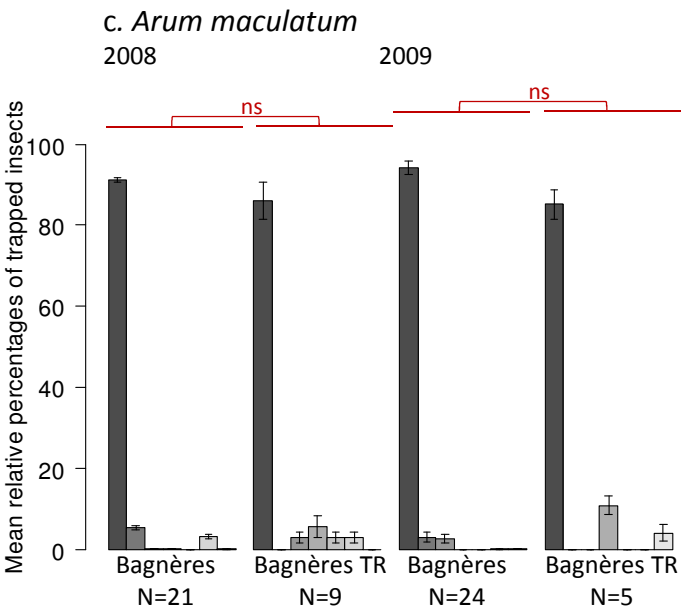


Figure 6 Figure 7

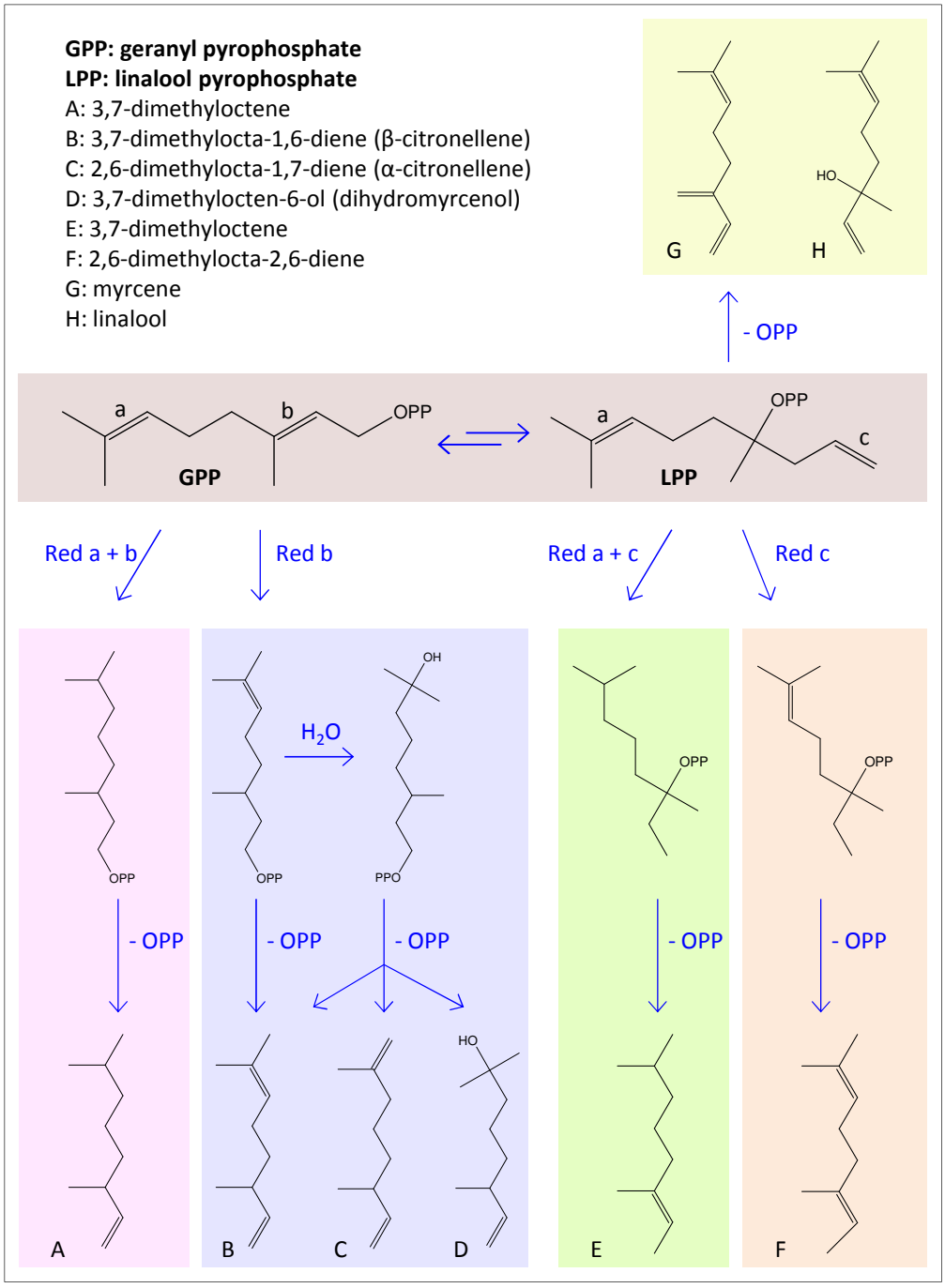

GPP: geranyl pyrophosphate
LPP: linalool pyrophosphate
A: 3,7-dimethyloctene
B: 3,7-dimethylocta-1,6-diene (β-citronellene)
C: 2,6-dimethylocta-1,7-diene (α-citronellene)
D: 3,7-dimethylocten-6-ol (dihydromyrcenol)
E: 3,7-dimethyloctene
F: 2,6-dimethylocta-2,6-diene
G: myrcene
H: linalool
G
H
HO
- OPP
a
b
OPP
GPP
a
OPP
c
LPP
Red a + b
Red b
Red a + c
Red c
OPP
- OPP
H_2O
OH
PPO
HO
A
B
C
D
E
F

Figure 8

	Arum italicum									
	Bagnères		Smarves		Toulouse		Chantonnay		Pierrelatte	Igeldo
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2009
N	27	29	14	25	39	15	19	16	22	34
Psychodidae	13.41 ± 2.65	23.62 ± 4.53	48.57 ± 12.91	0.88 ± 0.3	0.31 ± 0.12	0 ± 0	4.47 ± 0.95	0.31 ± 0.2	4 ± 0.77	4.41 ± 1.54
Chironomidae	7.67 ± 2.51	48.69 ± 15.02	0.29 ± 0.16	0.28 ± 0.09	0.15 ± 0.08	0 ± 0	0.21 ± 0.12	0.56 ± 0.22	0 ± 0	8.06 ± 2.25
Others	0.95 ± 0.95	0.87 ± 0.87	0.37 ± 0.37	0.13 ± 0.13	0.3 ± 0.3	0.66 ± 0.66	0.6 ± 0.6	0.26 ± 0.26	0.45 ± 0.45	0.26 ± 0.26
Total	23.22 ± 4.84	75.86 ± 18.14	49.5 ± 13.16	1.48 ± 0.36	1.67 ± 0.36	1.13 ± 0.62	6.16 ± 1.39	1.38 ± 0.46	5.59 ± 1.04	13.12 ± 3.22

Table 1

	Arum maculatum				
	Bagnères		Smarves		Loubatière
	2009	2010	2009	2010	2009
N	24	24	11	34	14
Psychodidae	145.96 ± 52.94	212.25 ± 39.78	16.82 ± 7.57	0.53 ± 0.15	5.36 ± 1.4
Chironomidae	1.42 ± 0.96	1.12 ± 0.31	0.73 ± 0.41	0.35 ± 0.13	0.21 ± 0.11
Others	0.12 ± 0.12	1.15 ± 1.15	0.67 ± 0.67	0.19 ± 0.19	0.32 ± 0.32
Total	147.75 ± 52.88	216.12 ± 39.89	19.36 ± 7.56	1.47 ± 0.35	6.5 ± 1.44

Table 2

	npMANOVA:	F	r2	p-value
Arum italicum	Smarves	9.31	0.256	< 0.001
	Chantonnay	13.95	0.366	< 0.001
	Bagnères	9.87	0.157	0.002
	Toulouse	2.52	0.085	0.035
Arum maculatum	Smarves	3.10	0.094	0.025
	Bagnères	0.77	0.017	0.487 n.s.

Table 3

			Arum italicum																				
			Bagnères-de-Bigorre						Smarves						Igeldo			Toulouse			Chantonnay		
			2009 (N=10)			2010 (N=5)			2009 (N=7)			2010 (N=4)			2009 (N=10)			2009 (N=5)			2009 (N=7)		
VOCs	RI	RT	Mean	SE	O	Mean	SE	O	Mean	SE	O	Mean	SE	O	Mean	SE	O	Mean	SE	O	Mean	SE	O
BENZENOIDS																							
p-cresol*	1083	11,79	**7.53**	**3.02**	***7***	**13.73**	**1.85**	***5***	**19.91**	**3.48**	***7***	4.09	4.09	*1*	**7.89**	**2.33**	***9***	**22.58**	**7.87**	***5***	**6.07**	**1.78**	***6***
MONOTERPENOIDS																							
Linear																							
3,7-dimethyloctene*	908	6,71	**17.61**	**2.61**	***10***	4.28	0.61	*5*	**11.31**	**2.51**	***6***	**6.82**	**2.92**	***3***	**17.57**	**3.02**	***10***	2.71	0.97	*4*	**15.36**	**4.95**	***7***
2,6-dimethylocta-1,7-diene (α-citronellene)	928	7,28	3.48	0.44	*10*	2.26	0.5	*5*	3.49	0.49	*7*	1.5	1.5	*1*	2.83	0.49	*10*	2.55	1.38	*3*	3.15	0.5	*7*
3,7-dimethylocta-1,6-diene* (β-citronellene)	941	7,66	**36.87**	**2.76**	***10***	**18.38**	**2.04**	***5***	**32.34**	**3.13**	***7***	**47.63**	**10.16**	***4***	**31.25**	**2.41**	***10***	**15.05**	**5.49**	***5***	**37.35**	**4.25**	***7***
2,6-dimethyl-3-octene	965	8,31	**9.01**	**1.78**	***10***	3.07	0.95	*4*	1.47	0.28	*6*	2.43	1.74	*2*	**7.56**	**2.01**	***10***	1.59	1.59	*1*	**8.58**	**2.94**	***7***
myrcene	987	8,94	-	-	-	-	-	-	0.33	0.33	*1*	0.68	0.68	*1*	-	-	-	-	-	-	-	-	-
2,6-dimethylocta-2,6-diene*	995	9,15	-	-	-	1.16	0.53	*3*	-	-	-	4.39	4.39	*1*	-	-	-	-	-	-	-	-	-
3,7-dimethylocta-2,6-diene (E)	997	9,22	**5.93**	**1.6**	***9***	-	-	-	0.44	0.28	*2*	-	-	-	3.68	0.92	*7*	0.97	0.97	*1*	4.45	2	*6*
3,7-dimethylocten-6-ol* (dihydromyrcenol)	1074	11,52	1.62	0.78	*4*	-	-	-	0.78	0.4	*3*	-	-	-	2.11	0.75	*6*	0.54	0.33	*2*	0.84	0.42	*3*
linalool	1099	12,27	-	-	-	1.38	0.39	*4*	-	-	-	3.22	2.44	*2*	-	-	-	-	-	-	-	-	-
Cyclic																							
limonene*	1027	10,12	-	-	-	**9.29**	**2.24**	***5***	1.46	1.46	*1*	**11.54**	**7.98**	***3***	-	-	-	-	-	-	1.22	0.36	*5*
dihydromonoterpene (menthene?*)	1021	9,94	0.31	0.21	*2*	0.29	0.29	*1*	-	-	-	-	-	-	-	-	-	-	-	-	0.18	0.18	*1*
SESQUITERPENOIDS																							
isocaryophyllene	1404	20,77	1.43	0.54	*6*	4.76	1.23	*5*	0.17	0.17	*1*	0.41	0.41	*1*	2.1	0.59	*7*	4.87	1.53	*4*	1.38	0.66	*3*
β-caryophyllene*	1420	21,18	**8.85**	**2.46**	***9***	**27.14**	**3.6**	***5***	4.92	2.64	*5*	**5.66**	**3.08**	***4***	**12.81**	**2.72**	***10***	**24.54**	**11.78**	***5***	**10.03**	**3.45**	***7***
α-humulene*	1456	22,08	0.64	0.27	*4*	2.62	0.79	*4*	0.18	0.18	*1*	-	-	-	0.9	0.38	*4*	3.92	1.25	*4*	1.04	0.54	*3*
α-selinene	1497	23,09	-	-	-	0.22	0.22	*1*	-	-	-	0.52	0.52	*1*	-	-	-	0.6	0.37	*2*	-	-	-
δ-cadinene*	1517	23,58	0.12	0.12	*1*	0.87	0.36	*3*	0.44	0.44	*1*	-	-	-	-	-	-	1.79	0.57	*4*	0.21	0.21	*1*
spathulenol	1580	25,06	-	-	-	0.38	0.38	*1*	-	-	-	0.29	0.29	*1*	-	-	-	-	-	-	-	-	-
alloaromadendrene*	1461	22,19	-	-	-	0.43	0.43	*1*	-	-	-	-	-	-	-	-	-	0.67	0.41	*2*	0.16	0.16	*1*
tetrahydrosesquiterpene	1366	19,77	-	-	-	-	-	-	1.36	0.7	*3*	-	-	-	0.33	0.33	*1*	0.42	0.42	*1*	0.45	0.45	*1*
dihydrosesquiterpene	1395	20,56	4.63	1.84	*9*	**7.98**	**1.6**	***5***	**18.65**	**4.6**	***7***	**5.57**	**2.84**	***3***	**8.74**	**1.47**	***10***	**12.06**	**3.77**	***5***	**8.29**	**2**	***7***
2 sesquiterpenes	1476	22,56	-	-	-	0.37	0.37	*1*	-	-	-	-	-	-	-	-	-	0.28	0.28	*1*	-	-	-
CAROTNOID DERIVATIVES																							
6-methylhept-5-en-2-one*	987	8,93	-	-	-	-	-	-	0.47	0.47	*1*	0.49	0.49	*1*	0.15	0.15	*1*	1.03	1.03	*1*	-	-	-
FATTY ACID DERIVATIVES																							
2-heptanone*	893	6,33	1.84	0.8	*6*	0.36	0.36	*1*	1.2	0.39	*5*	-	-	-	2.08	0.58	*7*	2.18	0.78	*4*	1.07	0.54	*3*
2-methylundecane	1154	13,92	-	-	-	-	-	-	-	-	-	2.38	1.74	*2*	-	-	-	-	-	-	-	-	-
decanal	1203	15,36	-	-	-	-	-	-	-	-	-	1.12	1.12	*1*	-	-	-	0.56	0.56	*1*	-	-	-
UNKNOWN COMPOUNDS																							
unknown1	1645	26,52	0.12	0.12	*1*	-	-	-	0.18	0.18	*1*	-	-	-	-	-	-	-	-	-	-	-	-

Table 4

			Arum maculatum														
			Bagnères-de-Bigorre						Smarves						La Loubatière		
			2009 (N=9)			2010 (N=5)			2009 (N=8)			2010 (N=4)			2009 (N=7)		
VOCs	RI	RT	Mean	SE	O	Mean	SE	O	Mean	SE	O	Mean	SE	O	Mean	SE	O
BENZENOIDS																	
acetophenone	959	8,15	-	-	-	1.03	0.46	*3*	-	-	-	0.55	0.55	*1*	-	-	-
p-cresol*	1083	11,79	1.12	0.78	*3*	**6.88**	**4.21**	***3***	**5.16**	**3.4**	***2***	-	-	-	1.49	1.05	*2*
N-CONTAINING COMPOUNDS																	
indole*	1298	17,97	**26.88**	**7.61**	***7***	**20.85**	**11.15**	***5***	**26.77**	**8.33**	***7***	1.99	1.19	*2*	**15.99**	**4.55**	***6***
MONOTERPENOIDS																	
Linear																	
3,7-dimethyloctene	908	6,71	3.1	2.41	*4*	0.36	0.36	*1*	-	-	-	-	-	-	0.82	0.53	*2*
α-pinene*	933	7,42	**11.43**	**5.05**	***5***	2.4	0.73	*4*	**5.28**	**1.82**	***6***	1.36	0.79	*2*	**9.71**	**4.06**	***6***
3,7-dimethylocta-1,6-diene	941	7,66	**8.03**	**4.45**	***7***	4.8	0.82	*5*	3.86	1.28	*7*	3.05	1.78	*2*	0.97	0.49	*3*
myrcene*	987	8,94	0.5	0.38	*2*	1.51	0.11	*5*	1.48	0.64	*5*	0.41	0.41	*1*	1.7	0.63	*5*
3,7-dimethylocta-2,6-diene (E)	997	9,22	1.37	1.17	*2*	-	-	-	0.39	0.27	*2*	-	-	-	0.16	0.16	*1*
linalool	1099	12,27	-	-	-	4.62	1.18	*4*	-	-	-	**13.89**	**2.81**	***4***	-	-	-
Cyclic																	
limonene*	1027	10,12	**6.86**	**3.28**	***5***	**41.99**	**11.16**	***4***	4.29	2.16	*5*	**50.53**	**4.8**	***4***	**5.05**	**2.58**	***5***
α-pinene*	933	7,42	**11.43**	**5.05**	***5***	2.4	0.73	*4*	**5.28**	**1.82**	***6***	1.36	0.79	*2*	**9.71**	**4.06**	***6***
β-pinene	974	8,57	-	-	-	2.3	0.61	*4*	-	-	-	1.21	0.71	*2*	-	-	
SESQUITERPENOIDS																	
isocaryophyllene	1404	20,77	0.55	0.39	*2*	1.06	0.8	*2*	2.27	0.71	*7*	-	-	-	1.26	0.94	*2*
β-caryophyllene*	1420	21,18	2.44	0.96	*5*	1.54	1.54	*1*	**7.7**	**1.09**	***8***	-	-	-	**5.84**	**4.33**	***3***
α-humulene*	1456	22,08	2.36	0.95	*5*	-	-	-	**6.21**	**0.57**	***8***	-	-	-	1.54	0.87	*3*
α-copaene*	1375	20,01	0.29	0.29	*1*	-	-	-	1.74	0.57	*5*	-	-	-	0.23	0.23	*1*
α-selinene	1497	23,09	1.94	0.72	*5*	0.3	0.3	*1*	4.8	1.27	*7*	0.42	0.42	*1*	2.9	2.41	*2*
δ-cadinene*	1517	23,58	**5.95**	**3.25**	***6***	-	-	-	**5.34**	**0.64**	***8***	-	-	-	2.82	1.11	*5*
alloaromadendrene*	1461	22,19	2.01	0.82	*5*	0.24	0.24	*1*	4.18	1.21	*7*	-	-	-	0.67	0.67	*1*
2 sesquiterpenes	1476	22,56	1.8	0.72	*5*	-	-	-	4.34	0.79	*8*	-	-	-	0.3	0.3	*1*
dihydrosesquiterpene?	1395	20,56	**5.3**	**1.27**	***8***	3.25	1.02	*4*	1.8	0.83	*5*	-	-	-	1.98	0.84	*4*
CAROTENOID DERIVATIVES																	
geranylacetone*	1445	21,79	0.71	0.56	*2*	0.25	0.25	*1*	0.31	0.2	*2*	0.89	0.52	*2*	3.93	1.18	*6*
6-methylhept-5-en-2-one*	987	8,93	-	-	-	-	-	-	0.96	0.58	*3*	-	-	-	**11.63**	**3.83**	***7***
FATTY ACID DERIVATIVES																	
2-heptanone*	893	6,33	**5.26**	**3.76**	***7***	-	-	-	4.26	1.46	*7*	-	-	-	**8.48**	**3.55**	***5***
alcane	996	9,2	-	-	-	-	-	-	-	-	-	2.13	0.92	*3*	-	-	-
2-methylundecane	1154	13,92	-	-	-	-	-	-	**5.69**	**2.13**	***6***	**7.94**	**4.59**	***2***	**5.84**	**2.08**	***5***
nonanal	1006	9,48	-	-	-	-	-	-	-	-	-	-	-	-	2.04	0.92	*4*
decanal*	1203	15,36	**9.17**	**3.57**	***6***	1.94	0.5	*4*	1.98	0.64	*6*	4.93	0.67	*4*	**9.56**	**3.15**	***7***
alcane	1481	22,7	-	-	-	-	-	-	-	-	-	-	-	-	3.59	3.59	*1*
UNKNOWNS COMPOUNDS																	
unknown	1330	18,82	-	-	-	-	-	-	-	-	-	0.79	0.48	*2*	-	-	-
unknown	1342	19,14	1.63	1.63	*1*	-	-	-	-	-	-	3.83	2.41	*2*	1.07	1.07	*1*
unknown	1392	20,48	-	-	-	-	-	-	-	-	-	0.95	0.58	*2*	-	-	-
unknown	1401	20,71	-	-	-	1.02	0.43	*3*	-	-	-	1.04	0.6	*2*	-	-	-

Table 5

Chapitre 8

Pourquoi deux espèces d'*Arum* capables de s'hybrider ne s'excluent pas par compétition : rôle de l'attraction olfactive différentielle des pollinisateurs

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Why is exclusive competition not achieved between two hybridizing *Arum* species growing in sympatry: the role of differential olfactory pollinator attraction.

8.1 ABSTRACT

Background and Aims When growing in sympatric sites, related species sharing pollinators are likely to come into competition. In instances where resultant plant hybrids are not fertile, and thus constitute an indirect loss of fitness, there might in addition be selection for reproductive isolation between species. In these cases, character displacement in one of the species can override exclusive competition and make cohabitation possible. To this end, the role of pollinator-attractive odours in such a mechanisms is investigated in two sympatric *Arum* species sharing pollinators, *Arum italicum* and *A. maculatum*.

Methods First, the phenology and the period of pollinator attraction of the two *Arum* species were compared. Inflorescence odour, pollinators and floral traits were compared between the two species and their potential hybrids in two consecutive years, and manual cross-pollinations were performed to test inter-species cross-pollination compatibility. Then, we tested for pollinator constancy and attraction with biotests in a cage containing receptive and thus attractive inflorescences.

Key results Pre-pollination reproductive barriers were not complete as the two species flowered at the same time and shared one pollinator, *Psychoda phalaenoides*. Post-pollination barriers were also weak, as almost all manually inter-species pollinated inflorescences produced fruit. As hybrids are pentaploid, they are likely to be sterile, selecting for reproductive barriers. Hybrid floral odours and pollinators were more similar to *A. italicum*. According to correlative analyses between odour compounds and insects, and to the composition of the hybrids odour, certain compounds may play a role in the attraction of the different pollinators of *A. italicum* and *A. maculatum*, and cohabitation may be made possible by the high attractiveness and specificity for *P. phalaenoides* of the less frequent species, *A. maculatum*.

Conclusion When growing sympatrically, exclusive competition and detrimental partial hybridization can be overridden by differences in the pollinator-attractive floral odours in these two *Arum* species.

8.2 INTRODUCTION

Angiosperm diversification has been accompanied by extraordinary variations of floral features, mostly due to selective pressures associated with pollination (reviewed by Fenster *et al.* 2004, Manning and Goldblatt 2005, Johnson 2006). At the specific level, such selective pressures are likely to vary across species' distribution ranges. This may be illustrated by the concept of species defined as populations in constant local interactions, each of them in a different context, resulting in a geographic mosaic of coevolution (Thompson 1999, Levin 2000, Thompson 2005, Herrera *et al.* 2006, Aldridge and Campbell 2009). For instance, in entomophilous species, floral traits are likely to vary owing to local adaptations for pollinator preferences or pollinator availability (Herrera *et al.* 2006, Anderson and Johnson 2007, Schlumpberger *et al.* 2008, Gomez *et al.* 2009, M Chartier, unpubl. res.) or owing to interactions with other species sharing the same pollinators (e.g. Flanagan *et al.* 2011, reviewed by Geber and Moeller 2006). In this case, floral traits may be influenced by competition and/or hybridization if the species are interfertile.

When two species compete for the same resource, such as pollinators, exclusive competition is a mechanism by which the less frequent species is predicted to be excluded after a number of generations in the absence of counterbalancing forces (Hardin, 1960, Kuno, 1992). When applied to pollination biology, exclusive competition may be the result of competition for pollinators by two main processes: competition through pollen transfer or competition through pollinator visitation rates (Levin and Anderson 1970, Waser 1978, Vamosi *et al.* 2006, Mitchell *et al.* 2009). Such competitions can lead to a serious decrease of seed-set (Brown *et al.* 2002, Bell *et al.* 2005, Flanagan *et al.* 2011), or to the exclusion of the less frequent species (Takakura and Nishida 2009). Different mechanisms can be selected on plants to override competitive exclusion, like phenological shifts, patchiness repartition (of the rare species), increased autogamy rate, or higher pollinator constancy (Levin and Anderson 1970). In any event, the competitive exclusion model predicts that competitors should go through evolutionary differentiation to avoid competition. This may lead to floral (form, colour, odour) or phenological changes (Waser 1978, Mitchell *et al.* 2009). The more extreme and the more studied case of such differentiation is a shift in pollinator for at least one of the species, thus completely avoiding competition (e.g. Pick and Schleinwein 2011, Levin and Anderson 1970, Levin 2000).

Pollinator shifts are also selected when hybridization of closely related species is counter selected, because hybrids may not be very fertile or may even be sterile owing to hybrid depression, or chromosomal incompatibilities (Dujardin and Hanna 1988, Rieseberg and Willis

2007, Vereecken *et al.* 2008). In this case, reproductive barriers should appear between the parental species (Rieseberg and Willis 2007). Reproductive barriers have been classified depending on the stage they appear, and the type of mechanism involved (Grant, 1994, Campbell and Aldridge 2006, Andalo *et al.* 2010). Pre-pollination barriers occur before the deposit of pollen grains on the stigma and can be ethological, i.e. due to the morphology and behaviour of pollinators (Emms and Arnold 2000, Schiestl and Schlüter 2009), or seasonal i.e. plants flowering at different time periods (Raine *et al.* 2007). Post-pollination reproductive barriers consist in pollen-stigma/style (Pellegrino *et al.* 2010) or gamete (Costa *et al.* 2007) incompatibilities or in hybrid sterility (Vereecken *et al.* 2008, Cortis *et al.* 2009).

In the case of ethological pre-pollination reproductive isolation, floral odour variation has been shown to be efficient for pollinator segregation between species (Raguso 2008, Schaeffer and Ruxton 2011). Especially in orchids (Ayasse *et al.* 2011, Schiestl and Schlüter 2009, but see also Hossaert-McKey *et al.* 2010), it has been shown that pollinator shifts and thus reproductive isolation between two species could arise through a change in the relative amounts of floral blend compounds (Schiestl and Ayasse, 2002, Stölk *et al.* 2008) or in the blend composition (Mant *et al.* 2005, Waelti *et al.* 2008, Shuttleworth and Johnson 2010), sometimes in synergy with other cues (Salzmann and Schiestl 2007). Floral odour variations are thus likely to be selected in sympatric zones where reproductive isolation is selected (Aldridge and Campbell 2009, Suchet *et al.* 2011). This can happen through the combination of detrimental hybridization and competition for pollinators between closely related species growing in sympatry.

This is the case of two insect-deceiving European species, *Arum italicum* Mill. and *A. maculatum* L. (Araceae, Aroideae, Areae), growing on the forest floor in temperate and warm temperate woodlands (Boyce 1993, 2006). These two species largely overlap in their distribution (Boyce 1993, 2006, Linz *et al.* 2010), and their ecology is similar. Consequently, they sometimes grow sympatrically. Inflorescences of both species attract Diptera pollinators by mimicking the odour of their oviposition sites, and sequester them for almost a day to ensure pollination (Lack and Diaz 1991, Albre *et al.* 2003, Gibernau *et al.* 2004). Recent studies have shown that pollinators of *A. italicum* belong to different Diptera families (Psychodidae, Chironomidae, Ceratopogonidae) from the same functional group and that their relative abundances vary in time and space (Chartier *et al.* 2011). By contrast, *A. maculatum* is mainly pollinated by a Psychodidae species, *Psychoda phalaenoides*, across its main geographical range, and by a second species, *Psycha grisescens*, in some southern or western European populations (Espindola *et al.* 2011, M Chartier, unpubl. res.). *Arum italicum* also attracts *P. phalaenoides* and *P. grisescens* in

some populations, and are thus likely to compete for pollinators in sympatric sites (M Chartier, unpubl. res.). At one site, Bagnères-de-Bigorre (Pyrénées, France), potential hybrids have been observed (Chartier, pers. observation), suggesting incomplete reproductive isolation between the two species.

Here, we investigated how competitive exclusion through detrimental hybrid production can be overridden when reproductive barriers are not complete through different pollination strategies. Field work, biotests and chemical analyses were conducted on *A. italicum*, *A. maculatum* and their potential hybrids to answer the following questions: (1) Which reproductive barrier(s) result(s) in an incomplete reproductive isolation between the two studied species? (2) Is there potential selection to avoid hybridization between *A. italicum* and *A. maculatum* in Bagnères-de-Bigorre? (3) Is a competitive exclusion leading to a complete isolation of the two species? (4) What role does odour play in the isolation of the two species? We particularly studied the ecology and floral odours of the potential hybrids to better understand the role of floral odour as an ecological barrier. Inflorescence morphological terminology used below follows Boyce (1993).

8.3 MATERIALS AND METHODS

Pollination cycle of the studied species *Arum italicum* and *A. maculatum* are pollinated according to the same floral cycle, which lasts about 24 hours over two days. On the afternoon of the first day, the spathe begins to open above the constriction, revealing a sterile organ called the appendix (Fig. 1D). In the evening, the appendix begins to warm (Bermadinger and Bermadinger-Strabentheiner 1995, Albre *et al.* 2003) and emits the pollinator-attractive odour (Kite 1995). Insects mainly Diptera are attracted, land on the spathe and slide in the floral chamber (Fig. 1A-D, F; Lack and Diaz 1991, Albre *et al.* 2003, Gibernau *et al.* 2004). At this stage, female flowers are receptive, and can be pollinated if the insects carry fresh pollen. The insects remain captive within the floral chamber because a corona of sterile hair blocks the exit until the next day. On the second day afternoon, the pollen is released and the sterile hairs dry, allowing the insects to leave the inflorescence carrying fresh pollen.

Study sites, phenology and morphology All experiments were carried out in humid deciduous woodland near Bagnères-de-Bigorre (Midi-Pyrénées, France, 43 ° 04'N 0 ° 09'E). On this site, several tens of *Arum italicum* and *A. maculatum* grow mixed together, with individuals spaced from one to several meters. The total number of opening inflorescences of *A. maculatum*, *A. italicum* and their potential hybrids was daily recorded from 1st Apr. 2010 to 20 May 2010. *Arum italicum* and *A. maculatum* are separable at this site as they have respectively yellow or purple stamens and appendix (Fig. 1D and 1F). Potential hybrids were identified when the inflorescences presented purplish stamens with a yellowish appendix (Fig. 1E). Some potential hybrids had purple to pink stamens. The colour of the stamens and of the appendix was noted for 25 potential hybrids found at the site. In order to characterize the morphology of the potential hybrids, 16 inflorescences for each of the two species and their potential hybrids were measured. Morphologies such as the length of the whole spadix, the appendix, the male and female flower zone were recorded. The number of female and male flowers was also counted.

Tests for hybridization Receptive inflorescences of *Arum italicum* and *A. maculatum* were hand pollinated with fresh pollen harvested in the morning of the same day from three inflorescences at the male stage. Intra- and inter-specific cross pollinations were performed on at least three inflorescences per test. On the same days, at least 10 non-manipulated inflorescences of the two species and their potential hybrids were marked as control. One month later, all

manipulated and marked infructescences where harvested, and the percentage of developed fruits (berries) per infructescence calculated. Three inflorescences of potential hybrids were also pollinated with pollen of *A. italicum*, and two inflorescences of *A. italicum* with pollen from potential hybrids.

Pollinator diversity and trapping dynamics Insects visitors were collected in 2009 and 2010 from the inflorescences of potential hybrids and compared to the insects collected in inflorescences of *Arum italicum* and *A. maculatum* at the same site and years (data from M Chartier, unpubl. res.). Collections were made in the morning of the second day of flowering when the insects are captive in the inflorescences, by pouring ethanol (70%) into the floral chamber and then opening the spathes with a scalpel. Insects were conserved in 70% ethanol until determination to family level under a stereomicroscope, with assistance of Diptera taxonomist Prof. Alain Thomas. Psychodideae, the most numerous insects trapped, were identified to species level based on their genitalia and antenna shapes for at least eight Psychodidae per inflorescence from eight inflorescences for each taxon in 2009, and from 10 inflorescences in 2010 (Ježek 1990).

To compare the trapping dynamics between *A. italicum* and *A. maculatum*, insects were collected every hour from three inflorescences each of both species from 1800 h to 2200 h on the first day of flowering, and at 1000 h on the second day of flowering. Insects were collected with an aspirator through a small hole cut at the base of the spathe. The holes were closed with adhesive tape between each collection.

Pollinator constancy (cage experiment) Cross pollination between two different *Arum* species occurs when insects released from the inflorescence "source" of one species are caught in the inflorescence "receptor" of the other species. In order to test for pollinator constancy, insects were collected alive from inflorescences of *A. italicum* or *A. maculatum* in the morning before pollen release and kept in a plastic tube with a humidified piece of paper. At dusk (1900 h), they were released in a tulle cage (0.9 m x 0.5 m x 0.6 m) containing two odour-producing "receptor" *Arum* inflorescences of approximately the same size, both with empty floral chambers. Experiments were carried out in the field. The cage was wrapped with a black tissue during the test to avoid any bias from light. After one hour, all insects trapped into the floral chambers were collected and conserved in 70% ethanol.

Determination of insects was made at the species level for Psychodids, and family level for the others. All the combinations of "source" and "receptor" species were tested, alternating the

side of the inflorescences in the cage to avoid any directional bias.

Floral odour collection and Gas Chromatography-Mass Spectrometry analyses Odours of the potential hybrids were collected for five inflorescences in 2009 and 2010, and compared to the odour of *A. italicum* and *A. maculatum* at the same site and years (data from M Chartier, unpubl. res.). Inflorescence odours were collected in the field between 2000 h and 2300 h, when the spathe is widely open, the appendix warm, and the odour is strong. Inflorescences (spathe and spadix) were wrapped in a plastic inert bag (Nalophan NA colorless, calibre 90, available from ETS Charles-Frères, France) in order to create an "open static headspace". The bottom of the bag was closed below the floral chamber with a band, isolating the inflorescence from the leaves and soil. The top of the bag was kept open 10 cm above the spathe, to avoid any condensation due to the heating of the appendix. Volatile organic compounds (VOCs) were collected by solid phase microextraction (SPME). VOCs are absorbed and desorbed from a fiber attached within the needle of a modified syringe. StableFlexTM SPME fibers, 65μ m polydimethylsiloxane/divinylbenzene coating for manual holder (available from Supelco®) were used. For each inflorescence, a fiber was introduced in the nalophan bag through a little slit and maintained 0.5-1.0 cm distant from the appendix for 20 min. Closed empty bags containing ambient air from 3-4 m away the inflorescence were used as controls to discard putative VOCs not originating from inflorescences. Fibers were stored in a freezer (-20 $^\circ$C) until analyses by GC-MS.

GC-MS analyses were performed on the the Platform for Chemical Analyses in Ecology of the "SFR 119 Montpellier Environnement Biodiversité", at the "Centre d'Écologie Fonctionnelle et Évolutive (Montpellier, France)". SPME fibers were desorbed 5 minutes at 250 $^\circ$C into the 1177 Split/Splitless injector of a CP-3800 gas chromatograph (Varian Inc., Palo Alto, CA, USA) coupled with a Saturn 2000 ion trap spectrometer (Varian Inc.). The carrier gas was helium with a constant flow rate set close to 1.0 mL/min. A split ratio of 1:4 was used. The temperature of the column (fused silica capillary column, 30m x 0.25mm x 0.25 μm, CP-Sil 8 CB lowbleed MS, Varian Inc. in 2009, Optima 5 Accent, Macherey-Nagel, Düren, Deutschland in 2010) was maintained at 50 $^\circ$C for 2 min after injection, linearly increased to 200 $^\circ$C at a rate of 5 $^\circ$C/min, and then increased to 250 $^\circ$C at a rate of 10 $^\circ$C/min and maintained at 250 $^\circ$C for 1 min. Mass spectra were recorded in scan mode from 38 to 300 m/z with an electronic impact (EI) at 70 eV. The chemical compounds were identified by comparison with the mass spectral library NIST98 MS and Adams 2007, and retention indices found in libraries and published data (Adams 2007).

All chromatograms peaks were manually integrated, and the relative percentage area of each peak was calculated for each chromatogram (i.e. each inflorescence sampled). Only peaks representing more than 1% of the total peaks area per chromatogram were retained.

Statistical analyses Morphological differences between the three types of *Arum* (*A. italicum*, *A. maculatum*, and their potential hybrids) were assessed with ANOVAs and Kruskall-Wallis tests in instance where data were not normally distributed. We used the functions aov() and kruskal.test() with TukeyHDS() and kruskalmc() as post hoc tests from the packages *stats* and *pgirmess* in R (version 2.12.2). Pollinator diversity was compared between the two species and their potential hybrids using non parametric multivariate ANOVAs (npMANOVAs) with the function adonis() from *vegan* package in R. Ten thousand permutations were used to calculate the distribution of a pseudo F ratio under the null hypothesis (Anderson 2001). The same test was used post-hoc, with a Bonferroni correction.

In the cage experiments, the number of trapped insects (count data) per inflorescence was analyzed according to the "source" and "receptor" status to group treatments together with a generalized linear model using a Poisson error (χ^2 statistics; GLIM 1986). A model with all six treatments (six combinations of *Arum* plant species as "source" and/or "receptor") was fitted to the data (full models). Afterwards, simplified models grouping certain treatments were adjusted to the data, and only those statistically similar to the full model (Chi-square test) were retained (simplified models).

The floral odour of *A. italicum*, *A. maculatum* and the potential hybrids were compared in 2009 and 2010. The significance of the odour differences was assessed with a npMANOVA. The same test was used post-hoc, with a Bonferroni correction. The odour variability was then compared within the three taxa by comparing the mean Bray-Curtis distances per taxon with a Kruskall-Wallis test. Differences between inflorescences based on the relative percentages of their blend of odour compounds were represented with a non metric multidimentional scaling (nMDS) using the function metaMDS() from *vegan* package in R. This method maximizes the representation of the ranked difference between samples in a two dimensional plot using a distance matrix (we used the Bray-Curtis index). The goodness of the fit is estimated by the stress value, ranging from 0 (perfect solution) to 1 (worse solution) (Rabinowitz 1975).

The SPME method does not permit precise quantitative analyses. If the collection and analyses conditions are the same, and because the fiber capacity is sufficiently large to avoid saturation, it is possible to compare the relative quantity of compounds present in the headspace

during the odour collection by comparing the total areas of peaks in the chromatograms. Thus, we estimated the difference of total amount of emitted VOCs from *A. italicum*, *A. maculatum* and the potential hybrids inflorescences by comparing the mean total peak areas per chromatogram between the three taxa.

Correlations between the trapped insect taxa and the emitted VOCs were assessed with a co-inertia analysis on 15 inflorescences for which we had data on the odour and pollinators (six inflorescences of *A. italicum*, four of *A. maculatum*, and five of potential hybrids). To reduce the data set, 15 VOCs were kept in the analysis, the most common in term of occurrence and/or those significantly correlated to at least one of the insect groups (Pearson correlation, $p < 0.05$). Relative percentages of insects and odour compounds were arcsin squareroot transformed to approach normality. We then performed two principle component analyses (PCA), one for the insects, one for the odours (function dudi.pca() from *ade4* package in R) followed by a co-inertia analysis on both PCA (function coinertia() from *ade4* package in R). The significance of the association between insects and odour compounds was assessed with a Monte-Carlo test on the sum of the co-inertia eigenvalues (function RV.rtest() from *ade4* package in R).

8.4 RESULTS

Phenology and morphology From 1st Apr. 2010 until 9 May 2010, 322 inflorescences of *Arum maculatum* opened, 1367 of *A. italicum*, and 99 belonging to potential hybrids. *Arum maculatum* and *A. italicum* flowered following a unimodal pattern. A first phase with a low number of flowerings lasted about 12 days for *A. maculatum* and for 14 days for *A. italicum*. This was followed by a peak of flowering, reaching its maximum on 21 Apr. 2010 for *A. maculatum* with 36 simultaneously open inflorescences, and 29 Apr. 2010 for *A. italicum*, with 190 simultaneously open inflorescences (Fig. 2). *Arum maculatum* flowered less but earlier than *A. italicum*, with a 24 days overlapping period representing 83 % of the *A. maculatum* flowering period, and about 50% of the *A. italicum* flowering period. At the end of the investigation, the flowering periods of *A. maculatum* and the potential hybrids were finished, whereas on 11 May 2010, 42 inflorescences of *A. italicum* still remained unopened.

The potential hybrids flowered more continuously, with a maximum of 11 simultaneously open inflorescences on 19 Apr. 2010. Among the 25 inflorescences for which colour was recorded, six (24%) had purple stamens with a yellow appendix, eight (32%) had purple stamens yellowish at their base, and a yellow appendix (e.g. Fig. 1B), and three (12%) had purple stamens with a yellow appendix with a purplish stipe (e.g. appendix in Fig. 1C). The eight (32%) remaining inflorescences presented different combinations of pink (e.g. stamens in Fig. 1C), purple, and yellow, or purple stamens with a yellow, yellow and purple, or pink appendix.

In terms of inflorescence structure or number of flowers, potential hybrids were not different from *A. italicum*. On the other hand, *A. maculatum* had shorter inflorescences (fertile and sterile zones), and produced fewer male and female flowers (Table 1).

Tests for hybridization All pollinated and control inflorescences produced a high number of fruits, whereas a few inflorescences completely aborted (Table 2). There was no significant difference between the percentage of fructification of any of the modalities of crossing (KW: $\chi^2_2 = 1.89, p = 0.39$).

Pollinator diversity *Arum italicum* and *A. maculatum* trapped insects continuously between 1800 h and 2200 h on the first day of flowering, with a maximum of insects trapped per inflorescence between 1900 h and 2000 h of 5.3 ± 1.9 for *A. italicum* and 21.3 ± 10.3 for *A. maculatum*. The following morning after 12 hours, 2.3 ± 0.7 insects were collected in the

inflorescences of *A. italicum*, and 5.7 ± 1.2 in the inflorescences of *A. maculatum* (Fig. 3).

Diptera were the main insects trapped in the inflorescences in 2009 and 2010, and particularly those from the families Psychodidae (5776 insects trapped) and Chironomidae (1279 insects trapped). A few Brachycera (90) were also collected, as well as insects from the families Ceratopogonideae (57), Sciarideae (13) and Coleoptera: Staphylinideae (7). In both 2009 and 2010, inflorescences of *A. italicum* trapped on average significantly less insects than *A. maculatum* (24.5 ± 5.1 vs. 149.4 ± 53.3 in 2009; 75.9 ± 18.1 vs. 219.3 ± 39.9 in 2010; overall tests KW: $\chi^2_2 = 12.030, p = 0.002$ in 2009; $\chi^2_2 = 10.873, p = 0.004$ in 2010; post-hoc tests non shown). In 2009, inflorescences of the potential hybrids attracted on average as few insects (11.4 ± 5.7) as *A. italicum*, but in 2010 they trapped an intermediate number of insects (104.2 ± 33.5), which was not statistically different from the average number of insects trapped by neither *A. italicum* nor *A. maculatum*.

The diversity of insects caught varied among taxa in terms of family composition (npMANOVA: $F = 8.52, r^2 = 0.25, p = 0.001$ in 2009; $F = 31.19, r^2 = 0.47, p < 1.10^{-4}$ in 2010). During the study, inflorescences of *A. maculatum* attracted almost only Psychodidae (93% in 2009 and 96% in 2010, npMANOVA: $F = 0.77, r^2 = 0.017, p = 0.49$, Fig. 4A). The diversity of insects caught by *A. italicum* varied between 2009 and 2010 (npMANOVA: $F = 0.87, r^2 = 0.17, p = 0.001$) and was in both years significantly different from that of *A. maculatum* (Fig. 4A). In 2009, inflorescences of *A. italicum* attracted 63% Psychodidae and 23% Chironomidae, whereas in 2010 the proportion of Psychodidae was only 38% and Chironomidae represented 52% of the trapped insects (Fig. 4A). The diversity of insects trapped in inflorescences of the potential hybrids varied between years (npMANOVA: $F = 4.28, r^2 = 0.13, p = 0.021$). In 2009, a majority of Psychodidae (93%) were caught, and insect diversity was not significantly different from that of *A. italicum* and *A. maculatum* (Fig. 4A). In 2010, the potential hybrids insect diversity was intermediate between *A. italicum* and *A. maculatum*, but significantly different from both, with 58% Psychodidae and 30% Chironomidae (Fig. 4A).

Almost all the identified Psychodidae belonged to two species, *Psychoda phalaenoides* (369 individuals) and *Psycha grisescens* (164 individuals) (Ježek, 1990). Four specimens collected in the inflorescences of *Arum italicum* and the potential hybrids remained un-indentified (*Psychoda* sp.1 and *P.* sp.2).

The pattern of Psychodid species attracted by the inflorescences of *A. italicum* and *A. maculatum* were statistically different in 2009 and 2010 (npMANOVA: $F = 7.95, r^2 = 0.46, p = 0.004$ in 2009; $F = 33.8, r^2 = 0.715, p < 1.10^{-4}$ in 2010). *Arum italicum* trapped mainly *P.*

grisescens (66% in 2009 and 85% in 2010) whereas *A. maculatum* trapped almost exclusively *P. phalaenoides* (88% in 2009 and 96% in 2010, Fig. 4B).

The potential hybrids caught both *P. phalaenoides* and *P. grisescens*. In 2009, their Psychodid proportions were not significantly different from *A. italicum* (61% of *P. grisescens* and 35% of *P. phalaenoides*). In 2010, they were significantly different from both *A. italicum* and *A. maculatum* (56% of *P. phalaenoides* and 44% of *P. grisescens*, Fig. 4B).

Pollinator constancy (cage experiment) There was no significant difference between the insects trapped in the inflorescences placed on the right side or the left side of the cage for *Arum italicum* and *A. maculatum*, thus no side bias was detected in the experimental design (Wilcoxon: $V = 3.5, p = 1$ for *A. italicum*, $V = 7, p = 0.56$ for *A. maculatum*).

When *A. italicum* was at the same time the inflorescence "source" and "receptor", it trapped a mean of 1.70 ± 1.42 pollinators (Fig. 5). When *A. maculatum* was the inflorescence "source" and "receptor", it trapped a mean of 31.10 ± 37.35 insects, mainly *P. phalaenoides*.

When insects from a "source" inflorescence of *A. italicum* were presented to a choice between an *A. italicum* and an *A. maculatum* inflorescence as "receptor", both species trapped a significantly similar number of insects (3.20 ± 2.77 for *A. italicum* and 3.40 ± 1.34 for *A. maculatum*), but *A. italicum* trapped 81% of *P. grisescens*, no *P. phalaenoides*, and 19% of insects from the other categories. By comparison, *A. maculatum* trapped 38% of *P. phalaenoides*, 32% of *P. grisescens*, and 30% of insects from the other categories (Fig. 5). When insects from a "source" inflorescence of *A. maculatum* were released into a cage containing two "receptor" inflorescences, one of *A. italicum* and one of *A. maculatum*, *A. maculatum* trapped a higher number of insects (55.80 ± 37.78) than *A. italicum* (6.40 ± 5.50). *A. maculatum* trapped 99% of *P. phalaenoides* and 1% of insects from the other categories, whereas *A. italicum* trapped 82% of *P. phalaenoides*, 10% of *P. grisescens* and 10% of insects from the other categories (Fig. 5).

Comparison of the pollinator-attractive floral odours Forty-six different VOCs were found among all chromatograms, most of them being terpenoids (Table 3). *Arum italicum* emitted a total of 23 different VOCs, *A. maculatum* a total of 32 VOCs, and the potential hybrids a total of 34 VOCs. Three compounds were not identified and classified as "unknown compounds". In 2009 and 2010, each taxon emitted from four to eight compounds representing on average more than 5% of the compounds blends. In addition, four to 11 compounds per group occurred in more than 80% of the chromatograms; 40% of these common compounds represented

on average less than 5% or the compound blends (Table 3).

The potential hybrids shared 19 VOCs with *A. italicum* and 23 VOCs with *A. maculatum*. Among these compounds, α-citronellene, dihydromyrcenol, a dihydromonoterpene which may be menthene ($IR = 1021$) and 2,6-dimethylocta-2,6-diene were exclusively found in the odours of *A. italicum* and the potential hybrids. Indole, myrcene, α-copaene, bicyclogermacrene, decanal were exclusively found in the odours of *A. maculatum* and the potential hybrids and an unknown compound ($IR = 1486$) in the odour of the potential hybrids (Table 3).

The two nMDS gave reliable representations, with stress values of 11.53 in 2009 and 5.57 in 2010. For each year, the potential hybrids appeared to emit an overall odour closer to *A. italicum* and different to that *A. maculatum* (npMANOVA: $r^2 = 0.459, p = 1.10^{-4}$ in 2009; $r^2 = 0.580, p = 1.10^{-4}$ in 2010; Fig 6). The result was the same when adding the compounds presents in a single chromatogram.

By comparing the total chromatogram areas, it was possible to estimate that *A. italicum* and the potential hybrids emitted significantly higher quantities of VOCs than did *A. maculatum* (KW: $\chi^2_2 = 47.395, p < 1.10^{-10}$; Fig. 7).

Correlation between plants odours and pollinators The first two axes were retained from the co-inertia analysis, representing 70 and 21% of the total variance, and significantly representing the association between insects and odours (RV test: $RV = 0.53, p = 0.007$; Fig. 8). The attraction of *P. phalaenoides* was positively correlated with the emission of α-humulene, indole, α-selimene, alloaromadendrene and two mixted sesquiterpenes ($RT = 22.56$). The attraction of Chironomidae was positively correlated with the emission of 3,6-dimethyl-3-octene, 3,7-dimethylocta-2,6-diene, tetrahydromyrcene and β-citronellene. Finally, the attraction of *P. grisescens* and insects from the other categories was positively correlated with β-caryophyllene, menthene, isocaryophyllene and two unidentified compounds ($IR = 1330$ and 1342).

8.5 DISCUSSION

Is there potential selection to avoid hybridization between A. italicum and A. maculatum in Bagnères-de-Bigorre? In the studied population, a low proportion of potential hybrids (5.5% from the total recorded inflorescences) indicated that hybridization is either not frequent, and/or that hybrid fitness is low. A low rate of hybridization can be owing to pre-pollination reproductive barriers, whereas low fitness of hybrids constitutes a case of post-pollination reproductive barrier.

Hybrids of *Arum* species, especially *A. italicum* x *A.maculatum*, have already been found in nature or produced in botanical gardens, but are mentioned to be rare (Bedalov *et al.* 1998). As *A. italicum* is hexaploid ($2n = 6x = 84$) and *A. maculatum* tetraploid ($2n = 4x = 56$), their hybrids have been shown to be pentaploid ($2n = 5x = 70$) and to resemble more closely *A. italicum* (Beuret 1977, Bedalov 1984, Bedalov and Küpfer 2005). Pentaploid hybrids are likely to be sterile, owing to meiotic abnormalities (Rieseberg and Willis, 2007). The potential hybrids were able to attract Diptera and observations in the field confirmed that they were also able to release the attracted flies, and thus to pollinate other *Arum* inflorescences. Furthermore the hybrids were able to set fruits (Table 2). Fruits are known to have developed from pentaploid Arum hybrids, and Bedalov *et al.* (1998) mentions germination. Partial or complete sterility of F1 hybrids occurring after seed production may be the main reason of the low number of hybrids in the population. Tests for seed germination remain to be done to confirm this hypothesis.

A similar case of hybrids able to attract pollinators but not to reproduce has been described by Vereecken *et al.* (2008) on two sexually deceptive orchids (*Ophrys arachnitiformis* and *O. lupercalis*). In this case, the hybrids could potentially occupy a new ecological niche, as they produced an odour different from the parental species, and were able to attract a new pollinator. When it occurs, hybrid sterility is one of the primary factors selecting for floral isolation between species, as they constitute a loss of pollen and ovules (reviewed in Grant 1994, Levin 2000, Campbell and Aldridge 2006). As the observed *Arum* hybrids were able to set fruit, post-pollination reproductive barrier seems to be weak between *A. italicum* and *A. maculatum* (Table 2) and therefore pre-pollination reproductive barriers in all probability exist between *A. italicum* and *A. maculatum*, since only few hybrids were observed.

Pre-pollination reproductive barriers may be ethological (owing to insects behaviour), seasonal (owing to plants phenology), ecological (owing to plant ecology) or mechanical (due to plant morphology) (reviewed in Grant, 1994, Campbell and Aldridge, 2006). At the study site *A.*

italicum and *A. maculatum* flowering periods largely overlapped, with 50% - 80% of inflorescences open during their common flowering season (Fig. 2). The periods during anthesis that insects are attracted also overlap significantly (Fig. 3). Furthermore, inflorescences have similar shapes, even though *A. italicum* can be larger than *A. maculatum* (Chartier and Gibernau 2009), and all pollinator insects are able to enter inflorescences from both species, as demonstrated by the cage experiment. Thus, there must be no efficient seasonal, ecological or morphological reproductive isolation between the two species. The most likely pre-pollination isolation mechanism that could occur in this case is ethological isolation. This mechanism has been studied in different plant lineages (Schaeffer and Ruxton 2011) and has been shown to be possibly driven by differences in insect preferences among the plants pollinator-attractive morphologies (e.g. Bradshaw and Schemske 2003, Cunningham *et al.* 2004, Waelti *et al.* 2008, Suchet *et al.* 2011).

In both years, *A. italicum* trapped a significantly different pollinator guild than *A. maculatum* (Fig. 4). *Arum maculatum* trapped more than 90% of *Psychoda phalaenoides* (Psychodidae, Diptera), whereas *A. italicum* trapped species from at least 2 different insect families (Psychodidae and Chironomidae), with a high proportion of *Psycha grisescens*. This difference of specificity degrees has already been demonstrated in previous investigative studies (reviewed by Gibernau *et al.* 2004, see also Chartier *et al.* 2011, Espindola *et al.* 2011). In Bagnères-de-Bigorre, *A. italicum* trapped approximately 20% and 7% of *P. phalaenoides* in 2009 and 2010, whereas *A. maculatum* trapped 12% and 4% of *P. grisescens*. Consequently, and taking into account the trapping efficiency of both *Arum* species (Fig. 5), *A. italicum* and *A. maculatum* could potentially "exchange" up to 30% of their pollinators. The sharing of 20% of pollinators has previously been shown to be sufficient to provoke hybridization and then the introgression of hybrids between two *Ophrys* species (Orchidaceae) in Sardinia (Stölk *et al.* 2008). The ethological reproductive barrier is thus only partial between *A. italicum* and *A. maculatum*.

How do the two species override competitive exclusion? As they share *Psychoda phalaenoides* as a pollinator, there might be competition for pollinators between the two *Arum* species in Bagnères-de-Bigorre, potentially leading to competitive exclusion of the minor species: *A. maculatum* (Hardin, 1960, Levin and Anderson, 1970). Several ecological factors can in part explain the apparent stability of this system. First, it has been shown in other investigations that the specificity of *A. maculatum* for *P. phalaenoides* is not always so strong. For instance, in the west of France, inflorescences of *A. maculatum* trapped only 45% of psychodids, most of which were *Psycha grisescens* (M Chartier, unpubl. res.). *Psycha grisescens* has also been

recorded as the main pollinator in 12 populations of *A. maculatum* in the Mediterranean region, and four populations in north west France, and England (Espindola *et al.* 2011). In Smarves (Poitou-Charentes, France), where *A. italicum* and *A. maculatum* also grow in sympatry, *A. italicum* mainly trapped *Psychoda crassipenis*, even if a few *P. grisescens* were also attracted, whereas *A. maculatum* trapped mainly *P. grisescens* (M Chartier, unpubl. res.). The attraction of *P. grisescens* or *P. phalaenoides* as main pollinator by *A. maculatum* according to the site is likely to be due to the geographic distribution of both insect species, resulting from a climatic effect (Espindola *et al.* 2011). In sympatric populations where both Psychodid species occur, the shift in pollinator of *A. maculatum* could also be an adaptation overriding exclusive competition.

A second means to avoid competitive exclusion is an increase of pollinator constancy for the minor species (Hardin, 1960, Levin and Anderson, 1970). In the case of deception, this can be achieved by increasing pollinator preference through plant attractiveness. This is the situation with *A. maculatum*, which may afford a weak percentage of pollen loss because it is strongly attractive and relies exclusively on one pollinator species. In Bagnères-de-Bigorre, the mean number of insects attracted per inflorescence of *A. maculatum* was very high (149 in 2009 and 219 in 2010). In a large scale study across Europe investigating fifty-six populations of *A. maculatum*, the mean number of insects caught in inflorescences varied between populations between 1.50 and 425, with only six populations attracting more than 100 insects in average (Espindola *et al.* 2011). Thus, attractiveness in Bagnères-de-Bigorre is high for the species. In addition, *A. maculatum* is here shown to be significantly more attractive for *Psychoda phalaenoides* than is *A. italicum* in all cases of the cage experiment. Contrary to *A. maculatum*, the opportunistic mode of pollination of *A. italicum* and its high density in the site are likely to increase its probability of trapping "good" pollinators, even if some *P. phalaenoides* are also trapped.

Pollinator variations between *Arum* populations, as described by Espindola *et al.* (2011) and M Chartier (unpubl. res.) are likely to be the result of varying climatic conditions, but also to some extent local selective forces, such as selection for reproductive barriers owing to hybrid sterility, or to competitive exclusion. These selective forces are combined among species populations, creating a geographical mosaic of coevolution (Thompson 2005).

How can odour help maintain cohabitation of two species? In *Arum*, the major pollinator attractive feature is known to be the attractive dung-, carrion-, or urine-like odour emitted by the spadix appendix. *Arum italicum* and *A. maculatum* both produce odours mimicking dung/urine (Lack and Diaz, 1991, reviewed by Gibernau *et al.* 2004 and Urru *et al.*

2011). The volatile odour of the two *Arum* species, and their potential hybrids, in Bagnères-de-Bigorre was mostly composed of monoterpenes, sesquiterpenes and hydrocarbons already identified in previous work (Kite, 1995, Kite *et al.* 1998, Diaz and Kite, 2002, reviewed in Urru *et al.* 2011, M Chartier, unpubl. res.). All taxa also produced various proportions of 2-heptanone (a ketone), p-cresol (a benzenoid). Indole, a nitrogen-containing compound, was only found in *A. maculatum* and the potential hybrids. These three compounds have been successfully tested for their attractiveness to psychodid flies (Kite *et al.* 1998). In addition, some of the emitted compounds, like p-cresol, indole, α-pinene, limonene or β-caryophyllene, where also found in sheep, cow, horse, or boar dung odours (Kite 1995, Dormont *et al.* 2010, Johnson and Jürgens 2010), as well as in the odour of deceptive asclepiads (Apocynaceae: Asclepiadoideae) (Jürgens *et al.* 2006). Among these compounds, some are very common components of flower scent, e.g. α-pinene, limonene and β-caryophyllene (Knudsen *et al.* 2006).

Among plants in which the main pollinator-attractant is odour, pre-pollination reproductive barriers have been mostly studied for deceptive orchid species (reviewed by Schaeffer and Ruxton 2011), where the odour has been shown to be an efficient and easily modified trait (Salzmann *et al.* 2006, Stökl *et al.* 2009). Odour can regulate and filter pollinator attraction in both quantity and diversity by variations of the number, the quality, or relative amount of compounds, or in synergy with other cues (Raguso 2008). For example, in *Hesperis matronalis* L. (Brassicaceae), an increase of the quantity of total emitted odour lead to an increase of pollinator attraction (Majetic *et al.* 2007). On the contrary, the odour in *A. maculatum* was estimated to be emitted in much less quantity than the odour in *A. italicum*, but *A. maculatum* trapped on average 20 times more insects. The difference in attractiveness of both species is more likely to be owing to qualitative more than quantitative differences in the odours, related to the ability of the insects to perceive the attractive signal (Knudsen *et al.* 2006, Raguso 2008).

In deceptive systems, odour has been shown to be highly variable, in order to avoid learning or selective responses from insects (Moya and Ackerman 1993, Knudsen *et al.* 2006, Ackerman *et al.* 2011). This is the case on *A. italicum* and *A. maculatum*, in which inter-individual odour variations are greater than inter-population variations (M Chartier, unpubl. res.). This variation could furthermore be explained if the dung odour they mimic was naturally variable, which remains to be shown (Dormont *et al.* 2010). Despite these variations, some characteristics of the signal keep it efficient, as evidenced by few inflorescences of either species found empty of insects in the field. As often in chemical ecology studies, understanding the accurate functioning of the attraction remains challenging without having conducted biotests on the insects (like electro-

antennography or behavioural tests). Nonetheless, this descriptive and correlative approach permitted some hypotheses generation on the mechanisms shaping the different attractiveness strategies of *A. italicum* and *A. maculatum*.

Despite *A. italicum* and *A. maculatum* sharing a large amount of odour compounds, most of their pollinators are different. Compounds are known to act in synergy (Raguso 2008, Kite 1995) and the important number of terpenes shared by the odour of *A. italicum* and *A. maculatum*, while they are not closely related species (Linz *et al.* 2010), may be explained by the fact that some of their pollinator insects breed in the same type of substrate (Gibernau *et al.* 2004) and are attracted by the same compounds. Furthermore, co-inertia analysis revealed that some compounds were correlated with some insects in particular, some of which occur in more than 80% of the chromatograms of one of the taxa, and thus are likely to be under positive selection. Such compounds are good candidates for the specificity of the different signals: indole in *A. maculatum* may be an efficient attractant of *Psychoda phalaenoides*, but some other compounds produced by *A. italicum* may also attract this species. In *A. italicum*, β-caryophyllene appears to be a major attractant for Chironomidae, whereas β-citronellene, tetrahydromyrcene and 2,6-dimethyl-3-octene seem to be the main attractants for *Psycha grisescens* and the other insect categories. This correlative approach is anyway not sufficient, as 2-heptanone and p-cresol, not highlighted by the analysis, are known to be attractive at least for *Psychoda phalaenoides* (Kite *et al.* 1998).

Interestingly, the hybrid floral odour was more similar to that of *A. italicum* than to that of *A. maculatum* in both quantity and diversity, but the hybrids attracted a higher percentage of *P. phalaenoides* (the favoured pollinator for *A. maculatum*), especially in 2010. Careful examination of odour changes in hybrids between 2009 and 2010, revealed no major changes except (in 2010) the production of limonene, also present in the floral odour of *A. maculatum* and *A. italicum* (Table 3). In fact, this increase in attractiveness of the hybrids towards *P. phalaenoides* in 2010 is probably owing to the slight increase in the proportions of all the floral odour compounds correlated to the number of *P. phalaenoides*: α-humulene, indole, α-selimene, alloaromadendrene, and two mixed sesquiterpenes (Table 3, Fig. 8). This suggests that the relative abundances of compounds in the odour may produce a quantitative effect on insect attraction. From a morphological point of view, the potential hybrids were not different from *A. italicum*: both produced larger inflorescences with more female and male flowers than *A. maculatum*. The only noticeable morphological difference was the colour of the stamens, and of the appendix stipe. Intermediate coloration of hybrids has already been observed in *Caladenia* (Orchidaceae)

(Salzmann *et al.* 2006) and *Mimulus* (Scrophulariaceae) (Schemske and Bradshaw 1999) where a change in coloration was linked with pollinator preferences. This is not be the case in *Arum*: whereas most potential hybrids have a yellow appendix as in *A. italicum*, and attract a diversity of pollinators more similar to *A. italicum*, the visual cue has been shown to be mainly due to the spathe, and this largely minor in the attraction compared with olfactory cues (Lack and Diaz 1991). Thus, the similitude of pollinator attraction between *A. italicum* and the potential hybrids is more likely owing to a similitude in floral odour.

This leads to the hypothesis that the production of indole, alloaromadendrene and decanal, three compounds exclusively produced by *A. maculatum* and the potential hybrids, is sufficient to increase the attraction of *Psychoda phalaenoides*. In the same way, *A. maculatum* lacks the production of dihydromyrcene and 2,6-dimethyl-3-octene, which may cause the attraction of the pollinators of *A. italicum* and potential hybrids. It has already been shown that few compounds can suppress the reproductive ethological isolation between two closely relative species of *Silene* (Caryophyllaceae) (Waelti *et al.* 2008).

In conclusion, the attractive odour of *A. italicum* and *A. maculatum* may be composed of a mix of compounds mimicking various dung, sometimes common to the two species, and of compounds selected to be constant as they are linked to the attraction of more-or-less specific pollinators. In *A. maculatum*, the specialized species, the odour fits to mimic the oviposition site (cow dung) of its major pollinator (*Psychoda phalaenoides*) whereas in *A. italicum*, an opportunist/generalist species, the odour seems more variable and/or diverse and thus attracting a wider range of Diptera. Theses qualitative and quantitative differences of attractiveness between the two species seem sufficient to prevent the exclusion of *A. maculatum* through competition with the more numerous *A. italicum*. Further studies on insect preferences and olfactory receptors are needed to better understand which compounds of the floral odours of Arum may be under selection and whether such selection is directional or balanced. These compounds also lead to a difference of attractiveness between the two species, *A. maculatum* being far more attractive to its specific pollinator, *P. phalaenoides*.

In addition, floral traits of natural hybrids are here shown to potentially help understanding the ecology of their parental species, at least concerning the pollinator-attractive features of the inflorescences. They may also be helpful in future studies on the genetic basis of the odour compound blend composition variations.

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8.8 FIGURE LEGENDS

Figure 1. A. *Arum maculatum* in its habit. Insects were collected from *A. italicum* (B.) and *A. maculatum* (C.) by pouring ethanol in the floral chamber. D. Inflorescence of *Arum italicum*. E. Inflorescence of the potential hybrids. F. Inflorescence of *A. maculatum*. Spathes have been cut to show the floral parts: 1. appendix, 2. male flowers, 3. female flowers. Note that pictures scales are different between D.-F.. Please refer to Table 1 for accurate floral parts measures.

Figure 2. Phenology of *A. italicum*, *A. maculatum* and their potential hybrids in Bagnères-de-Bigorre (Hautes-Pyrenees, France). Records lasted from 1st Apr. to 29 May 2010.

Figure 3. A. Diversity of the inflorescence visitors of *Arum italicum*, *A. maculatum* and their potential hybrids in Bagnères-de-Bigorre in 2009 and 2010 (mean percentages ± standard error). B. Specific composition of the psychodids caught, estimated on 8 insects per inflorescence (when enough insects caught). n = number of sampled inflorescences. n.s. = groups non-significantly different (npMANOVAs).

Figure 4. Pollinator capture dynamic of *Arum italicum* and *A. maculatum* recorded on 3 inflorescences of each species between 1800 h and 1000 h in the next morning.

Figure 5. Box plot of the log transformed mean number of insects trapped in "receptor" inflorescence of *Arum italicum* and *A. maculatum* in the cage experiment. Receptor=inflorescences placed in the cage, Source=inflorescences from which the insects released in the cage were collected, t = *A. italicum*, m = *A. maculatum*. Pies indicate the estimated proportions of insects trapped in the inflorescences (see legend). Capital letters indicate groups non-significantly different in insect diversity. Lower case letters indicates groups non-significantly different in insect quantity.

Figure 6. Box plot of the log transformed total area per chromatogram for inflorescences of *Arum italicum* ($N = 48$), *A. maculatum* ($N = 34$) and their potential hybrids ($N = 9$) in 2009 and 2010.

Figure 7. Non Metric Multi-Dimensional Scaling representation of the odours of *Arum italicum* (empty symbols), *A. maculatum* (black symbols) and their potential hybrids (crossed symbols) in Bagnères-de-Bigorre in 2009 and 2010. Letters indicate groups when post-hoc test values were significant.

Figure 8. Co-inertia canonical weights highlighting the correlation between trapped insects and emitted VOCs in the odour of *A. italicum*, *A. maculatum* and their potential hybrids. Compounds in bold occur in more than 80% of the blends from one taxa.

Table 1. Floral part lengths and flower numbers (mean ± standard error) for *A. italicum*, *A. maculatum* and their potential hybrids in Bagnères-de-Bigorre (Hautes-Pyrenees, France). Statistics and p-values are given for ANOVAs and Kruskall-Wallis overall tests. N=number of inflorescences sampled. *=Post-hoc test p-value < 0.05, ** < 0.01 and *** < 0.001.

Table 2. Percentages of fructification for hand-pollinated inflorescences of *Arum italicum*, *A. maculatum* and their potential hybrids in Bagnères-de-Bigorre (Hautes-Pyrenees, France). Controls consisted of non-manipulated infructescences. Crossings are coded as follows: pollen donor x pollen receiver.

Table 3. Mean relative amounts of VOCs produced by *Arum italicum*, *A. maculatum* and their potential hybrids in Bagnères-de-Bigorre in 2009 and 2010. RI = retention index, RT = retention time, N = number of sampled inflorescences, SE = standard error, CV = coefficient of variance (standard deviation divided by the mean), O = number of occurrences of the molecule in the sample.

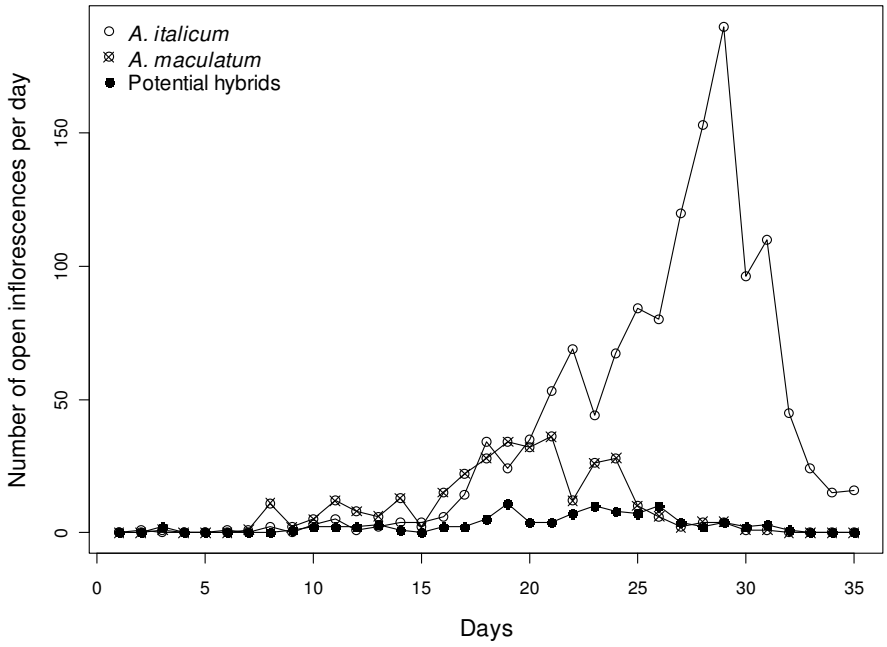


Figure 1
Figure 2

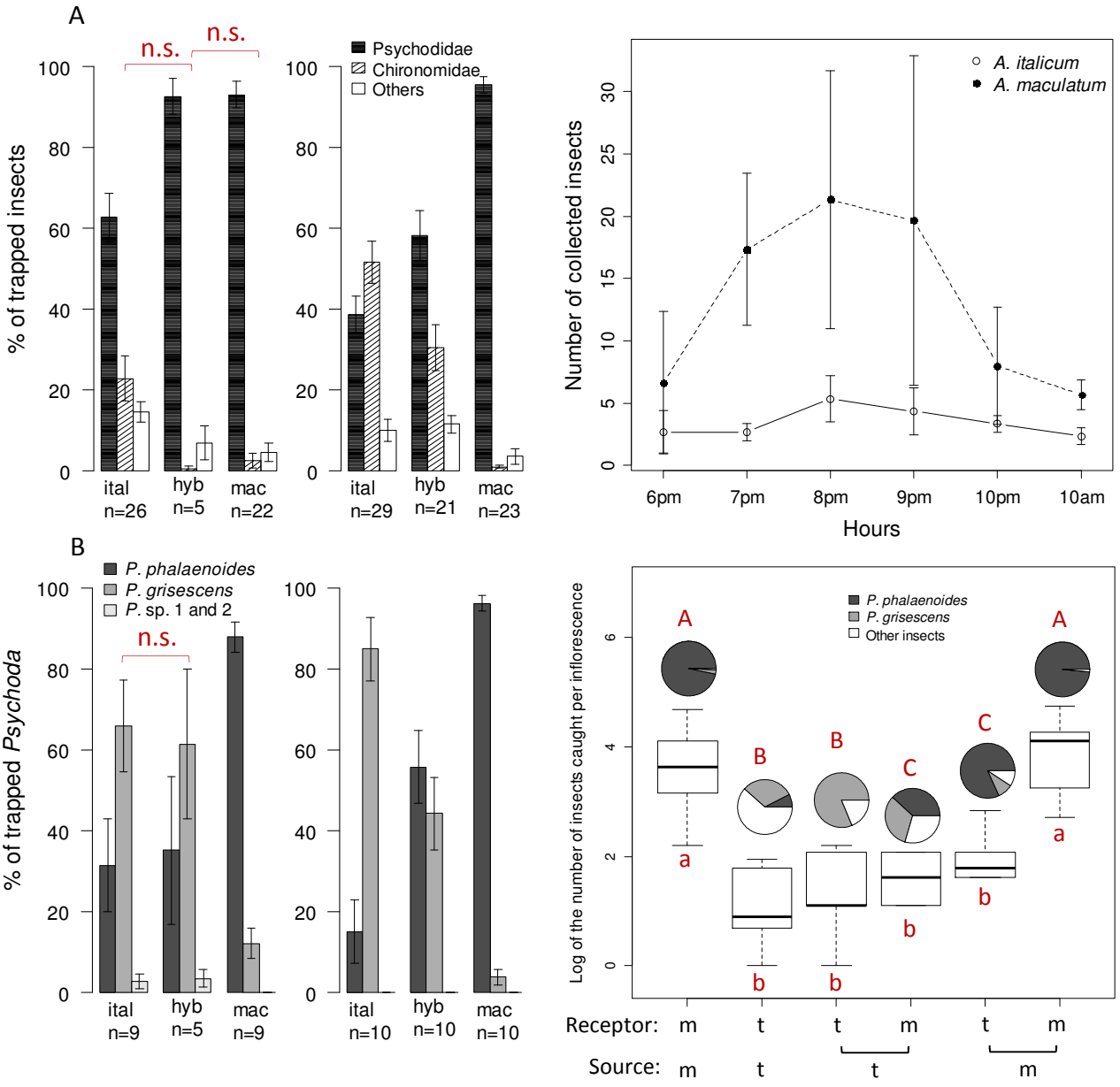
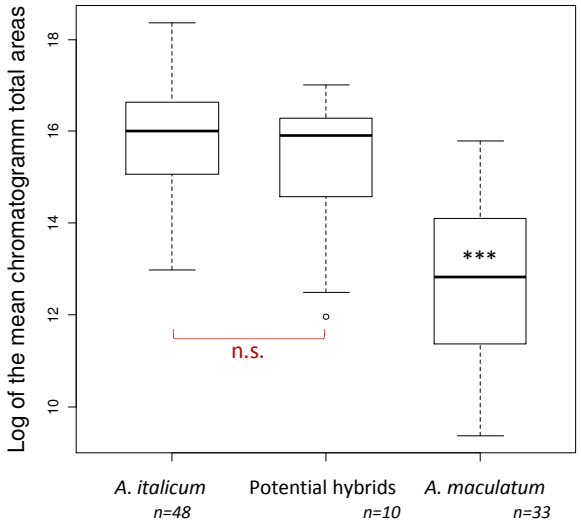


Figure 3A Figure 4
Figure 3B Figure 5



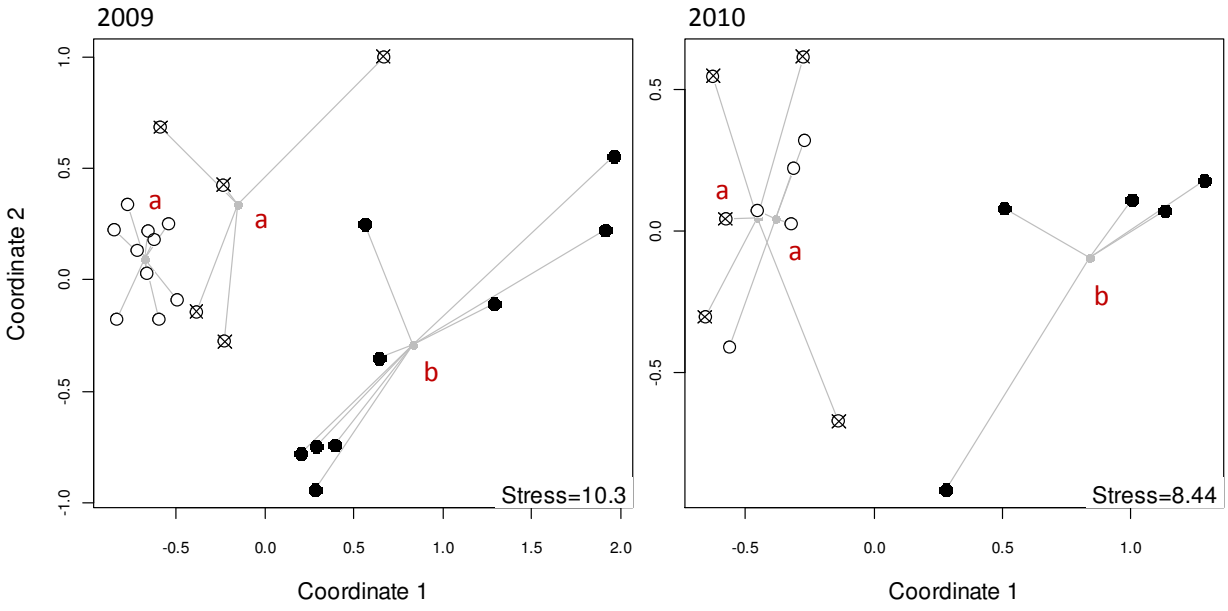


Figure 6

Figure 7

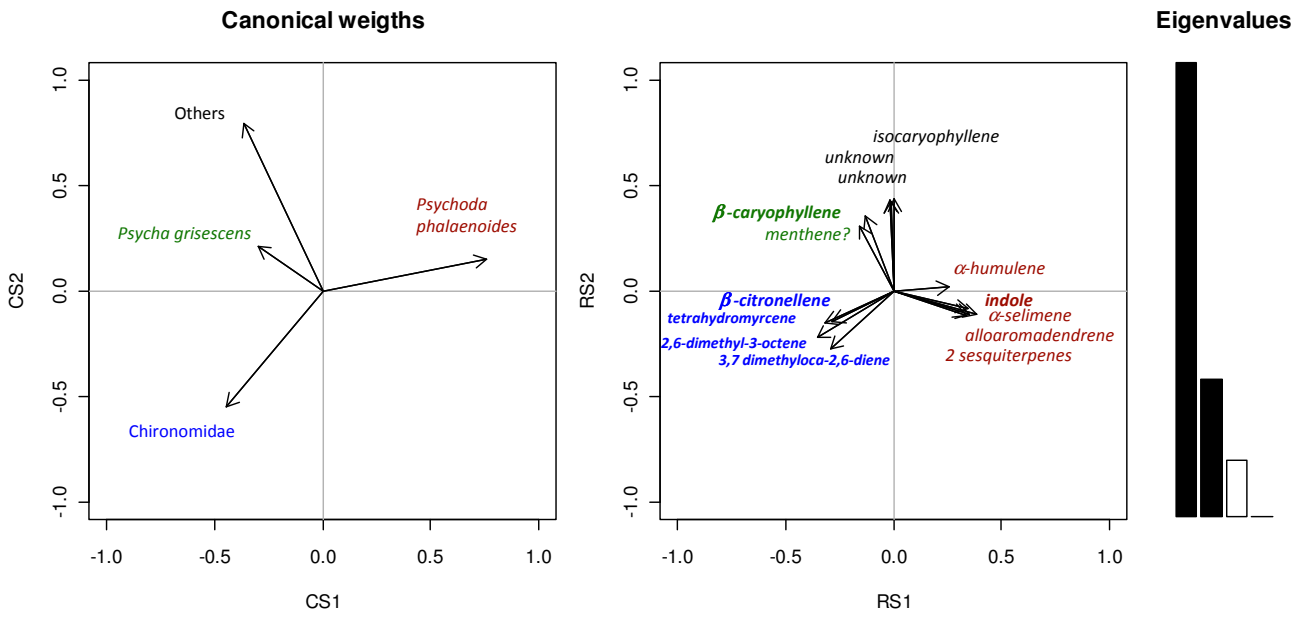

Canonical weigths
Eigenvalues
Others
Psycha grisescens
Psychoda
phalaenoides
Chironomidae
CS1
CS2
isocaryophyllene
unknown
unknown
β-caryophyllene
menthene?
α-humulene
β-citronellene
tetrahydromyrcene
2,6-dimethyl-3-octene
3,7 dimethyloca-2,6-diene
indole
α-selimene
alloaromadendrene
2 sesquiterpenes
RS1
RS2

Figure 8

Taxa	*Arum italicum* N=15	Potential hybrids N=16	*Arum maculatum* N=16	Statistic	P-value
Spadix length (mm)	93.1 ± 4.9	96.9 ± 3.6	70.7 ± 3.3 **	$F_{2.44}$=13.14	$3.35.10^{-5}$
Appendix length (mm)	38.9 ± 2.8	38.8 ± 2.4	27.1 ± 109 *	$F_{2.44}$=7.82	0.001
Male zone length (mm)	7.3 ± 0.3	6.9 ± 0.2	6 ± 0.2 *	$F_{2.44}$=8.67	$6.69.10^{-4}$
Female zone length	13.3 ± 0.6	12.1 ± 0.7	8.4 ± 0.5***	$F_{2.44}$=19.10	$1.07.10^{-6}$
Male flower number	159.3 ± 9.2	155.6 ± 7.9	103.2 ± 6.1 *	X^2_2=21.87	$1.79.10^{-5}$
Female flower number	56.7 ± 2.9	48.1 ± 2.8	32.7 ± 2.8 **	$F_{2.44}$=17.49	$2.58.10^{-6}$

Table 1

	Classes of % of fructification			Mean % of fructification
	[0-20]	]50-80]	>80	
Control *A. maculatum*	0	0	11	99.5
Control *A. italicum*	3	1	11	76.8
Control hybrids	1	1	10	87.1
A. maculatum x *A. maculatum*	0	0	3	100
A. italicum x *A. italicum*	1	0	6	84.4
A. maculatum x *A. italicum*	1	0	5	82.8
A. italicum x *A. maculatum*	0	0	11	99.7
A. italicum x hybrids	1	0	2	66.7
Hybrids x *A. italicum*	0	0	2	100

Table 2

			Arum italicum						Potential hybrids						*Arum maculatum*					
			2009 (n=10)			2010 (n=5)			2009 (n=4)			2010 (n=5)			2009 (n=10)			2010 (n=5)		
COVs	RI	RT	Mean ± s.e.	CV	O	Mean ± s.e.	CV	O	Mean ± s.e.	CV	O	Mean ± s.e.	CV	O	Mean ± s.e.	CV	O	Mean ± s.e.	CV	O
BENZENOIDS																				
acetophenone	959	8.15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.03 ± 0.46	1	3
p-cresol	1083	11.79	**7.53 ± 3.02**	**1.27**	**7**	**13.73 ± 1.85**	**0.3**	**5**	**5.86 ± 4.71**	**1.61**	**2**	**11.63 ± 3.79**	**0.73**	**5**	1.12 ± 0.78	2.21	3	**6.88 ± 4.21**	**1.37**	**3**
N-CONTAINING COMPOUNDS																				
indole	1298	17.97	-	-	-	-	-	-	2.8 ± 1.78	1.27	2	3.94 ± 2.84	1.61	3	**26.88 ± 7.61**	**0.9**	**7**	**20.85 ± 11.15**	**1.2**	**5**
MONOTERPENOIDS																				
Tetrahydromyrcene	908	6.71	**17.61 ± 2.61**	**0.47**	**10**	4.28 ± 0.61	0.32	5	**8.4 ± 3.11**	**0.74**	**3**	**9.21 ± 3.33**	**0.81**	**4**	3.10 ± 2.41	2.46	4	-	-	-
α-citronellene	928	7.28	3.48 ± 0.44	0.4	10	2.26 ± 0.5	0.5	5	2.8 ± 1.31	0.94	3	3.34 ± 1.1	0.74	4	-	-	-	-	-	-
α-pinene	933	7.42	-	-	-	-	-	-	-	-	-	-	-	-	**11.43 ± 5.05**	**1.4**	**5**	2.40 ± 0.73	0.68	4
β-citronellene	941	7.66	**36.87 ± 2.76**	**0.24**	**10**	**18.38 ± 2.04**	**0.25**	**5**	**20 ± 10.11**	**1.01**	**4**	**22.75 ± 4.36**	**0.43**	**5**	**8.03 ± 4.45**	**1.75**	**7**	**4.80 ± 0.82**	**0.38**	**5**
2,6-dimethyl-3-octene	965	8.31	**9.01 ± 1.78**	**0.62**	**10**	3.07 ± 0.95	0.69	4	**7.05 ± 3.77**	**1.07**	**3**	**6.36 ± 2.40**	**0.84**	**4**	-	-	-	-	-	-
β-pinene	974	8.57	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.30 ± 0.61	0.6	4
myrcene	987	8.94	-	-	-	-	-	-	-	-	-	-	-	-	0.50 ± 0.38	2.41	2	1.51 ± 0.11	0.17	5
2,6-dimethylocta-2,6-diene	995	9.15	-	-	-	1.16 ± 0.53	1.03	3	-	-	-	2.39 ± 1.08	1.01	3	-	-	-	-	-	-
3,7-dimethylocta-2,6-diene	997	9.22	**5.93 ± 1.6**	**0.85**	**9**	-	-	-	2.37 ± 0.94	0.8	3	-	-	-	1.37 ± 1.17	2.7	2	-	-	-
menthene?	1021	9.94	0.31 ± 0.21	2.14	2	-	-	-	-	-	-	0.7 ± 0.45	1.45	2	-	-	-	-	-	-
limonene	1027	10.12	-	-	-	**9.29 ± 2.24**	**0.54**	**5**	**-**	**-**	**-**	**6.66 ± 1.67**	**0.56**	**5**	**6.86 ± 3.28**	**1.51**	**5**	**41.99 ± 11.16**	**0.59**	**4**
dihydromyrcenol	1074	11.52	1.62 ± 0.78	1.53	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
linalool	1099	12.27	-	-	-	1.38 ± 0.39	0.63	4	-	-	-	-	-	-	-	-	-	4.62 ± 1.18	0.57	4
SESQUITERPENOIDS																				
dihydrosesquiterpene	1395	20.56	4.63 ± 1.84	1.26	9	**7.98 ± 1.6**	**0.45**	**5**	**5.76 ± 2.02**	**0.7**	**3**	**13.46 ± 1.03**	**0.17**	**5**	**5.30 ± 1.27**	**0.76**	**8**	3.25 ± 1.02	0.7	4
isocaryophyllene	1404	20.77	1.43 ± 0.54	1.19	6	4.76 ± 1.23	0.58	5	3.49 ± 1.4	0.8	4	0.97 ± 0.41	0.95	3	0.55 ± 0.39	2.25	2	1.06 ± 0.80	1.68	2
β-caryophyllene	1420	21.18	**8.85 ± 2.46**	**0.88**	**9**	**27.14 ± 3.6**	**0.3**	**5**	**12.8 ± 5.54**	**0.87**	**4**	**7.35 ± 3.36**	**1.02**	**4**	2.44 ± 0.96	1.25	5	-	-	-
α-humulene	1456	22.08	0.64 ± 0.27	1.31	4	2.62 ± 0.79	0.68	4	0.88 ± 0.52	1.17	2	2.05 ± 1.00	1.09	3	2.36 ± 0.95	1.28	5	-	-	-
alloaromadendrene	1461	22.19	-	-	-	-	-	-	0.7 ± 0.42	1.19	2	1.36 ± 0.64	1.05	3	2.01 ± 0.82	1.29	5	-	-	-
2 sesquiterpenes	1476	22.56	-	-	-	-	-	-	-	-	-	1.53 ± 0.94	1.37	2	1.80 ± 0.72	1.27	5	-	-	-
α-selinene	1497	23.09	-	-	-	-	-	-	-	-	-	0.67 ± 0.41	1.37	2	1.94 ± 0.72	1.17	5	-	-	-
δ-cadinene	1517	23.58	-	-	-	0.87 ± 0.36	0.93	3	1.39 ± 0.51	0.73	3	1.84 ± 0.67	0.81	4	**5.95 ± 3.25**	**1.73**	**6**	-	-	-
FATTY ACID DERIVATIVE																				
2-heptanone	893	6.33	1.84 ± 0.8	1.37	6	-	-	-	1.68 ± 1.35	1.61	2	-	-	-	**5.26 ± 3.76**	**2.26**	**7**	-	-	-
decanal	1203	15.36	-	-	-	-	-	-	2.29 ± 1.73	1.51	2	-	-	-	**9.17 ± 3.57**	**1.23**	**6**	1.94 ± 0.50	0.57	4
UNIDENTIFIED COMPOUNDS																				
unknown	1330	18.82	-	-	-	-	-	-	2.85 ± 7.86	1.3	2	-	-	-	-	-	-	-	-	-
unknown	1401	20.71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.02 ± 0.43	0.95	3
unknown	1486	22.82	-	-	-	-	-	-	**10.6 ± 6.64**	**1.25**	**2**	-	-	-	-	-	-	-	-	-

Table 3

Quatrième partie

CONCLUSION GÉNÉRALE ET PERSPECTIVES

La majorité des espèces animales, fongiques ou végétales évoluent en interaction les unes avec les autres dans des écosystèmes plus ou moins interconnectés. Ces interactions, combinées aux variations des paramètres abiotiques des milieux dans lesquelles les espèces sont réparties, jouent un rôle important dans l'évolution et dans la différenciation des espèces (Thompson 2005).

Nous avons étudié ici le cas très répandu chez les plantes à fleurs des interactions de pollinisation entomophile. Les insectes, ainsi que certains mammifères et oiseaux, ont certainement joué un rôle majeur dans la formation de nouvelles espèces de plantes, par la spécialisation de certains groupes de plantes à certains groupes de pollinisateurs entrainant des divergences entre des populations par isolement floral. Cet isolement peut mener à la spéciation lorsqu'il devient trop important, et s'il est conjugué à d'autres facteurs, par exemple géographiques (révisé par Kay et Sargent 2009).

Chez les Aracées, la plupart des espèces renseignées en termes de pollinisation sont spécialisées au moins à un ordre d'insectes (Gibernau *et al.* 2010). Comme nous l'avons vu au cours de cette étude, une grande partie des traits floraux de ces espèces ont un rôle important lié à la pollinisation : la possession d'une spathe formant des chambres florales plus ou moins refermées autour du spadice, les cycles de floraison longs ou courts selon le type d'attraction des pollinisateurs, la formation d'appendices variés permettant l'émission des odeurs attractives ou encore la thermogenèse des inflorescences. L'étude du CHAPITRE 2, par des analyses corrélatives, a montré dans un premier temps qu'il était possible de classer les Aracées selon un certain nombre de traits floraux dans des groupes correspondant aux différents ordres auxquels appartenaient leurs pollinisateurs. Ainsi, il existe bien des syndromes de pollinisation chez les Aracées. Par exemples, un nombre important de fleurs, ou une taille élevée des grains de pollen, se sont avérés significativement corrélés respectivement à la pollinisation par les mouches et par les coléoptères. Ce résultat est intéressant car il permet d'assigner un pollinisateur potentiel à des genres non renseignés. Ainsi, il est probable que les écologues montrent dans le futur que *Scindapsus hederaceus* est pollinisé par des abeilles, ou qu'*Ulearum sagittatum* est pollinisé par des mouches, comme prédit par l'analyse multivariée du CHAPITRE 2. Une telle étude corrélative était nécessaire dans un premier temps, mais incomplète du point de vue évolutif : en effet, elle ne permet pas de prendre en compte les liens de parenté entre les espèces, et donc le fait que deux espèces proches, qu'elles soient pollinisées par le même pollinisateur ou pas, ont une probabilité plus élevée de partager des traits morphologiques que deux espèces phylogénétiquement éloignées (Felsenstein 1985). Pour pallier à cela, l'étude du CHAPITRE 4 a permis d'inclure une inférence phylogénétique aux tests de corrélations, ce qui a confirmé

que certains traits, comme par exemple la relation entre le nombre de fleurs, ou leur sexualité, avaient évolué en corrélation avec certaines modalités de pollinisation. L'apport des données phylogénétiques a également permis de retracer l'histoire de la pollinisation chez les Aracées, et donc, en plus de les décrire, de comprendre comment ces modalités de pollinisation ont évolué les unes par rapport aux autres, et quels traits floraux leurs étaient associés. Ainsi, chez les Aracées, des systèmes de pollinisation spécialisés à des abeilles, des coléoptères ou des mouches seraient apparus à partir d'un ancêtre commun généraliste du point de vue de la pollinisation, en corrélation avec des modifications successives des inflorescences ayant parfois même évolué vers des interactions de duperie fortement spécialisée et nécessitant la possession d'un piège floral.

Une telle étude au niveau de la famille a été possible parce qu'elle intégrait des traits partagés par un grand nombre d'espèces, et ne variant pas trop au sein des genres (l'unité taxonomique opérationnelle de l'étude). Pour aller plus loin, il serait maintenant intéressant de descendre à un niveau taxonomique inférieur, comme la sous-famille ou le genre, et/ou d'étudier plus en détail les changements intervenant entre des espèces proches pollinisées par différents ordres d'insectes. Ceci permettrait de prendre en compte des traits morphologiques différents et de comprendre plus finement les pressions de sélection pesant sur l'évolution des traits préalablement étudiés. Certains traits floraux quantitatifs se sont avérés, dans l'étude du CHAPITRE 4, évoluer en corrélation avec des traits qualitatifs eux même liés à la pollinisation, mais pas directement avec le mode de pollinisation lors de l'étude faite au niveau de la famille. Par exemple, la quantité de grains de pollen produites par la plante peut être une mesure de l'efficacité de l'exportation de pollen de certains pollinisateurs, mais dans un système ou le pollen sert de récompense au pollinisateur, un pollinisateur très efficace en terme d'exportation pourrait être associé à une production importante de pollen par la plante s'il en consomme un certain pourcentage, et au contraire à une quantité faible s'il est récompensé autrement ou dupé. De plus, l'interaction de pollinisation n'est pas le seul facteur façonnant les traits floraux. Des contraintes liées aux prédateurs (florivores), à l'ontogénie, aux interactions avec d'autres partenaires (fourmis) ou à la répartition des ressources, peuvent engendrer des réponses de différentes amplitudes au niveau des variations de ces traits (Galen 1999). Par exemple, si le nombre de gamètes produits peut varier selon le comportement du pollinisateur, il existe chez certaines Aracées des compromis dans la production de pollen et d'ovules par les inflorescences selon les réserves dont la plante dispose (e.g. Chartier et Gibernau 2009) pouvant aussi jouer sur ce nombre. La solution pour mettre en évidence ce phénomène est de travailler à plus faible échelle taxonomique, dans des unités partageant déjà la même organisation d'inflorescence.

Un exemple intéressant serait celui des sous-familles des Monsteroideae et Pothoideae. Les systèmes de pollinisation de ces sous-familles proches n'ont principalement été étudiés qu'en Amérique du Sud. Ainsi, certaines espèces sont pollinisées par des abeilles mélipones attirées par une récompense alimentaire (pollen) ou par des abeilles euglossines, qui récoltent des cires odorantes leur permettant de fabriquer un parfum pour attirer les femelles et peut-être repousser les autres mâles (Hentrich *et al.* 2007, 2010), mais certains genres sont également pollinisés par des mouches ou des coléoptères (Gibernau *et al.* 2003, 2011, Chouteau *et al.* 2007). Produire une quantité plus importante de pollen ou des molécules olfactives spécialisées doit engendrer des compromis morphologiques/biochimiques différents engendrant des corrélations entre traits floraux et pollinisation qu'il serait très intéressant d'étudier à ce niveau taxonomique. Par ailleurs, les espèces de ces sous-familles sont réparties en Asie tropicale, Afrique et Amérique du Sud, et certains genres sur deux continents (*Spathiphyllum* en Asie et Amérique du Sud, *Raphidophora* et en Asie et Afrique et *Pothos* en Asie et à Madagascar). Il a été mentionné par exemple que le genre *Rhaphidophora* est pollinisé par des scarabées en Afrique et par des abeilles en Asie (Gibernau 2011). Il serait donc très intéressant de comparer l'évolution des systèmes de pollinisation de ces espèces, pour voir si certaines d'entre elles ont évolué de la même manière dans les différents continents au contact de pollinisateurs appartenant aux mêmes groupes fonctionnels, ou si elles se sont diversifiées différemment au contact de pollinisateurs appartenant à des groupes fonctionnels différents, et à quel point cette diversification a été contrainte par l'inertie phylogénétique (i.e. l'état ancestral des inflorescences).

Au sein de la sous-famille des Monsteroideae, il a été observé que certaines espèces du genre *Monstera* étaient pollinisées par des abeilles (Gibernau *et al.* 2003) mais une espèce, *Monstera obliqua*, est pollinisée par des scarabées de la famille des Nitidulideae (Chouteau *et al.* 2007). De la même manière, certaines espèces ont été classées dans le CHAPITRE 2 à la fois dans le groupe des espèces pollinisées par des mouches ou des scarabées, représentant des systèmes de pollinisation « mixtes », car elles n'ont pas de traits floraux bien adaptés en un ensemble différencié. Ces espèces sont intéressantes car elles pourraient être actuellement en évolution dynamique, à la transition entre la pollinisation par un type ou un autre d'insectes. Dans ce cas, une étude des variations géographiques des interactions accompagnée de manipulations de la fréquence relative des pollinisateurs ou de tests de transplantations suivis de mesures du succès reproducteur (taux de fructification) permettrait de comprendre comment sont exercées les pressions de sélections locales et de quelle manière les espèces sont susceptibles de changer de pollinisateur et de diverger dans certaines populations.

Un type de travail similaire a été commencé sur le genre européen *Arum* (PARTIE 3 de la thèse), par la comparaison de deux espèces du même genre dont les modalités de pollinisation sont maintenant bien connues. En effet, si les études phylogénétiques à grande échelle taxonomique permettent de comprendre les grands schémas d'évolution des interactions plantes-pollinisateurs et leur impact sur la diversification des espèces, les mécanismes précis de changement de pollinisateurs sont peu connus (Kay et Sargent 2009). Les tests de transplantations entre différentes populations d'*Arum* effectués au cours de cette étude constituent un premier moyen efficace de mettre en évidence l'adaptation locale des plantes et les sites dans lesquels les pressions de sélection sont différentes, menant potentiellement à la divergence de certaines populations. Ainsi le CHAPITRE 7 a permis de montrer que les causes des variations locales des pollinisateurs entre les populations étaient variables d'une espèce d'*Arum* à l'autre. Dans le cas d'*Arum italicum*, ces variations sont dues, non pas à l'adaptation locale de la plante, mais aux variations géographiques des pollinisateurs, et l'odeur des inflorescences, peu différenciée entre les populations, a la même attractivité dans tous les sites étudiés. Au contraire, chez *A. maculatum*, une adaptation de l'odeur à l'un ou l'autre de ses deux pollinisateurs selon la population étudiée est fortement soupçonnée, et pourra être démontrée par des tests de transplantations supplémentaires ou des tests de choix sur les insectes. Enfin, l'étude des sites de sympatrie dans lesquels les deux espèces sont en compétition pour le(s) même(s) insecte(s) pollinisateur(s) ont permis de mettre en évidence une influence sur l'interaction de la structuration de la communauté dans laquelle les populations évoluent. La prochaine étape de l'étude des variations géographiques des interactions *Arum*-pollinisateurs sera de mettre en relation les variations morphologiques et physiologiques jusque là étudiées avec les variations génétiques des populations. Ce genre d'étude permettra dans le futur de comprendre la base génétique des variations florales induites par les pressions de sélections des pollinisateurs. Dans ce but, une grande quantité de matériel végétal a déjà été récolté dans toutes les populations d'*Arum* étudiées au cours de cette thèse.

En conclusion, les études multi-échelles de type entonnoir, allant de l'évolution à long terme des interactions au sein d'une famille jusqu'à l'étude fine des variations géographiques dans les différentes populations d'une espèce, permettent, à leur terme, de comprendre les étapes évolutives successives ayant mené à la diversification des espèces de plantes. La famille des Aracées, par ses nombreuses adaptions florales et sa diversité de modalités de pollinisation, constitue un modèle particulièrement recommandable dans le futur pour ce genre d'études. Certains systèmes de pollinisation sont maintenant bien renseignés, comme chez le genre *Arum* en Europe, *Colocasia* en Asie, ou *Philodendron* en Amérique du Sud, et les Aracées constituent

le sujet d'étude d'une importante communauté de chercheurs dynamique et répartie dans le monde entier (Aroid.com).

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