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L'écologie évolutive des interactions symbiotiques vue à travers les plantes à fourmis

Mémoire d'Habilitation à Diriger des Recherches

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Synthèse des recherches et des encadrements d'étudiants

Note : Les références à mes articles sont précédées d'un « A » et sont visibles dans le CV (liste complète des publications). Les autres références sont visibles dans la section Bibliographie.

1. Cadre général

Après une formation en biologie du comportement, je me suis progressivement dirigé vers l'étude des interactions durables entre espèces, et en particulier la coévolution entre les fourmis dites « esclavagistes » et leurs hôtes. Ces fourmis prélèvent des nymphes dans des nids d'autres espèces de fourmis lors de raids afin d'augmenter la force de travail de leur propre colonie, constituant un cas de parasitisme social. J'ai travaillé sur les aspects comportementaux de ces interactions particulières, tout d'abord lors de mon stage post-doctoral en 2001 et 2002, puis au cours des premières années qui ont suivi mon recrutement comme chargé de recherche au CNRS, de 2003 à 2005, dans le Laboratoire d'Ethologie Expérimentale et Comparée. Ma carrière a pris un virage thématique, conceptuel et méthodologique en 2006 lorsque j'ai intégré le Centre d'Ecologie Fonctionnelle et Evolutive (CEFE) dans le but d'aborder les dimensions écologiques et évolutives des interactions entre espèces. J'ai alors démarré un nouveau projet de recherche ayant comme modèle les interactions symbiotiques entre plantes et fourmis. Ce projet fait l'objet de la présente synthèse. Je n'aborderai pas mes travaux sur le parasitisme social, les considérant trop éloignés de mes activités actuelles, aussi bien conceptuellement que temporellement. Toutefois, veuillez noter qu'au cours de ces travaux (les trois premières années en tant que chargé de recherche) j'ai encadré trois étudiants de Master, dont deux avec lesquels j'ai cosigné des publications (publications A13 et A30 de la liste complète). Durant cette période précédant mon arrivée au CEFE j'ai aussi cosigné des publications avec trois étudiants en thèse dans l'encadrement desquels j'ai été impliqué (publications A14, A17 et A30).

Mes activités de recherche se situent dans le cadre général des interactions biotiques et de la coévolution. L'intégration des interactions mutualistes bi-partenaires comme des maillons élémentaires dans un réseau complexe d'interactions apparait de plus en plus comme une clé pour comprendre l'organisation des communautés (Vazquez et al., 2009). J'aborde donc la dynamique des interactions biotiques mutualistes en intégrant différents niveaux d'organisation et en explorant différents aspects complémentaires. En disséquant le fonctionnement de ces interactions, mes recherches contribuent aux grands thèmes actuels de l'écologie évolutive : dynamique de la biodiversité, structuration des écosystèmes (à travers les relations fonctionnelles entre espèces), évolution des communautés, réponse des écosystèmes aux changements globaux. J'aborde ces grands thèmes à travers quatre axes majeurs détaillés ci-dessous.

2. Axe 1. Evolution des stratégies alimentaires dans les interactions plantes-fourmis

Je définis la symbiose comme une association entre individus d'espèces différentes qui passent ensemble une partie importante de leur cycle de vie. Cette définition présente une part de subjectivité et est sujette à débat. Elle inclue des interactions de natures aussi diverses que le parasitisme, le commensalisme et le mutualisme. Les symbioses ont longtemps été perçues comme des interactions entre deux partenaires, mais il devient évident que les interactions symbiotiques bipartites sont encastrées dans des réseaux symbiotiques plus vastes et que la compréhension de l'écologie, de l'évolution et du rôle de la symbiose dépend de la compréhension de ces réseaux. Les recherches sur les plantes à fourmis illustrent bien cette évolution conceptuelle. Les plantes à fourmis, aussi appelées plantes myrmécophytes, présentent des structures creuses (tiges, feuilles, stipules, etc.), appelées domaties, dans lesquelles logent des fourmis (Figure 1). En plus du gîte, certaines de ces plantes produisent des ressources nutritives pour leurs fourmis (corps nourriciers, nectar extra-floral, figure 2). De leur côté, les fourmis protègent la plante des herbivores et la plante obtient des nutriments résultants de leur activité (Rico-Gray and Oliveira, 2007; Rosumek et al., 2009). Certaines espèces de fourmis élèvent dans les domaties des hémiptères suceurs de sève dont elles consomment le miellat. Même si cette troisième catégorie de partenaires est connue depuis longtemps, son effet sur l'interaction entre la plante et la fourmi commence tout juste à être appréhendé (Gaume et al., 1998; Frederickson et al., 2012). Les fourmis à plante, comme la plupart des fourmis arboricoles, ont un régime alimentaire composé essentiellement de substances sucrées pauvres en lipides et en acides aminés, et sont donc confrontées à un déficit d'azote (Davidson et al., 2003). L'importance de bactéries symbiotiques dans l'écologie nutritionnelle des fourmis arboricoles est un sujet d'actualité (Russell et al., 2009; Kautz et al., 2013), et le cas des fourmis à plante commence à être abordé (Eilmus and Heil, 2009; Seipke et al., 2012).

Figure 1 : Domatie de *Tococa guianensis* à la base du limbe (Guyane).

Figure 2 : Ouvrières de *Petalomyrmex phylax* récoltant le nectar foliaire de leur plante à fourmis hôte, *Leonardoxa africana* (Cameroun).

C'est dans ce contexte que j'ai focalisé mon attention sur l'étude de champignons présents dans les domaties des plantes à fourmis. Bien que leur existence ait été décrite il y a longtemps (Miehe, 1911; Bailey, 1920), leur identité et la nature de leur interaction avec la plante et la fourmi n'étaient jusque-là pas connues.

a. Vers une approche intégrée des réseaux d'interactions symbiotiques pour comprendre l'écologie nutritionnelle des associations plantes-fourmis

Les données de la littérature et surtout l'observation d'un grand nombre d'individus de plantes à fourmis m'ont permis de détecter la présence de champignons dans les domaties de plus de 30 genres de plantes [A20, A24]. A chaque fois, ces champignons sont présents avec

Figure 3 : Domatie de *Leonardoxa africana* ouverte pour montrer le patch de champignon (zone noire au centre) présent sur la paroi interne de la domatie (Cameroun).

les fourmis, sous forme d'un tapis noir bien délimité d'hyphes fongiques (Figure 3). J'ai confié l'étude des relations entre le champignon et les autres partenaires à un étudiant de Master 2, Emmanuel Defossez, au cours d'un stage focalisé sur la symbiose entre la plante *Leonardoxa africana* et la fourmi *Petalomyrmex phylax*, incluant un mois de terrain au Cameroun. Le stage était structuré en deux parties, l'une visant à décrire la fréquence de l'association et le comportement des fourmis envers le champignon *in natura* grâce à un endoscope monté sur un caméscope, et l'autre visant à enrichir les fourmis avec des isotopes stables du carbone et de l'azote pour suivre les flux trophiques dans le système. Je connaissais déjà l'étudiant pour ses activités de cinéaste animalier amateur, ce qui m'a incité à lui proposer un sujet techniquement complexe et audacieux. En intégrant ses observations à des données que j'avais obtenues précédemment, il a pu démontrer le caractère symbiotique de l'association entre la fourmi et le champignon. Par exemple, il a mis en évidence que le champignon était systématiquement et uniquement présent avec cette espèce de fourmis. Il a aussi montré l'existence de comportements spécifiques des fourmis envers les champignons dans les domaties. Son expérience de marquage isotopique a mis en évidence un flux trophique de la fourmi vers le champignon, et de la fourmi vers la plante. Les résultats de son stage ont été publiés dans deux articles dont il est premier auteur dans de très bonnes revues (*New Phytologist* [A18] et *Proceedings of the Royal Society B* [A22]).

J'ai par la suite complété cette étude par le marquage isotopique et par colorant du champignon dans trois symbioses plantes-fourmis-champignon, montrant que les fourmis utilisent le champignon comme source de nourriture [A29]. La description des interactions entre les trois partenaires nous a permis de comprendre que les fourmis cultivent un champignon qu'elles utilisent comme source de nourriture et qu'elles transfèrent à la plante une partie des nutriments auxquels elles ont accès. Le suivi des isotopes dans le système pendant quatre ans a montré un recyclage très important de l'azote, mais pas du carbone, ce dernier étant probablement respiré par la plante in fine.

L'écologie nutritionnelle des symbioses plantes-fourmis est complexe (Figure 4) et encore très mal comprise. L'accès aux ressources nutritives est pourtant un facteur fondamental dans la structure des écosystèmes et influence de manière décisive l'évolution des stratégies d'histoire de vie des espèces. Presque toutes les interactions symbiotiques ont évoluées en réponse à des

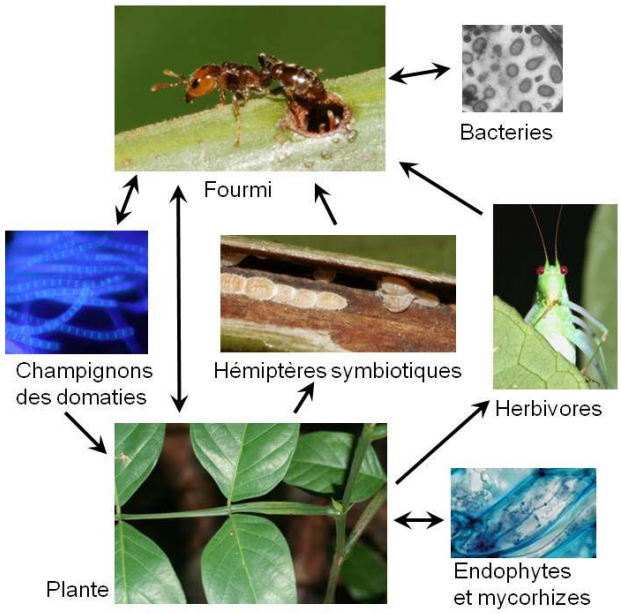

Figure 4 : Flux trophiques démontrés ou attendus entre les partenaires des symbioses plantes-fourmis.

contraintes nutritives. Notre compréhension du fonctionnement des écosystèmes en général, et le pouvoir prédictif des scénarios face aux changements globaux en particulier, ne seront améliorés qu'à travers une meilleure compréhension des flux trophiques dans les réseaux d'interactions symbiotiques. Afin de contribuer à cette compréhension, j'envisage d'étendre progressivement mes investigations dans le domaine des flux trophiques aux organismes les plus susceptibles de jouer un rôle clé dans les symbioses plantes-fourmis : bactéries, nématodes, mycorhizes et endophytes. Ces derniers sont présents dans les tissus des plantes, et sont considérés asymptomatiques. Leurs interactions avec les plantes sont inconnues, mais il a été montré récemment qu'ils pouvaient avoir un rôle dans la nutrition des plantes (Behie et al., 2012). On s'attend par exemple à ce que les communautés d'endophytes et de mycorhizes varient en fonction des autres sources de nutriments disponibles (plantes à fourmis versus plantes libres par exemple). Une approche fonctionnelle intégrée de tous les partenaires symbiotiques me permettra de poser de nouvelles hypothèses sur les modalités d'évolution des stratégies d'approvisionnement chez les plantes.

b. Spécificité des associations

Dans les symbioses plantes-fourmis certaines espèces de plantes et de fourmis sont associées de manière très spécifique. C'est le cas par exemple des plantes endémiques d'Afrique centrale *Leonardoxa africana* et *Barteria fistulosa* qui sont occupées respectivement par les fourmis *Petalomyrmex phylax* et *Tetraponera aethiops*. J'ai choisi ces deux modèles pour tester la spécificité du champignon entre les systèmes et au sein de chacun d'eux par une approche de type code-barres génétique basé sur la séquence des ITS (Internal Transcribed Spacer) de l'ARN ribosomique (Schoch et al., 2012), d'une part parce que l'association entre la fourmi et la plante est très spécifique, et d'autre part parce que mes travaux précédents avaient montré que ces fourmis cultivent les champignons symbiotiques comme source de nourriture, et qu'il s'agissait donc de nouveaux cas d'agriculture par les insectes [A29]. J'ai constitué un échantillonnage conséquent au cours de différentes missions et confié en partie le sujet à deux étudiants successifs. La première, Sarah Debaud, en DUT Génie Biologique (équivalent L2), a eu en charge la mise au point technique de l'approche, en cohérence avec les débouchés de son cursus : optimisation de l'extraction d'ADN et des PCR pour palier à une présence importante d'inhibiteurs dans les champignons étudiés. Elle a aussi contribué de manière importante à la production des données pour la symbiose entre *Leonardoxa* et *Petalomyrmex*. Le second étudiant, Alex Salas-Lopez, en Master 1, a contribué à la production des données pour la symbiose entre *Barteria* et *Tetraponera*. Ces deux systèmes sont phylogénétiquement indépendants (les deux plantes, comme les deux fourmis, sont phylogénétiquement très éloignées), mais le patron de spécificité s'est montré très similaire. Dans chacun des cas, nous avons identifié une espèce de champignon dominante, appelée symbiote primaire, et une ou deux espèces moins fréquentes, appelées symbiotes secondaires. Symbiotes primaires et secondaires sont souvent présents simultanément dans un même patch d'hyphes, mais les espèces sont strictement spécifiques à chaque système et connues nulle part ailleurs. Ce résultat a fait l'objet d'une publication dans *Plos One* [A34] à laquelle les deux étudiants ont été associés. Cette structure dans l'interaction champignon-insecte est similaire à celle qu'on trouve dans d'autres cas de fongiculture, tels que celle pratiquée par certains coléoptères de la famille des Scolytidae (Batra, 1985). Etant donné que les plantes à fourmis représentent de nombreux cas d'évolution indépendante de la symbiose avec différents états de spécificité (Davidson and McKey, 1993), l'étude de la spécificité du champignon sur un large panel de plantes à fourmis devrait permettre de mieux comprendre les modalités d'évolution de l'agriculture chez les insectes.

c. Identité et phylogénie des champignons associés aux symbioses plantes-fourmis

La caractérisation des séquences d'ADN ribosomique des champignons trouvés dans près d'une dizaine de symbioses plantes-fourmis a permis de préciser leur origine taxinomique. Tous ces champignons sont des espèces nouvelles pour la science, appartenant à l'ordre des Chaetothyriales [A24], champignons à croissance très lente, jusqu'alors surtout connu pour leurs représentants extrêmophiles. L'intérieur d'une domatie, avec sa température et son hygrométrie stables et dans des gammes favorables à la croissance de n'importe quel champignon (tels que les *Aspergillus* et *Penicillium*, à croissance très rapide), ne semble pas constituer un milieu extrême et pourtant, seuls les Chaetothyriales spécifiques se développent en présence des fourmis. Une hypothèse à tester est que les fourmis sélectionnent le champignon symbiotique par les composés chimiques qu'elles produisent. Cette hypothèse est vraisemblable car les Chaetothyriales sont connus pour leurs capacités métaboliques hors du commun. Certaines espèces par exemple utilisent les hydrocarbures aromatiques comme source de carbone (Prenafeta-Boldu et al., 2006), tandis que d'autres utilisent la radioactivité comme source d'énergie (Dadachova et Casadevall, 2008) au même titre que les végétaux utilisent la lumière. L'identification des composés impliqués dans la sélection des champignons associés aux fourmis est une piste prometteuse pour comprendre d'évolution de la spécificité dans ces symbioses, tout en ouvrant des débouchés sur la découverte et l'exploitation de nouvelles substances antifongiques.

En collaboration avec des laboratoires étrangers (Sybren De Hoog du CBS-KNAW Fungal Biodiversity Centre aux Pays Bas, Veronika Mayer et Hermann Voglmayr de l'Université de Vienne en Autriche) nous avons récemment initié la réalisation d'une phylogénie des champignons de l'ordre des Chaetothyriales visant, entre autres, à élucider l'origine évolutive des espèces associées aux fourmis. J'ai encadré une étudiante de 5ème année d'école d'ingénieur agronome, Marie Vasse, sur cette thématique. Ses résultats ont montré qu'il n'y avait pas de relation entre la phylogénie et la distribution géographique des espèces associées aux fourmis, et qu'elles forment un nombre réduit de clades monophylétiques, suggérant une radiation de ces espèces faisant suite à un nombre réduit d'évolutions de l'association fourmi-champignon (Figure 5). Cependant, de nouvelles espèces de Chaetothyriales découvertes récemment dans les communautés fongiques épiphylles en milieu

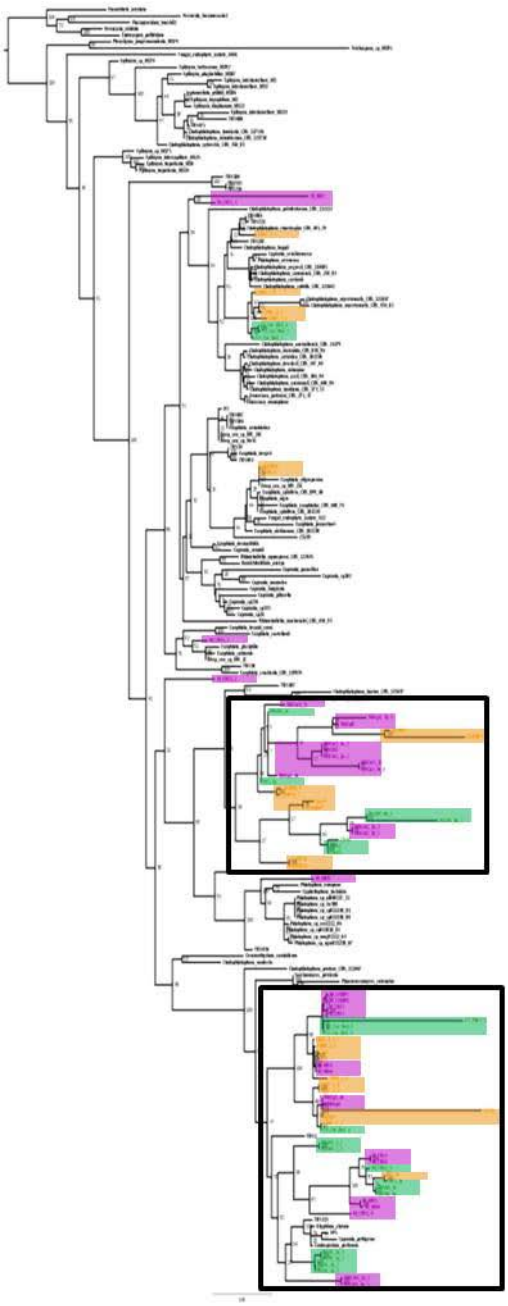

Figure 5 : Phylogramme de 200 souches de Chaetothyriales, dont 80 associées aux fourmis, fondé sur trois marqueurs. Les souches associées aux fourmis sont colorées selon leur origine géographique : rose = Asie, vert = Afrique, orange = Amérique. Les deux grands clades monophylétiques sont encadrés en noir.

tropical se placent dans l'un des clades d'espèces associées aux fourmis. Une meilleure couverture de la diversité des Chaetothyriales sera nécessaire pour s'assurer que l'apparente radiation des espèces associées aux fourmis ne résulte pas d'un manque d'échantillonnage (dans les communautés d'épiphylles en particulier). Le travail de Marie Vasse a été intégré dans le vaste projet de phylogénie des Chaetothyriales et n'a donc pas encore été publié.

3. Axe 2. Evolution de la communication chimique dans les symbioses plantes-fourmis

Les plantes à fourmis, comme la plupart des plantes, émettent des composés organiques volatiles lorsqu'elles sont attaquées par les insectes phytophages [A21]. Chez certaines plantes ces composés attirent les ennemis naturels des insectes phytophages. Chez les plantes myrmécophytes ce sont les fourmis résidentes qui sont attirées. Par une approche en écologie chimique, j'ai montré que, parmi les composés émis par la plante à fourmis *Leonardoxa africana* subsp. *africana*, seul le salicylate de méthyle induit une réponse de la fourmi mutualiste *Petalomyrmex phylax* [A19]. Afin de comprendre les modalités d'évolution du signal nous avons caractérisé les émissions chez les autres sous-espèces de *Leonardoxa africana* dans le cadre du stage de Master 2 de Marion Vittecoq. Elle a ainsi montré par chromatographie en phase gazeuse et spectrométrie de masse que trois sous-espèces, *L. a. africana*, *L. a. letouzeyi* (myrmécophyte) et *L. a. gracilicaulis* (non myrmécophyte), émettent les mêmes composés lorsqu'elles sont blessées. Grace à la technique d'éléctroantennographie qui consiste à mesurer l'influx nerveux dans les antennes, elle a montré que les composés émis par ces plantes sont perçus par une large gamme de fourmis, dont les symbiotes des plantes étudiées. Par contre, suite à des tests comportementaux sur le terrain (Figure 6), elle a démontré que les fourmis *Aphomomyrmex afer*, mutualistes de *L. a. letouzeyi*, répondent à l'hexanal mais pas au salicylate de méthyle, et elle a confirmé que les fourmis *P. phylax*, mutualistes de *L. a. africana*, répondent surtout au salicylate de méthyle [A23]. Ces résultats indiquent qu'à partir d'un même pool de composés émis par les plantes, un composé différent a été sélectionné dans chacune des deux symbioses, illustrant un cas probable d'évolution par contingence (Gould et Lewontin, 1979). Le stage de Marion Vittecoq a permis la publication de deux articles dont elle est première auteure [A23, A25]. L'un de ces articles concerne une expérience annexe faite sur le terrain en parallèle au sujet principal.

Figure 6 : dispositif expérimental mis en œuvre sur le terrain permettant de mesurer l'effet des composés volatils émis par la plante sur l'activité des fourmis. Les composés de synthèse purs sont déposés sur l'une des pastilles, l'autre recevant uniquement le solvant (témoin), et le nombre de fourmis attirées sur chaque pastille est compté.

Il existe peu de données sur la nature du signal chimique que les fourmis vivant sur les plantes utilisent pour localiser la source de stress, mais les travaux que j'ai dirigé sur ce thème suggèrent que ces signaux sont des molécules communément émises par de nombreuses plantes soumises au stress, et dont la fonction primaire est généralement antiseptique. L'évolution de la communication chez les plantes à fourmis résulterait donc d'un « bricolage évolutif » parcimonieux. A l'inverse, les plantes qui n'hébergent pas de fourmis émettent des signaux qui attirent spécifiquement les ennemis des insectes phytophages présents (Clavijo McCormick et al., 2012). Ce contraste est probablement lié au fait que chez les plantes à

fourmis, les ennemis des insectes phytophages, c'est-à-dire les fourmis, sont déjà localisées sur la plante et ont un intérêt à débarrasser la plante de tout type d'herbivore. Pas besoin, donc, d'un signal spécifique. Les grandes lignes de l'évolution des signaux de stress chez les plantes résulteraient donc d'une optimisation sélective minutieuse en fonction de la nature des interactions biotiques. Afin d'évaluer la pertinence de cette hypothèse j'envisage de poursuivre la caractérisation des signaux de communication chez les plantes à fourmis car elles représentent de nombreux cas indépendants d'évolution de la communication en présence de prédateurs généralistes.

Mes travaux sur la communication entre plantes et fourmis ont contribué à la compréhension des grands mécanismes d'évolution de la communication, et ont abouti à la rédaction d'une synthèse sur le sujet [A21].

4. Axe 3. Facteurs de spéciation et de structuration génétique en Afrique centrale : l'apport de l'approche comparée entre partenaires symbiotiques

Le domaine forestier d'Afrique Centrale est la deuxième zone la plus étendue de forêt tropicale dans le monde. Dans cette zone il n'y a que quelques barrières géographiques majeures qui puissent limiter la dispersion des organismes forestiers et les processus qui façonnent la distribution spatiale de la diversité génétique sont encore mal compris. Les fluctuations climatiques passées ont provoqué des cycles d'expansion, de contraction et de fragmentation du bloc forestier au profit de la savane au cours du quaternaire, et ce jusqu'à très récemment (Vincens et al., 1999; Hessler et al., 2010; Lezine et al., 2013). De nombreuses populations actuelles des plantes de la forêt tropicale humide d'Afrique centrale résultent donc d'une recolonisation récente. L'étude de la structure génétique des populations d'espèces interdépendantes associées dans des mutualismes symbiotiques offre des opportunités uniques en phylogéographie comparative. Je me suis focalisé sur deux échelles spatiales différentes : l'échelle locale et l'échelle du paysage.

a. Expansion récente d'aire de distribution et plasticité des interactions mutualistes : le modèle Leonardoxa

Quand je suis arrivé au CEFE en 2006, Doyle McKey s'intéressait déjà aux patrons de diversité génétique en Afrique Centrale et il co-encadrait, avec Finn Kjellberg, la thèse de Guillaume Léotard sur ce thème. J'ai donc pris le train en marche et ai participé à l'encadrement de cette thèse qui portait sur les facteurs responsables des grands patrons de biodiversité par l'étude du complexe de *Leonardoxa africana*, plante à fourmis des forêts pluviales du Cameroun.

Les études de structuration génétique des populations ont montré que la plante myrmécophyte *Leonardoxa africana* subsp. *africana* et ses deux fourmis symbiotiques *Petalomyrmex phylax* (mutualiste) et *Cataulacus mckeyi* (parasite du mutualisme) ont subi une expansion récente (probablement au cours de l'holocène) avec un front de colonisation où le phénotype des fourmis présente une meilleure efficacité de dispersion : taille et proportion relative des femelles ailées plus importantes (Dalecky et al., 2007; Léotard et al., 2009). Par conséquent les colonies du front de colonisation investissent moins dans les ouvrières et donc moins dans la défense de la plante. Avec une étudiante de Master 2 que j'encadrais, Marion Vittecoq, nous avons montré que les ouvrières en front de colonisation s'investissent

individuellement moins dans la défense de plante, ce qui renforce le caractère moins mutualiste de leur phénotype [25]. L'existence de fronts de colonisation liés aux fluctuations d'aires induites par les changements climatiques a donc un effet potentiellement déstabilisateur du mutualisme. Cependant, notre modèle d'étude semble être résilient à ces perturbations car aucune réponse de la plante n'a pu être mise en évidence sur le front de colonisation (Léotard et al., 2009), probablement parce que les caractères moins mutualistes chez la fourmi sont transitoires et que le taux d'évolution est beaucoup plus lent chez la plante que chez la fourmi, ralentissant la sélection d'une stratégie moins mutualiste en retour.

J'ai encadré la thèse de Guillaume Léotard plus spécifiquement sur la question du rôle des grands fleuves dans la structure génétique de la plante et de sa fourmi mutualiste. J'ai aussi encadré un étudiant de Master 1, Emmanuel Defossez, qui a travaillé de concert avec Guillaume Léotard. Des travaux précédents avaient montré chez la fourmi mutualiste un changement de structure sociale et de paramètres biométriques liés aux capacités de dispersion de part et d'autre d'un fleuve, d'où l'hypothèse d'un rôle des fleuves dans la dynamique des populations au cours de l'expansion récente de l'aire de distribution (Dalecky, 2003). Les données de Guillaume Léotard et d'Emmanuel Defossez, basées sur des marqueurs microsatellites, ont montré que le fleuve n'avait aucun effet sur la structure génétique spatiale de la plante et de la fourmi, invalidant l'hypothèse de départ. Ces résultats ont fait l'objet d'un article avec Guillaume Léotard (en premier auteur) et Emmanuel Defossez [A16].

b. Evolution du myrmécophytisme dans le genre Barteria

Le genre *Barteria* compte quatre espèces endémiques d'Afrique Centrale, dont trois sont myrmécophytes (Breteler, 1999). *Barteria solida* est une espèce restreinte aux forêts humides de l'étage collinéen et connue que de quelques localités seulement. Ses branches sont pleines et elle n'héberge donc pas de fourmis. *Barteria nigritana* est restreinte aux formations dunaires sableuses de la frange du littoral atlantique. Chaque branche porte, à sa base, une petite cavité creuse qui héberge des fourmis opportunistes non spécifiques dont l'efficacité de protection est variable (Djiéto-Lordon et al., 2004). *Barteria fistulosa* est largement distribuée en Afrique Centrale, mais restreinte aux forêts de plaines. Elle présente des branches renflées et creuses sur toute leur longueur, qui abritent la fourmi spécifique *Tetraponera aethiops* (et dans une moindre mesure sa sœur jumelle *T. latifrons*). Ces grosses fourmis à la piqûre douloureuse assurent à la plante une protection efficace contre les herbivores invertébrés et vertébrés et contre la végétation environnante (Janzen, 1972; McKey, 1974; Dejean et al., 2008). *Barteria dewevrei* a la même distribution que *B. fistulosa*, mais présente une forte variation de la taille des domaties. Les domaties les plus étroites sont occupées par des fourmis non spécifiques du genre *Crematogaster*, et les plus larges sont occupées par les fourmis spécifiques des *Barteria* : *T. aethiops* ou *T. latifrons*. Il existe donc dans le genre *Barteria* un gradient important d'investissement dans les traits myrmécophytes et nous avons construit une phylogénie du genre afin de comprendre les modalités d'évolution de ces traits. Pour ce faire j'ai coordonnée le travail de personnels techniques du laboratoire et d'un post-doctorant, Jean Peccoud, travail auquel a aussi contribué un étudiant en Master 2, Finn Piatscheck, que j'ai encadré. Bien que nous ayons testé plus d'une dizaine de marqueurs habituellement utilisés comme code-barres génétique ou en phylogénie chez les plantes, incluant ceux réputés pour être les plus variables, les séquences des différentes espèces de *Barteria* n'ont pas montré de variabilité, sauf pour l'ITS nucléaire. Nous avons donc développé une banque de marqueurs microsatellites [A27] et avons génotypé près de 700 individus de *Barteria* pour définir des groupes génétiques et juger de leur congruence avec les groupes morphologiques. Nous avons ensuite construit une phylogénie à partir des génotypes microsatellites et des séquences ITS. Les deux types de marqueurs reconnaissent une première

dichotomie entre le groupe *B. fistulosa* + *B. solida* d'une part et le groupe *B. dewevrei* + *B. nigritana* d'autre part. *Barteria nigritana* semble former un clade monophylétique dérivé de *B. dewevrei*, et correspond probablement à un évènement de spéciation récent. La spéciation écologique est le processus par lequel des barrières aux flux de gènes évoluent entre populations comme conséquence d'une sélection divergente basée sur des facteurs écologiques (Rundle et Nosil, 2005). Etant donnée la niche si particulière de *B. nigritana*, il est possible que la divergence entre *B. dewevrei* et *B. nigritana* résulte d'un tel processus. Dans le groupe *B. fistulosa* + *B. solida*, il existe des clades intraspécifiques aussi distants les uns des autres que les clades interspécifiques. C'est le cas en particulier du clade *B. solida* du Cameroun qui est aussi proche du clade *B. solida* du Gabon que du clade *B. fistulosa* du Gabon (Figure 7). De plus, l'enracinement des arbres phylétiques par les plus proches parents du genre *Barteria*, qui ne sont pas myrmécophytes, montre que l'espèce *B. solida* (non myrmécophyte) n'est pas basale, mais dérive probablement de *B. fistulosa*. Il est possible que le taxon *B. solida* résulte d'un (ou plusieurs) évènements d'isolement par spécialisation écologique à l'altitude à partir du taxon *B. fistulosa* qui lui est plutôt restreint aux forêts de plaine. La perte des traits myrmécophytes chez *B. solida* serait liée au fait que la diversité des fourmis et des insectes phytophages chute rapidement avec l'altitude, et que donc les coûts de production des traits myrmécophytes dépassent les bénéfices dans cet environnement. Quoi qu'il en soit nous pouvons affirmer avec certitude que les traits myrmécophytes sont très labiles d'un point de vue évolutif et que leur évolution ne suit pas un gradient de spécialisation croissante dans le genre *Barteria* (Figure 7). Les travaux sur la phylogénie des *Barteria* ont fait l'objet de deux publications (dont une note technique) auxquelles Finn Piatscheck (étudiant Master 2) et Jean Peccoud (post-doctorant) ont été associés [A27, A31].

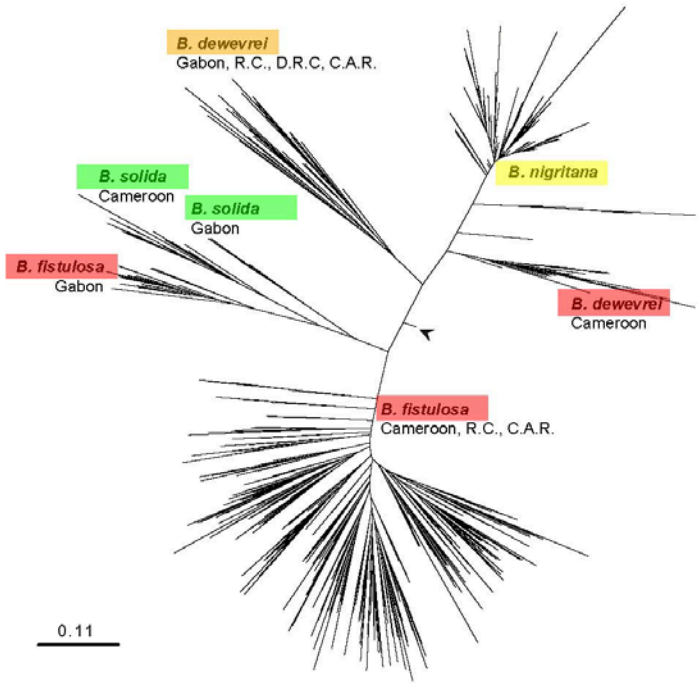

Figure 7 : Arbre basé sur les génotypes microsatellites de 700 individus des quatre espèces de *Barteria*. Les noms des clades sont surlignées en fonction du degré de spécialisation des traits myrmécophytes : vert : pas de domaties ; jaunes : domaties courtes colonisées par des fourmis non spécifiques ; orange : domaties longues mais de diamètre variable et occupées par des fourmis spécifiques ou non ; rouge : domaties longues et larges occupées par des fourmis spécifiques. RC : Congo ; DRC : République Démocratique du Congo ; CAR : Centrafrique.

c. *Origine de la diversité et structuration génétique spatiale à l'échelle du paysage : le modèle* Barteria

Par les questionnements qu'elle a fait surgir, la phylogénie du genre *Barteria* a ouvert des perspectives nouvelles sur la dynamique de la spéciation en Afrique Centrale. En particulier, les génotypes aux marqueurs microsatellites obtenus pour cette phylogénie ont montré une différentiation génétique importante entre les populations du nord et du sud dans le domaine de la Basse Guinée (Cameroun et Gabon). Pourtant, dans cette zone il n'existe aucune barrière géographique susceptible d'expliquer une interruption des flux de gènes. Les hypothèses proposées pour expliquer l'origine de la diversité en Afrique Centrale sont: (i) l'existence de refuges forestiers isolés durant les périodes sèches du Quaternaire, (ii) une inversion de saisonnalité entre le nord et le sud d'une charnière climatique proche de l'équateur, (iii) un gradient de pluviométrie est-ouest et (iv) l'adaptation divergente à des facteurs édaphiques. Il existe cependant très peu d'études visant à tester ces hypothèses. Une synthèse récente montre que les patrons de structure génétique des arbres de forêt tropicale humide dans cette région sont variables d'une espèce à l'autre, hormis la différentiation nord-sud qui est présente chez la moitié des espèces étudiées (Hardy et al., in press). Pour évaluer la pertinence des différentes hypothèses et pour tenter de comprendre la dynamique de la diversité dans cette région du globe, j'ai entrepris une étude de génétique des populations comparée entre les trois partenaires symbiotiques, la plante *B. fistulosa*, sa fourmi associée *T. aethiops* et le champignon qu'elle cultive dans les domaties (le taxon CTeY1, espèce de l'ordre des Chaetothyriales non encore décrite), à partir d'un échantillonnage couvrant le domaine de la Basse Guinée et incluant un transect nord-sud afin de caractériser en détail la structure génétique chez la plante au niveau de la charnière climatique.

J'ai obtenu près de 800 échantillons de *B. fistulosa*, la plupart avec leurs symbiotes, accumulés lors de différentes missions de terrain et grâce aux contributions de nombreux collègues, et j'ai encadré une petite équipe de deux post-docs et un étudiant en Master 2, secondés les personnels techniques du laboratoire. Ce travail a été initié par Finn Piatscheck lors de son stage de Master 2 au cours duquel il s'est intéressé à la co-structuration entre la plante et la fourmi. Il a été épaulé dans cette tâche par Jean Peccoud, en post-doc sur la phylogénie du genre *Barteria*. Enfin, Céline Born, en post-doc, s'est focalisée sur le partenaire fongique et sur la structure génétique chez la plante au niveau de la charnière climatique. Nous avons mis au point une banque de marqueurs microsatellites pour chacune des trois espèces [A26, A27, A33] et génotypé l'ensemble des individus. Les trois espèces présentent des structures génétiques congruentes. Dans la zone d'étude, le domaine de la Basse Guinée, trois groupes génétiques ont été identifiés et correspondent à des entités géographiques bien délimitées (Figure 8) : un groupe correspondant aux populations de la ligne volcanique camerounaise (LVC) et deux groupes de part et d'autre de la latitude ~1°N (groupe Nord et groupe Sud). Pour la fourmi et le champignon la différentiation entre ces trois groupes est modérée (Fst ~ 0.05) et les groupes montrent une introgression marquée. Pour la plante, la différentiation entre le groupe de la LVC et le groupe nord est identique à ce qui est observé chez les autres symbiotes (Fst ~ 0.05), mais la différentiation entre les groupes nord et sud est beaucoup plus forte (Fst ~ 0.25). La transition entre ces deux groupes est très abrupte (environ 40 km) et montre très peu d'hybrides (Figure 9). Etant donné un tel degré de différentiation sur une si courte distance il est clair que les deux groupes présentent des incompatibilités reproductives.

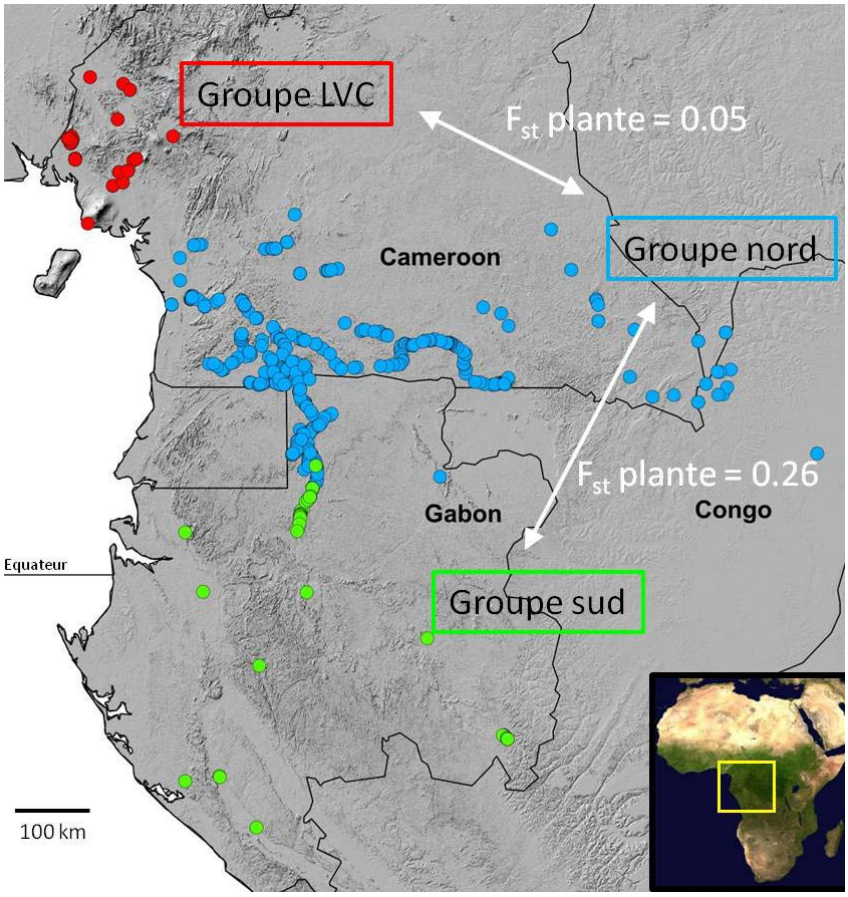

Figure 8 : Distribution des trois groupes génétiques identifiés pour chacune des trois espèces en symbiose (plante, fourmi, champignon), avec les valeurs de différentiation génétique pour la plante.

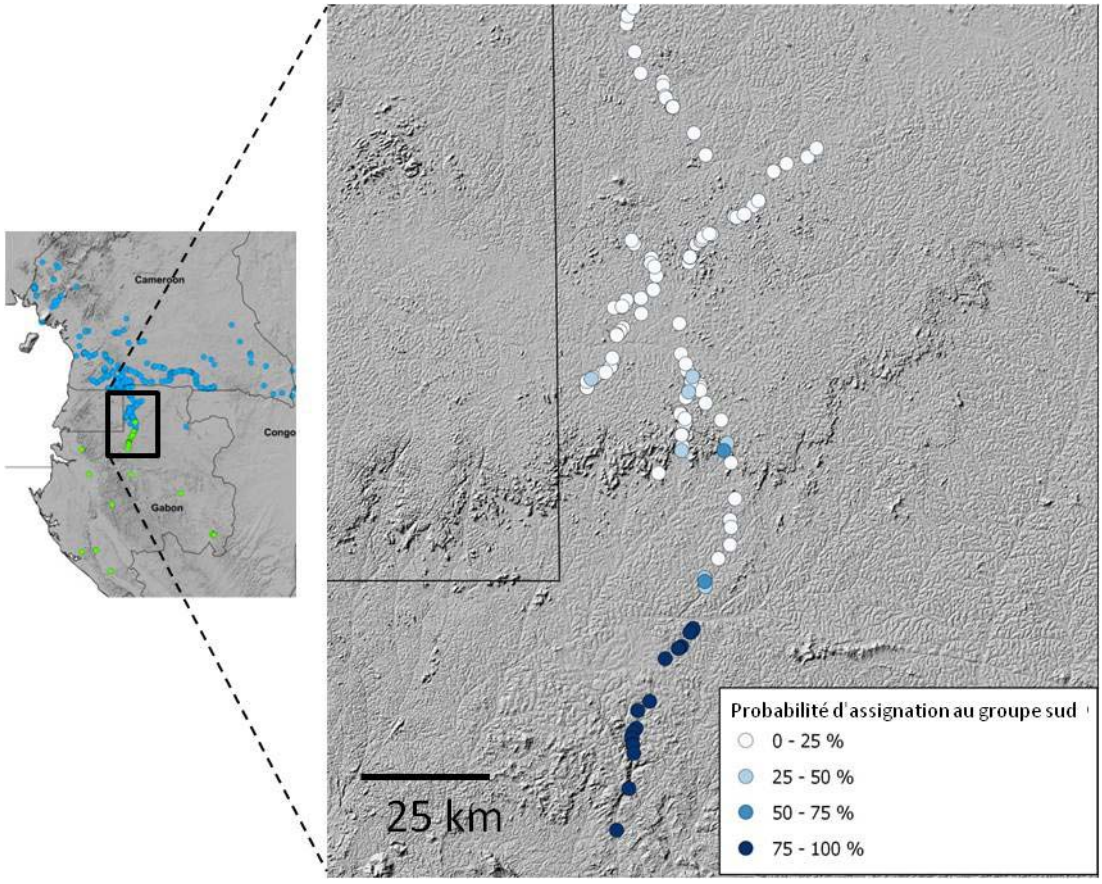

Figure 9 : Zone de transition entre les groupes nord et sud pour la plante. Les probabilités d'assignation ont été calculées par l'algorithme STRUCTURE.

La répartition des trois groupes génétiques reconnus pour chaque espèce, et le fait que ces trois groupes soient géographiquement congruents entre les espèces, sont des arguments en faveur de l'hypothèse des refuges forestiers. Les fluctuations climatiques du Quaternaire ont provoqué des phases de sécheresse en Afrique Centrale, induisant des contractions et des fragmentations répétées du bloc de forêt tropicale humide. Selon l'hypothèse de Maley, les refuges forestiers auraient été les mêmes pour tous les organismes forestiers (Maley, 1996). La ligne volcanique camerounaise serait l'un de ces refuges. D'autres ont été identifiés au Cameroun et au Gabon. Il est donc envisageable que les trois groupes génétiques que nous avons reconnus chez les trois organismes symbiotiques correspondent à des populations isolées par le passé et qui sont entrées en contact secondaire suite à la recolonisation. Par contre, cette hypothèse n'explique pas pourquoi la différentiation entre les groupes nord et sud est beaucoup plus marquée chez la plante que chez les deux autres symbiotes.

Dans le domaine de la Basse Guinée la saisonnalité est inversée entre le nord et le sud d'une charnière climatique située à environ 2°N (Hardy et al., 2013). Les données de floraison des *Barteria fistulosa* que nous avons pu compiler indiquent un décalage phénologique de 6 mois entre le nord et le sud de cette charnière (Figure 10).

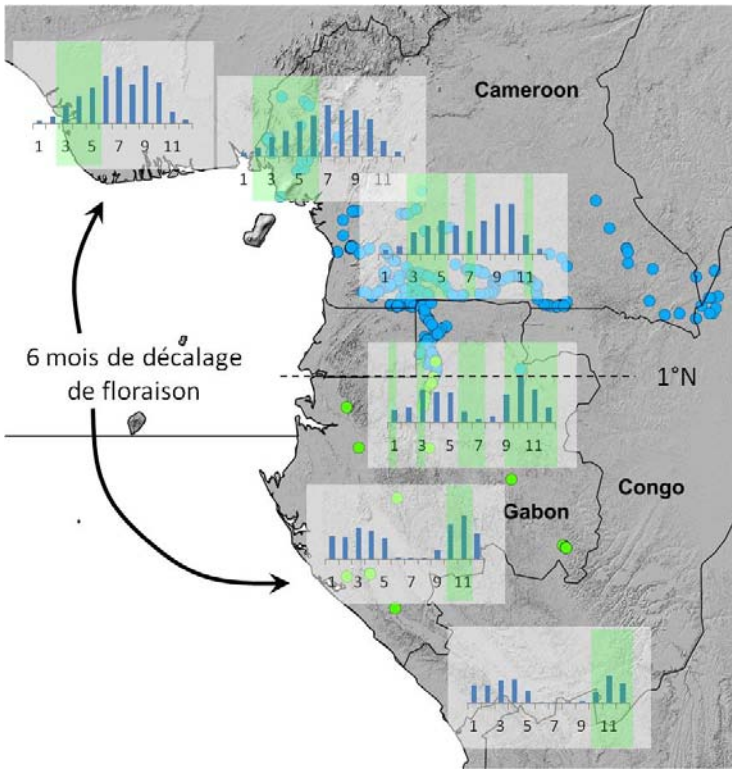

Figure 10 : Inversion de saisonnalité et décalage phénologique. Les barres bleues des diagrammes correspondent aux pluviométries mensuelles calculées pour six sites à partir du modèle WorldClim. Les zones colorées en vert sur les diagrammes correspondent aux périodes de floraison observées ou déduites des données d'herbiers. Au niveau de la charnière climatique (~2°N) on observe un chevauchement substantiel des périodes de floraison. Les deux groupes génétiques pour la plante sont représentés, ainsi que la position de la zone de transition (~1°N).

Comme cette charnière correspond grossièrement à la zone de tension entre les deux groupes génétiques, il est tentant de penser que la plus forte différentiation observée chez la plante est liée à une interruption au flux de pollen, et donc de gènes, correspondant au décalage de floraison entre le nord et le sud. Cette hypothèse n'est cependant pas satisfaisante car la zone de tension ne correspond pas exactement à la charnière climatique, et le décalage phénologique est progressif au niveau de cette charnière, n'excluant pas complètement la possibilité de flux de gènes. De plus les flux de graines ne sont pas interrompus par le décalage phénologique. Par contre il est fort possible que ce décalage phénologique ait joué

un rôle plus important lors des périodes sèches. En effet, les populations de plantes au nord et au sud de cette charnière subissaient l'isolement géographique, comme pour les autres types d'organismes (insectes, champignons), mais aussi un isolement temporel puisqu'aucun refuge n'est suspecté au niveau de la charnière. De plus, pendant les phases plus sèches l'inversion de saisonnalité devait être plus marquée. Les évènements de flux de pollen qui auraient pu avoir lieu entre les refuges n'auraient donc pas abouti à des flux de gènes. Les populations de plante au nord et au sud de la charnière auraient donc divergé beaucoup plus fortement que les autres, et que les populations de fourmis et de champignons. Ce processus aurait induit une incompatibilité génétique entre les populations de plante du nord et du sud qui expliquerait l'absence de flux de gènes entre les groupes même après mise en contact secondaire. La position de la zone de tension pourrait s'expliquer par sa « capture » sur un gradient environnemental. En effet, les données de terrain et la modélisation montrent que les zones de tension se fixent sur des transitions environnementales ou écologiques et peuvent être renforcées par adaptation locale de part et d'autre de la transition (Bierne et al., 2011). La transition entre les *Barteria* du nord et du sud pourrait donc correspondre soit à la transition climatique, soit à une transition dans les facteurs édaphiques.

De ce travail récent, seules les trois notes techniques ont été publiées, avec l'étudiant de Master 2 et les deux post-docs comme coauteurs [A26, A27, A33]. L'étudiant et les post-docs sont aussi coauteurs d'un article en préparation.

Ce travail a fourni un terreau fertile au développement de nouvelles perspectives prometteuses pour la compréhension des mécanismes de spéciation et de diversification sous les tropiques. Dans l'avenir j'envisage de tester la généralité du scénario présenté ci-dessus sur d'autres organismes modèles. J'envisage aussi de différentier le rôle des facteurs édaphiques et climatiques actuels sur la position de la zone de tension entre les groupes nord et sud de *B. fistulosa*. Un suivi temporel de cette zone et des expériences de transplantation devraient apporter des informations utiles. Il me semble aussi indispensable d'améliorer la fiabilité des modèles climatiques dans cette région, par exemple en utilisant les images satellites pour extraire certaines données climatiques.

5. Axe 4. Effet de synergie entre les activités passées de l'Homme et les ingénieurs d'écosystème

Les savanes côtières de Guyane sont couvertes de milliers de buttes de terre qui sont les vestiges d'anciens champs surélevés précolombiens abandonnés depuis près de 500 à 1000 ans, mais qui ont perduré depuis (McKey et al., 2010). De par mes compétences sur les interactions entre espèces et sur l'écologie des insectes sociaux, j'ai été amené à participer à l'encadrement de la thèse de Delphine Renard (dirigée par Doyle McKey) qui visait à coupler l'histoire de ces populations natives d'Amérique du Sud et de leurs activités agricoles avec l'étude de l'écologie actuelle de ces paysages de buttes saisonnièrement inondés (Figure 11).

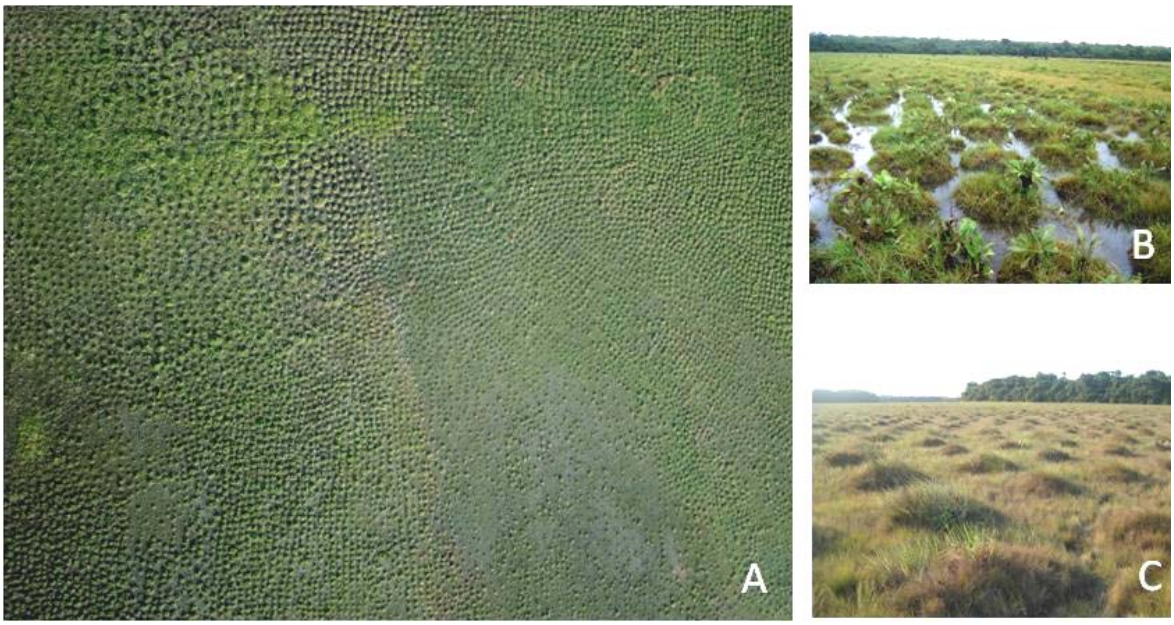

Figure 11 : Paysage de buttes dans les savanes saisonnièrement inondées de Guyane. A : Vue aérienne ; B : Saison des pluies ; C : Saison sèche. (Photos : Delphine Renard et Doyle McKey)

La partie de la thèse dans laquelle j'ai joué un rôle consistait à caractériser la contribution des ingénieurs d'écosystèmes dans le maintien des buttes. J'ai épaulé et conseillé Delphine Renard dans la définition des protocoles expérimentaux et lors des relevés de terrain. Ce travail a montré que les caractéristiques du sol dans les buttes leur confèrent une meilleure résistance à l'érosion qu'au sol de la matrice (espace entre les buttes). La différence de sol entre buttes et matrice est corrélée à une différence d'activité des ingénieurs d'écosystème: fourmis, termites et vers de terre. Les nids de fourmis et de termites sont concentrés sur les buttes, et l'activité des vers de terre est très faible pendant la saison sèche et concentrée sur les buttes pendant la saison des pluies. Les communautés de fourmis sur les buttes varient de manière substantielle, avec des espèces endogées qui remontent vers la surface en saison des pluies. De plus, Delphine Renard avait montré pendant son stage de Master 2, une dispersion des graines par les fourmis dirigée vers les buttes. Les flux de matière organique dans ces paysages se font donc de manière horizontale et verticale et sont concentrés vers la partie supérieure des buttes. En créant de l'hétérogénéité spatiale au sein des paysages de savanes inondées, l'Homme a initié des boucles de rétroactions qui ont permis le maintien de ces buttes même après leur abandon par l'Homme. Ce travail a fait l'objet d'un article cosigné avec la doctorante [A32]. Un autre article est en cours de préparation.

6. Perspectives

Même si mes travaux sur les symbioses plantes-fourmis s'inscrivent dans la lignée de ceux conduits par Doyle McKey et ses étudiants depuis plus de 20 ans, mon approche légèrement différente et résolument mécaniste m'a permis de découvrir des pistes complètement nouvelles et que j'ai à peine commencé à explorer. Celles qui me paraissent avoir l'intérêt scientifique le plus large et que je compte développer dans les années à venir sont présentées ci-dessous.

Une meilleure connaissance des flux de ressources entre partenaires symbiotiques mutualistes est importante pour comprendre à la fois l'origine et le maintien des ces

interactions au cours de l'évolution, et les flux de nutriments dans les écosystèmes. La découverte de champignons comme partenaires trophiques dans les symbioses plantes-fourmis sert de catalyseur à une nouvelle approche des stratégies d'approvisionnement des plantes que je suis en train de mettre en place à travers divers collaborations. Même si les champignons présents dans les domaties servent de nourriture aux fourmis, j'ai de bonnes raisons de penser qu'ils facilitent et renforcent les flux trophiques des activités des fourmis vers la plante. J'envisage donc d'étudier les capacités métaboliques de ces champignons en relation avec les substrats qui leur sont fournis par les fourmis pour tester l'hypothèse qu'ils sont responsables de la dégradation de macromolécules (ex. : chitine) en molécules plus simples directement assimilables par la plante. Selon cette hypothèse ces champignons permettraient aux plantes à fourmis d'accéder à une source d'azote et de phosphore sans passer par la décomposition de la matière organique dans le sol, à l'instar des plantes carnivores et des plantes pratiquant la nodulation. J'envisage par ailleurs de conduire une étude comparative de la structure anatomique des domaties pour détecter d'éventuelles adaptations à l'absorption de molécules simples. La présence d'un sclérenchyme canaliculé à la surface interne des domaties de plusieurs plantes à fourmis est une piste prometteuse.

L'ordre des Chaetothyriales, dont font partie les champignons associés aux fourmis, est surtout connu pour ses représentants pathogènes chez les humains. En effet, certaines espèces s'attaquent à la peau ou au système nerveux et provoquent des maladies graves. Ce sont probablement des espèces opportunistes qui trouvent dans ces substrats du corps humain un contexte favorable à leur développement. Une meilleure connaissance de la diversité et de l'écologie des Chaetothyriales pourrait permettre de mieux comprendre le déterminisme de la virulence des souches pathogènes. Une piste que j'envisage de développer en collaboration avec Sybren de Hoog (CBS-KNAW Fungal Biodiversity Centre, Pays Bas), spécialiste des Chaetothyriales et en particulier des souches pathogènes, est la recherche d'une éventuelle analogie entre les conditions que rencontrent ces champignons dans le système nerveux et celles qu'ils rencontrent dans les domaties occupées par les fourmis. Cette analogie résiderait dans la similitude entre la structure chimique des composés présents dans le système nerveux et celle des composés produits par les fourmis et déposés sur les patchs de champignons pour sélectionner les souches symbiotiques. Ces deux types de composés constitueraient à la fois une source de carbone (les Chaetothyriales sont oligotrophes et capables de métaboliser des composés toxiques pour la plupart des organismes) et une protection contre les compétiteurs par leur action toxique (les Chaetothyriales sont de très mauvais compétiteurs).

J'ai relativement peu œuvré dans le domaine de la communication chimique entre plantes et insectes, mais les quelques travaux que j'ai dirigé constituent une base pour un projet plus vaste sur l'évolution de la spécificité du signal chimique. L'approche comparative (entre espèces) est rarement utilisée dans ce contexte, et pourtant elle permet de comprendre les conditions d'évolution de la spécificité du signal, avec des conséquences pour la théorie de l'information. Il semble que le signal d'herbivorie émis par les plantes à fourmis soit peu spécifique. Pour comprendre les contraintes qui s'exercent sur l'évolution de ce signal il serait judicieux de tester l'effet sur la nature du signal de la combinaison de facteurs tels que (i) la nature des composés volatiles émis par la communauté végétale (risque de brouillage du signal), (ii) les contraintes métaboliques des plantes, (iii) la composition de la communauté d'herbivores, en particulier la nature des herbivores qui engendrent le plus de dommages, qui pourrait expliquer l'existence de signaux spécifiques.

L'origine de la diversité dans les régions tropicales est un sujet qui me fascine. Mes travaux sur la structure génétique des *Barteria* et de leurs symbiotes en Afrique Centrale suggèrent que l'inversion de saisonnalité de part et d'autre de l'équateur, combinée à d'autres facteurs, pourrait être un moteur important de la diversification, et peut-être de la spéciation. De plus, cette inversion de saisonnalité a un impact sur l'intensité et la direction des flux de

ressources végétales (fruits en particulier) échangées entre les populations locales. Même si je ne compte pas aborder les aspects socio-économiques de l'inversion de saisonnalité, une meilleure compréhension de la réponse des organismes à ce phénomène pourrait avoir des retombées dans ce domaine.

Les mutualismes sont à l'origine de divers services écosystémiques et il apparait évident que les changements globaux actuels ont des effets délétères sur ces mutualismes. La compréhension des facteurs qui stabilisent ou déstabilisent les mutualismes est donc particulièrement d'actualité. Je pense que la symbiose plante-fourmi est un modèle très pertinent pour aborder cette question car cette interaction me parait peu stable à l'échelle évolutive, ce qui devrait la rendre particulièrement sensible aux changements globaux actuels, et donc permettre de détecter des effets plus rapidement qu'avec d'autres modèles de mutualisme. En effet, les symbioses plantes-fourmis sont apparues de nombreuses fois indépendamment, mais ont rarement donné lieu à des radiations d'espèces. Ceci suggère que les traits morphologiques et fonctionnels liés à cette interaction sont facilement sélectionnés mais sont aussi facilement perdus, soit par extinction des lignées (cul-de-sac évolutif), soit par perte secondaire des caractères. Les travaux que j'ai mené sur le genre *Barteria* (Passifloraceae) vont dans ce sens. Chez ces plantes, le degré de spécialisation des traits myrmécophytes semble plus lié à l'écologie qu'aux relations phylogénétiques, et des clades phylogénétiquement très proches montrent des contrastes extrêmes du degré d'association avec les fourmis. L'acquisition ou la perte de traits myrmécophytes se ferait donc rapidement, en fonction des variations du rapport coûts/bénéfices de l'interaction, lequel est fortement dépendant des conditions environnementales. Il est admis que les changements évolutifs peuvent apparaitre sur des temps courts. Les plantes à fourmis pourraient donc servir de modèle à l'étude des processus de microévolution résultant du changement global actuel. Décrire et comprendre la réaction des symbioses plantes-fourmis aux changements globaux actuels et passés me paraît donc une piste prometteuse.

7. Bilan scientifique

Mon approche des interactions biotiques à différentes échelles – des molécules au paysage – me permet d'avoir une vision globale de l'importance que les interactions jouent dans le fonctionnement des écosystèmes. Dans un contexte sociétal où changements globaux et services écosystémiques sont au cœur de l'actualité, il me semble nécessaire de renforcer la recherche fondamentale sur les mécanismes du fonctionnement des écosystèmes. Deux approches me paraissent importantes : l'étude des propriétés des réseaux d'interactions et la dissection des mécanismes qui régissent les interactions elles-mêmes. C'est clairement cette seconde approche que j'ai adopté et avec laquelle je compte poursuivre, car, même si elle semble moins en vogue que la première, elle est tout aussi importante pour comprendre, à terme, l'effet des changements globaux sur les écosystèmes.

8. Bilan de l'activité d'encadrement depuis 2006 (arrivée au CEFE, Montpellier)

Nom	Diplôme préparé	Année	Articles publiés avec l'étudiant	Situation actuelle
Encadrement de stagiaires				
Pierre Arnal	Licence 3 (UM2)	2013		En Master 1
Marie Vasse	Ingénieur Agronome (Rennes)	2012		En thèse
Honoré Agbazahou	Master 1 (UM2)	2012		Fin Master 2
Alex Salas-Lopez	Master 1 (UM2)	2011	A34	En thèse
Finn Piatscheck	Master 2 (UM2)	2011	A26, A27, A31	CDD Mexique
Sarah Debaud	DUT Génie Biologique	2010	A34	
Marion Vittecoq	Master 2 (UM2)	2009	A23, A25	En post-doc
Emmanuel Defossez	Master 1 et 2 (Grenoble)	2007 et 2008	A16, A18, A22	En post-doc
Delphine Renard	Master 1 (UM2)	2006	A32	En post-doc
Participation à l'encadrement de doctorants				
Guillaume Léotard	Doctorat	2006-2007	A16	Expert indépendant en études environnementales
Delphine Renard	Doctorat	2008-2010	A28	En post-doc
Encadrement de Post-doctorants				
Jean Peccoud		2011	A26, A27, A31	En post-doc
Céline Born		2012-2013	A31, A33, A34, A35	Enseignante

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Dejean, A., Djiéto-Lordon, C., Orivel, J., 2008. The plant ant *Tetraponera aethiops* (Pseudomyrmecinae) protects its host myrmecophyte *Barteria fistulosa* (Passifloraceae) through aggressiveness and predation. Biol. J. Linn. Soc. 93, 63-69.

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Vincens, A., Schwartz, D., Elenga, H., Reynaud-Farrera, I., Alexandre, A., Bertaux, J., Mariotti, A., Martin, L., et al., 1999. Forest response to climate changes in Atlantic Equatorial Africa during the last 4000 years BP and inheritance on the modern landscapes. J. Biogeogr. 26, 879-885.

Curriculum Vitae complet

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Mes activités de recherche se situent dans le cadre général des interactions biotiques et de la coévolution. J'aborde la dynamique des interactions biotiques mutualistes en intégrant différents niveaux d'organisation et en explorant divers aspects complémentaires. En disséquant le fonctionnement de ces interactions, mes recherches contribuent aux grands thèmes actuels de l'écologie évolutive : dynamique de la biodiversité, structuration des écosystèmes (à travers les relations fonctionnelles entre espèces), évolution des communautés, réponse des écosystèmes aux changements globaux. J'utilise comme modèle biologique principal les interactions obligatoires symbiotiques entre plantes et fourmis.

1. Expérience professionnelle et formation

Janvier 2007 - : Chargé de Recherche (CR1) au CNRS. Centre d'Ecologie Fonctionnelle et Evolutive, UMR 5175.
Octobre 2002 - Décembre 2006: Chargé de Recherche (CR2) au CNRS. Laboratoire d'Ethologie Expérimentale et Comparée, Université Paris 13, CNRS UMR 7153.
Janvier-septembre 2002: Financement National Science Foundation. Stage postdoctoral à l'Université du Colorado (USA).
Janvier 2001 - 2002: Boursier de la Fondation Fyssen. Stage postdoctoral à l'Université du Colorado (USA).
1997 - 2000 : Allocataire de recherche (Ministère de l'Education Nationale, de la Recherche et de la Technologie). Doctorat de l'Université Paris 13 (Biologie du Comportement)
1997 : DEA de biologie du comportement (Université Paris 13).
1996 : Maîtrise de biologie des populations et des écosystèmes (Université Paris 6).
1995 : Licence de biologie des organismes (Université Paris 6).
1994 : DEUG Sciences de la nature et de la vie (Université Paris 7).

2. Contrats de recherche

Bibliothèque Du Vivant : *Phylogénie et spécificité des partenaires fongiques dans une multitude de symbioses entre fourmis et champignons* (PHYLOSYMB). Coordonateur. Novembre 2011 - Avril 2013.

ANR 6ème extinction: *Et si la 6ème extinction avait déjà eu lieu? Causes et conséquences de la dernière grande « crise » environnementale (3000 ans BP) sur les écosystèmes forestiers d'Afrique équatoriale atlantique* (C3A). Implication: 30%. Janvier 2010-décembre 2013.

ANR Jeunes Chercheuses Jeunes Chercheurs : *Dynamique de la coévolution, le cas des symbioses plantes-fourmis* (CoSy). Implication : Coordonateur, 90%. Janvier 2007-décembre 2009.

ANR Biodiversité : Les îles forestières africaines, modèle d'une nouvelle approche de la dynamique de structuration de la biodiversité (IFORA). Implication : 5%. Janvier 2007- décembre 2009.

3. Enseignement

- Ecologie comportementale. Master 2 BGAE, Université Montpellier 2. 2006-2013.
- Diversité et biogéographie des écosystèmes. Master I Ecologie Biodiversité, Université Montpellier 2. 2012-2013.
- Ecology in English. Master 1 BGAE, Université Montpellier 2. 2011.
- Méthodes comportementales et biochimiques de la reconnaissance sociale chez les fourmis. Master 2 Biologie du comportement, Université Paris 13. 2005.
- L'Homme dans le flux du vivant. Licence de Psychologie, Université Paris 13. 2003.
- Informatique. DEUG de Communication, Université Paris 13. 1998-1999.

4. Participation aux tâches collectives

Référé pour *Acta Oecologica, Animal Behaviour, Asian Myrmecology, Behavioral Ecology, Biology Letters, Biotropica, Ethology, European Journal of Entomology, Insects, Journal of Agricultural and Biological Sciences, Journal of Animal Ecology, Journal of Evolutionary Biology, Microbial Ecology, Molecular Ecology, New Phytologist, Sociobiology, Symbiosis.*

Expertise de projets:
National Science Foundation, USA (2011).
Agence Nationale de la Recherche. Programme blanc International (2012).

Participation à des jurys :
2011 : HDR Laurence Gaume
2011 : Jury de concours AI n°215 BAP A

Participation à la vie du laboratoire :
Membre de la commission « Terrain d'expérience »
Membre de la commission « Bibliothèque »
Membre de la commission « Hygiène et sécurité »

Actions de vulgarisation scientifique
- Fête de la biodiversité, Montpellier, 2013 (Animation pour scolaires et grand public)
- Salon Champignons et plantes d'automne, Montpellier, 2012 (Conférence)

- Festival Nature, Parc National des Cévennes, 2010 (Conférence)
- Darwin 2009, Montpellier, 2009 (Animation pour scolaires et grand public)
- Fête de la Science, Montpellier, 2006 (Conférence)
- Savante Banlieue, région parisienne, 2002 - 2005 (Conférence)
- La main à la pâte, région parisienne, 1998-1999 (Animation pour scolaires)
- Aide à la réalisation de Travaux Personnels Encadrés (Lycées et classes prépa), 2004-2008.

5. Bilan des publications de rang A

Nom de la revue	Nombre d'articles	Facteur d'impact (JCR 2012)
Current Biology	1	9,5
Molecular Ecology Resources (et Molecular Ecology Notes)	4	7,4
New Phytologist	1	6,7
Molecular Ecology	3	6,3
Proceedings of the Royal Society B	2	5,7
Molecular Phylogenetics and Evolution	1	4,1
Frontiers in Zoology	2	3,9
Plos One	1	3,7
Soil Biology & Biochemistry	1	3,7
Annals of Botany	1	3,4
Animal Behaviour	1	3,1
Behavioral Ecology and Sociobiology	1	2,8
Journal of Chemical Ecology	3	2,5
Evolutionary Biology	1	2,4
Biological Journal of the Linnean Society	1	2,4
Fungal Biology	1	2,1
Plant Signaling and Behavior	1	2,0
Acta Oecologica	1	1,6
Comptes Rendus Geoscience	1	1,4
Insectes Sociaux	2	1,3
Journal of Ethology	1	1,0
Journal of Insect Behavior	1	0,9
Sociobiology	2	0,6

6. Liste complète des publications

Publications de rang A
PDF disponibles sur http://www.cefe.cnrs.fr/interaction-biotiques/rumsais-blatrix
Les noms surlignés en gris sont ceux des étudiants et post-docs que j'ai encadrés ou dont j'ai participé à l'encadrement.

A35. Blatrix R., McKey D., Born C., 2013. Consequences of past climate change for species engaged in obligatory interactions. Comptes Rendus Geoscience, 345, 306-315.

A34. Blatrix R., Debaud S., Salas-Lopez A., Born C., Benoit L., McKey D., Atteke C., Djiéto-Lordon C., 2013. Repeated evolution of fungal cultivar specificity in independently evolved ant-plant-fungus symbioses. Plos One, 8, e68101.

A33. Molecular Ecology Resources Primer Development Consortium, 2013. Permanent genetic resources added to Molecular Ecology Resources database 1 February 2013-31 March 2013. Molecular Ecology Resources, 13, 760-762. (Benoit L., Blatrix R., Djiéto-Lordon C., Atteke C., Mezui-M'Eko J., Dubois M.-P., McKey D., Born C. Characterization of microsatellite loci for a fungal symbiont (Ascomycota, Chaetothyriales) in an ant-plant-fungus symbiosis)

A32. Renard D., Birk J. J., Zangerlé A., Lavelle P., Glaser B., Blatrix R., McKey D., 2013. Ancient human agriculture practices can promote activities of contemporary non-human soil ecosystem engineers: a case study in coastal savannas of French Guiana. Soil Biology & Biochemistry, 62, 46-56.

A31. Peccoud J., Piatscheck F., Yockteng R., Garcia M., Sauve M., Djiéto-Lordon C., Harris D. J., Wieringa J. J., Breteler F. J., Born C., McKey D., Blatrix R., 2013. Multi-locus phylogenies of the genus *Barteria* (Passifloraceae) portray complex patterns in the evolution of myrmecophytism. Molecular Phylogenetics and Evolution, 66, 824-832.

A30. Delattre O., Blatrix R., Châline N., Chameron S., Fédou A., Leroy C., Jaisson P., 2012. Do host species evolve a specific response to slave-making ants? Frontiers in Zoology, 9, 38.

A29. Blatrix R., Djiéto Lordon C., Mondolot L., La Fisca P., Voglmayr H., McKey D., 2012. Plant-ants use symbiotic fungi as a food source: new insight into the nutritional ecology of ant-plant interactions. Proceedings of the Royal Society B, 279, 3940-3947.

A28. Blatrix R., Renard D., Djiéto-Lordon C., McKey D., 2012. The cost of myrmecophytism: insights from allometry of stem secondary growth. Annals of Botany, 110, 943-951.

A27. Molecular Ecology Resources Primer Development Consortium, 2012. Permanent genetic resources added to Molecular Ecology Resources database 1 December 2011-31 January 2012. Molecular Ecology Resources, 12, 570-572. (Sauve M., Garcia M., Djiéto-Lordon C., Peccoud J., Piatscheck F., Dubois M. P., McKey D., Harris D. J., Blatrix R. Isolation and characterisation of 17 microsatellite loci for the ant-plant *Barteria fistulosa* (Passifloraceae) and cross-amplification in the other species of the genus)

A26. Molecular Ecology Resources Primer Development Consortium, 2012. Permanent genetic resources added to Molecular Ecology Resources database 1 August 2011-30 September 2011. Molecular Ecology Resources, 12, 185-189. (Piatscheck F., Djiéto-Lordon C., Garcia M., Sauve M., Peccoud J., Dubois M. P., McKey D., Blatrix R. Isolation and characterisation of 14 polymorphic microsatellite loci for the plant-associated ant *Tetraponera aethiops* (Hymenoptera: Formicidae) and cross-amplification in a closely related species)

A25. Vittecoq M., Djiéto-Lordon C., McKey D., Blatrix R., 2012. Range expansion induces variation in a behavioural trait in an ant-plant mutualism. Acta Oecologica, 38, 84-88.

A24. Voglmayr H., Mayer V., Maschwitz U., Moog J., Djiéto-Lordon C., Blatrix R., 2011. The diversity of ant-associated black yeasts: Insights into a newly discovered world of symbiotic interactions. Fungal Biology, 115, 1077-1091.

A23. Vittecoq M., Djiéto-Lordon C., Buatois B., Dormont L., McKey D., Blatrix R., 2011. The evolution of communication in two ant-plant mutualisms. Evolutionary Biology, 38, 360-369.

A22. Defossez E., Djiéto-Lordon C., McKey D., Selosse M.A., Blatrix R., 2011. Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. Proceedings of the Royal Society B, 278, 1419-1426.
A21. Blatrix R., Mayer V. 2010. Communication in ant-plant symbioses. Pp. 127-158. In: Baluska F. and Ninkovic V. eds. *Plant communication from an ecological perspective*. Springer, Berlin.
A20. Blatrix R., Bouamer S., Morand S., Selosse M.A., 2009. Ant-plant mutualisms should be viewed as symbiotic communities. Plant Signaling and Behavior, 4, 554-556.
A19. Schatz B., Djiéto-Lordon C., Dormont L., Bessière J.M., McKey D., Blatrix R., 2009. A simple non-specific chemical signal mediates defence behaviour in a specialised ant-plant mutualism. Current Biology, 19, 361-362.
A18. Defossez E., Selosse M.-A., Dubois M.-P., Mondolot L., Faccio A., Djiéto-Lordon C., McKey D., Blatrix R., 2009. Ant-plants and fungi: a new threeway symbiosis. New Phytologist, 182, 942-949.
A17. Hora R. R., Blatrix R., Fresneau D., Fénéron R., 2009. Social interactions between an inquiline ant, *Ectatomma parasiticum*, and its host *Ectatomma tuberculatum* (Formicidae, Ectatomminae). Journal of Ethology, 27, 285-288.
A16. Léotard G., Defossez E., Debain C., McKey D., Kjellberg F., Blatrix R., 2008. Local genetic co-structuring of the ant *Petalomyrmex phylax* and its host plant *Leonardoxa a. africana*: no role for a sixty meter river width in separating social forms. Sociobiology, 51, 363-371.
A15. Bono J. M., Blatrix R., Antolin M. F., Herbers J. M., 2007. Pirate ants (*Polyergus breviceps*) and sympatric hosts (*Formica occulta* and *Formica* sp. cf. *argentea*): host specificity and coevolutionary dynamics. Biological Journal of the Linnean Society, 91, 565-572.
A14. Denis D., Blatrix R., Fresneau D., 2006. How an ant manages to display individual and colonial signals using the same channel. Journal of Chemical Ecology, 32, 1647-1661.
A13. Blatrix R., Sermage C., 2005. Role of early experience in ant enslavement: a comparative analysis of a host and a non-host species. Frontiers in Zoology, 2:13.
A12. Genton B. J., Jonot O., Thevenet D., Fournier E., Blatrix R., Vautrin D., Solignac M., Giraud T., 2005. Isolation of five polymorphic microsatellite loci in the invasive weed *Ambrosia artemisiifolia* (Asteraceae) using an enrichment protocol. Molecular Ecology Notes, 5, 381-383.
A11. Blatrix R., Herbers J. M., 2004. Intracolonial conflict in the slave-making ant *Protomognathus americanus*: dominance hierarchies and individual reproductive success. Insectes Sociaux, 51, 131-138.
A10. Blatrix R., Herbers J. M., 2003. Coevolution between slave-making ants and their hosts: host specificity and geographical variation. Molecular Ecology, 12, 2809-2816.
A9. Blatrix R., Jaisson P., 2002. Absence of kin discrimination in a ponerine ant. Animal Behaviour, 64, 261-268.
A8. Blatrix R., Schulz C. M., Jaisson P., Francke W., Hefetz A., 2002. Trail pheromone of ponerine ant *Gnamptogenys striatula*: 4-methylgeranyl esters from Dufour's gland. Journal of Chemical Ecology, 28, 2557-2567.
A7. Schulz C. M., Lehmann L., Blatrix R., Jaisson P., Hefetz A., Francke W., 2002. Identification of new homoterpene esters from Dufour's gland of the ponerine ant *Gnamptogenys striatula*. Journal of Chemical Ecology, 28, 2541-2555.
A6. Blatrix R., Jaisson P., 2001. Reproductive strategy of the ponerine ant *Gnamptogenys striatula* Mayr (Hymenoptera: Formicidae). Sociobiology, 37, 147-161.

A5. Giraud T., Blatrix R., Poteaux C., Solignac M., Jaisson P., 2001. High genetic relatedness among nestmate queens in the polygynous ponerine ant *Gnamptogenys striatula* in Brazil. Behavioral Ecology and Sociobiology, 49, 128-134.
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A3. Blatrix R., Jaisson P., 2000. Optional gamergates in the queenright ponerine ant *Gnamptogenys striatula* Mayr. Insectes Sociaux, 47, 193-197.
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7. Cinq publications représentatives de mon parcours

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Multi-locus phylogenies of the genus *Barteria* (Passifloraceae) portray complex patterns in the evolution of myrmecophytism

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ABSTRACT

The four species of the central African genus *Barteria* show variation in habitat and in degree of association with ants. Whereas *B. solida*, restricted to submontane forests, attracts opportunistic ants to extrafloral nectar, the three other species, found in lowland rainforests (*B. fistulosa*, *B. dewevrei*) and in littoral scrub (*B. nigritana*), possess stem domatia of varying shapes and degrees of specialisation, hosting either non-specific arboreal ants (*B. nigritana*, some *B. dewevrei*) or two large species of ants of the genus *Tetraponera* Smith, 1852 that are specific to some species of *Barteria* (*B. fistulosa*, some *B. dewevrei*). We aimed to investigate whether this variation represents an evolutionary trend toward increasing specialisation of mutualism or the reduction or loss of myrmecophytic traits. For this, we determined phylogenetic relationships within the genus using DNA sequences (primarily nuclear ITS) and microsatellite genotypes (11 loci) on a large sample of individuals, mostly from Cameroon and Gabon. The two types of markers support an initial dichotomy that groups *B. dewevrei* with *B. nigritana* and *B. fistulosa* with *B. solida* respectively. Within these pairs, species do not appear reciprocally monophyletic. At microsatellite loci, *B. nigritana* forms a clade embedded within *B. dewevrei*; and within both *B. solida* and *B. fistulosa*, geographical populations show levels of differentiation similar to that observed between populations of *B. solida* and *B. fistulosa*. Geographic distance alone does not account for genetic differentiation between species, which indicates reproductive isolation. Divergence in each of the two pairs implies evolutionary transitions in habitat and in myrmecophytism. Specialised mutualism with specific ant species of the genus *Tetraponera* has been lost in species found in more marginal habitats.

1. Introduction

The tree genus *Barteria* Hook. f. (Passifloraceae) is endemic to the forests of Lower Guinea and the Congo basin. It comprises four recognised species of small trees that show variable degrees of symbiotic association with ants. Three species are myrmecophytic: they have swollen, hollow cavities in lateral branches, called domatia, that are used by ants as nesting sites. The four species were recognised as such only recently (Breteler, 1999). Photographs in Breteler (1999) illustrate the considerable variation in stem morphology related to myrmecophytism among these taxa. *Barteria solida* Breteler, the most recently described species, is essentially restricted to submontane forest in a few isolated sites in the Lower Guinea forest block (from extreme south-eastern Nigeria through Cameroon and Equatorial Guinea to Gabon). This species produces extrafloral nectar on its leaf margins and on the decurrent leaf extensions along the branches, which attracts opportunistic ants, but does not display specialised structures for hosting ants. *Barteria nigritana* Hook. f. is mostly restricted to a very narrow band of land along the Atlantic coast, where it grows on sandy beaches and other Holocene deposits. In southern Gabon, however, it also occurs in the interior, reaching 650 m altitude (So-

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sef et al., 2006; JW, unpubl. obs.). In the west of Cameroon it occurs as far inland as Mamfe at an altitude of 170 m. It is also recorded as more than 100 km inland in Nigeria and Democratic Republic of the Congo (Breteler, 1999). Each of its lateral branches bears at its base a swollen, spindle-shaped, hollow cavity, narrow and less than 15 cm long, that is used as a nesting site by various tree-dwelling ants, none of them specialist inhabitants of ant-plants (Djiéto-Lordon et al., 2004). Ants of the genus *Crematogaster* Lund, 1831 are its most frequent occupants. *Barteria fistulosa* Mast. is widely distributed in lowland forest over the whole Lower Guinea-Congo basin forest block. It is a light-loving tree that is very common in forest gaps. Every lateral branch is hollow and swollen over its entire length. Its swollen stems are wider than those of *B. nigritana*. Each individual tree is occupied by a single colony of one of two ant species of *Tetraponera*, *T. aethiops* F. Smith, 1877 or *T. latifrons* (Emery, 1912). These species are the largest (~1 cm long) of their genus (Wheeler, 1922) and are only found in association with individuals of *Barteria* presenting wide (broad) domatia. *Barteria fistulosa* also depends strongly on these ants, which protect their host tree against herbivores and prune plants adjacent to their host tree, and vines growing on it (Dejean et al., 2008; Janzen, 1972; McKey, 1974). Only rarely are trees of this species found without a colony of *Tetraponera* ants. The distribution of the fourth species, *B. dewevrei* De Wild. & T. Durand broadly overlaps with that of *B. fistulosa*, although the former species was previously thought to be absent from Cameroon and Gabon (Breteler, 1999). Lateral branches of *B. dewevrei* are hollow over their entire length, but the diameter of the cavity is more variable than in *B. fistulosa*. In the eastern part of its range it seems that most individuals are occupied by small, non-specialist ants of the genus *Crematogaster* (~4 mm long), whereas in the western part the large ants of the genus *Tetraponera* (*T. aethiops* and *T. latifrons*) are by far the most frequent inhabitants. In the course of this study, we discovered the presence of *B. dewevrei* much further west (Cameroon and north-western Gabon) than its previously known range. In these western populations, its domatia are very similar to those of *B. fistulosa*. Whether the morphotype with wide domatia occupied by *Tetraponera* occurs outside Cameroon, Gabon and Central African Republic still remains to be investigated. Fig. 1 illustrates the differences in myrmecophytic traits between species.

Obligate symbiosis with ants has appeared many times in the evolution of angiosperms, but myrmecophytes do not constitute large clades (Davidson and McKey, 1993). This suggests frequent acquisitions of myrmecophytic traits. It also suggests their frequent disappearance, which could result from two quite different processes. The first is the "evolutionary dead end scenario", whereby ecological specialisations, such as species-specific obligate mutualisms, arise and evolve but eventually end in extinction (for discussion see Althoff et al., 2012; Tripp and Manos, 2008). If this is so, myrmecophytic specialisation may be irreversible, and specialised myrmecophytes may be prone to extinction when conditions no longer favour them. Alternatively, specialisation may be reversible; in this case the lineages may survive but lose their myrmecophytic traits (Davidson and McKey, 1993; Janzen, 1974). Phylogenetic analyses suggest instances of reversal from obligate myrmecophytism to nonmyrmecophytism, and loss of mutualistic specificity in myrmecophytes, in two of the largest myrmecophyte radiations, *Macaranga* (Euphorbiaceae) (Blattner et al., 2001; Davies et al., 2001) and *Neonauclea* (Rubiaceae) (Razafimandimbison et al., 2005), but not in a third, neotropical *Acacia* (Fabaceae: Mimosoideae) (Gomez-Acevedo et al., 2010). The evolutionary scenarios and environmental conditions leading to gains and losses of myrmecophytic traits are largely unknown and must be investigated by reconstructing phylogenies of closely related species. The contrasting degree of association with ants in *Barteria* makes this genus a good model system for investigating such questions.

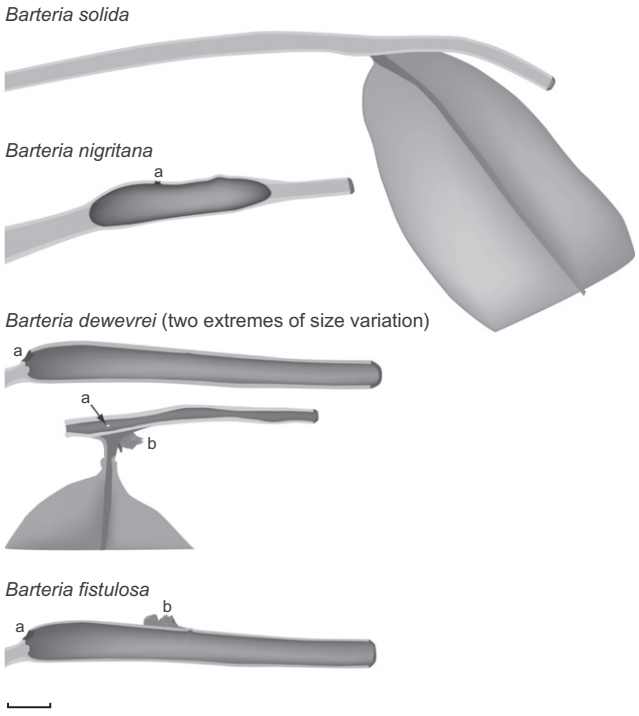

Fig. 1. Schematic representation of longitudinal sections of the base of plagiotropic branches of the four *Barteria* species showing shape of domatia (dark grey shading). *Barteria solida* has no domatia. *Barteria nigritana* has lateral branches swollen and hollow only on a short proximal section. *Barteria dewevrei* has lateral branches hollow throughout their length, more or less swollen depending upon the individual. *Barteria fistulosa* has swollen lateral branches hollow throughout their length. (a) Entrance holes made by ants; (b) dry flower bracts (post-fruiting). Scale bar = 1 cm. Drawings by Simon Benateau.

Currently, there is no hypothesis regarding the phylogeny of the four currently recognised taxa of *Barteria*, whose status as biological species (in terms of reproductive isolation) is not strictly established. We thus determined phylogenetic relationships within the genus using both DNA sequences and microsatellite data on a large sample of individuals, mostly from Cameroon and Gabon. The phylogenies obtained allowed us to highlight an unexpectedly complex pattern of evolution of myrmecophytic traits.

2. Materials and methods

2.1. Sample collection

Large sample sizes are required to clarify the species status and relatedness of taxa for which reproductive isolation has not been tested specifically. For this study, we obtained genotypes for 696 specimens of the four recognised species of *Barteria*. Out of the 53 specimens provided by herbaria, 40 were used in this study (we could not obtain genotypes for the others). Samples of leaves for 656 individual trees were collected in the field by the authors or their colleagues specifically for genetic analyses. These samples consisted in a piece of leaf 10 × 5 cm that was dried in silica gel immediately after collection. A very small fraction of each sample was used for DNA extraction. The remaining material is stored in the Centre d'Ecologie Fonctionnelle et Evolutive (Montpellier, France). Apart from the four species names currently recognised in the genus *Barteria* (Breteler, 1999), four others have been published. *Barteria acuminata* Baker f. and *B. stuhlmannii* Engl. & Gilg., both junior synonyms of *B. dewevrei*, were described respectively

from Uganda and Tanzania. We did not include the type specimen of *B. acuminata* in our analysis, but we included a specimen collected in the same area of Tanzania as the neotype of *B. stuhlmannii*. *Barteria braunii* Engl., a junior synonym of *B. nigritana*, was described from the village of Batanga, Cameroon. For this study we collected 30 specimens in Batanga, the type locality of *B. braunii*. *Barteria urophylla* Mildbr. is a *nomen nudum*. Fig. 2 shows the geographical origin of each *Barteria* used. The genus *Barteria* belongs to the tribe Paropsieae, which is composed of six genera. We obtained specimens of two other genera in the Paropsieae: *Paropsia edulis* Thou. and *Androsiphonia adenostegia* Stapf. We also obtained specimens of three other genera in the Passifloraceae to be used as outgroups: *Adenia cynanchifolia* Harms, *Efulensia clematoides* C.H. Wright and *Passiflora viridescens* L.K. Escobar. Table 1 summarises the samples used. A Supplementary Table (Table S1) provides detailed information on individual samples.

2.2. Molecular methods

For each specimen, DNA was isolated from 0.2 g of dry leaves. Extractions were completed using the DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) or the Extract-N-Amp PCR ReadyMix (Sigma–Aldrich, St. Louis, USA) following the manufacturer's instructions.

DNA sequence variation was investigated at nuclear (the Internal Transcribed Spacer locus comprising part of ITS1, 5.8S and ITS2) and plastid markers (matK and spacer 5'trnK-matk, trnH-psbA, rbcL, trnL), using standard Polymerase Chain Reaction (PCR) and Sanger sequencing. Table 1 indicates only those markers whose variation is detailed in Section 3. Table 2 presents the set of all markers investigated (including those in which variation was too low for a meaningful analysis), together with the primers used to amplify them.

Amplification of the ITS marker with primers "itsRYf" and "itsRYr" used 50 µl of solution containing 1X buffer (Q-Biogene, Montreal, Canada), 0.2 U of Taq polymerase (Q-Biogene), 2.5 mM of $MgCl_2$, 0.25 mM of dNTPs (Promega Corp, Fitchburg, USA.), 0.2 µM of each primer and 2 µl of DNA template. Amplifications took place in a thermal cycler programmed for an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 60 s at 94 °C, 45 s at 50–55 °C and an elongation step of 60 s at 72 °C.

Sequences obtained with this procedure allowed designing more specific internal primers for increased PCR yields (ITS-Bar2F and ITS-Bar2R, see Table 2). Amplifications were performed in a 30 µl solution containing 1X PCR mix (Qiagen multiplex kit), 0.2 µM of each primer and 2 µl of DNA template. They took place in a thermal cycler programmed for an initial denaturation step of 15 min at 94 °C, followed by 35 cycles of 60 s at 95 °C, 90 s at 58 °C and 60 s at 72 °C. The last elongation step lasted 11 min.

Products obtained with the "itsRYf" and "itsRYr" primers were purified by the PEG precipitation protocol (Rosenthal et al., 1993) and both strands were sequenced using the same primer combination as for PCR amplifications. Cycle sequencing products were run on ABI capillary sequencers (Applied Biosystems, Foster City, USA). Products obtained with the internal primers were purified and sequenced by Genoscreen (Lille, France).

Eleven microsatellite markers were genotyped for all individuals of *Barteria* according to a protocol described previously (Molecular Ecology Resources Primer Development Consortium et al., 2012). These markers, initially developed for *B. fistulosa*, showed reliable amplification in the other species (Molecular Ecology Resources Primer Development Consortium et al., 2012) and are: Bar6, Bar12, Bar16, Bar27, Bar50, Bar51, Bar53, Bar61, Bar62, Bar64 and Bar69. However, these markers failed to amplify in individuals belonging to the other genera.

2.3. Analysis of genetic data

Plastid markers did not show sufficient variation in *Barteria* for an informative phylogeny. Thus, we used only the ITS locus for phylogenetic reconstructions.

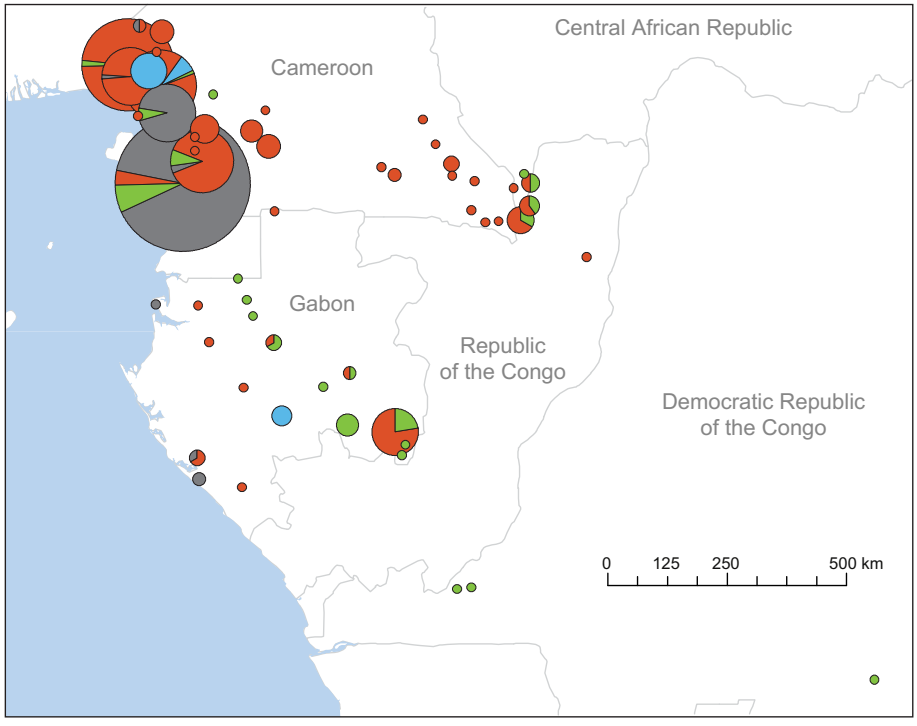

Fig. 2. Map of the collected samples. Each disc represents a group of sampling sites connected by less than 20 km. Size of the disc is proportional to the number of sampled individuals. Sectors of different colours represent *Barteria* species: red, *B. fistulosa*; grey, *B. nigritana*; green, *B. dewevrei*; blue, *B. solida*.

Table 1
Samples used in this study, with numbers of individuals successfully analysed at ITS sequences and microsatellite markers.

Taxon	Myrmecophytic traits	Country	ITS sequences	Microsatellite genotypes
Barteria dewevrei	Long, narrow to wide domatia, non-specialist ants of the genus Crematogaster or specialist ants of the genus *Tetraponera*	Cameroon	10	26
		Republic of the Congo	6	6
		Gabon	15	21
		Central African Republic	3	3
		Democratic Republic of the Congo	2	2
		Tanzania	1	1
Barteria fistulosa	Long, wide domatia, specialist ants of the genus *Tetraponera*	Cameroon	30	301
		Republic of the Congo	3	10
		Gabon	23	30
		Central African Republic		3
Barteria nigritana	Short domatia, diverse small, opportunistic ants	Cameroon	24	246
		Gabon	3	4
Barteria solida	No domatia	Cameroon	21	21
		Gabon	4	5
Adenia cynanchifolia	No domatia	Gabon	1	
Androsiphonia adenostegia	No domatia	Ivory Coast	1	
Efulensiaia clematoides	No domatia	Cameroon	1	
Paropsia edulis	No domatia	Madagascar	1	
Passiflora viridescens	No domatia	Equator	1	

Table 2
Sequence loci amplified in *Barteria* samples and outgroups. Only ITS showed sufficient variation in *Barteria* for an informative phylogeny.

Locus	Primer sequences (5′-3′)	Primer names	References
Nuclear internal transcribed spacer (ITS)	GGAAGTAGAAGTCGTAACAAG	itsRYf	(White et al., 1990)
	TCCTCCGCTTATTGATATGC	itsRYr	
	AGAACGACCCGCGAACAT	ITSBar2-F	This study
	TGGGGTCGCGACATAGAG	ITSBar2-R	
*mat*K and spacer 5′*trn*K-*mat*k	TGGGTTGCTAACTCAATG	*trnK*-3914 F	(Johnson and Soltis, 1994)
	AGAATGGATTTGCCTTGA	mat839 R	This study
*trn*H-*psb*A	CGCGCATGGTGGATTCACAATCC	trnH^{GUG}	(Tate and Simpson, 2003; Sang et al., 1997)
	GTTATGCATGAACGTAATGCTC	psbA	
*rbc*L	TCGCATGTACCTGCAGTAGC	rbcl724r	(Savolainen et al., 2000)
	ATGTCACCACAAACAGAAAC	rbcl1f	
*trn*L	CGAAATCGGTAGACGCTACG	trnL_C	(Taberlet et al., 1991)
	GGGGATAGAGGGACTTGAAC	trnL_D	
	GGTTCAAGTCCCTCTATCCC	trnL_E	(Taberlet et al., 1991)
	ATTTGAACTGGTGACACGAG	trnL_F	

Sequence data for the ITS marker were obtained for 145 individuals of *Barteria*. Sequence chromatograms were trimmed, assembled (for each individual), aligned using the Muscle algorithm and visually checked under Geneious Pro 5 (Drummond et al., 2011).

Many individuals showed ambiguities in base calling at one or several polymorphic sites. Ambiguities might have resulted from heterozygosity, but also from variation among the numerous repeats of ribosomal DNA gene clusters (Alvarez and Wendel, 2003). This uncertainty prevented us from inferring the gametic phase of the genotypes, and from recovering sequences of every gene copy via cloning of PCR products. We instead scored any ambiguous nucleotide in a given individual as such.

Maximum likelihood phylogenies were constructed with the PhyML (Guindon and Gascuel, 2003) Geneious plug-in and assumed the Hasegawa–Kishino–Yano (Hasegawa et al., 1985) + Gamma model of nucleotide evolution favoured by the Bayesian Information Criterion calculated with jModelTest (Posada, 2008). The PhyML algorithm was set to estimate transition/transversion ratios and the gamma parameter and to optimise the tree topology, branch lengths and the substitution rate. One thousand trees were generated by bootstrapping to test node support.

Bayesian inference phylogenies were constructed with the MrBayes (Ronquist and Huelsenbeck, 2003) Geneious plug-in and assumed the same model of evolution. The same sequence data were used as for reconstruction of maximum likelihood phylogeny. Four Markov chains were run simultaneously and sampled every 1000 iterations for a total of five million iterations. Stationarity was reached around 10^6 generations; the first 20% of trees generated were thus discarded.

Genotypes for microsatellite loci were obtained for the 696 individuals of *Barteria*. Microsatellite data were used primarily to identify genetic groups and assess their correspondence with morphologically defined species, and then to infer the phylogenetic relationships between these genetic groups in comparison to results obtained from ITS sequences. For this, we first built a Neighbour-Joining tree of all 11-locus genotypes based on Nei's minimum distance (Saitou and Nei, 1987), using the software Populations 1.2.32 (Langella, 2002–2011). This allowed us to delineate main genetic groups, all of which were consistent with taxon

membership and geographical origin. We then built a Neighbour-Joining phylogeny of these genetic groups using the same distance measure. We also used the Average Square Distance (Goldstein et al., 1995; Slatkin, 1995), which assumes closer relatedness between microsatellite alleles of similar sizes (the stepwise mutation model of microsatellite evolution, Ohta and Kimura, 1973). For each analysis, 1000 trees were generated by bootstrapping over loci.

Because some taxa of *Barteria* were not sampled from the same site (they grow in different habitats), genetic differentiation might merely reflect distance between sampled individuals and not necessarily reproductive isolation. To investigate barriers to gene flow beyond spatial distance, we examined whether genetic differentiation between any two individuals, as measured by Rousset's a_r (Rousset, 2000), was correlated with the natural logarithm of distance separating them. We then examined whether taxon membership could explain differentiation better than distance alone. We achieved this by correlating the a_r matrix to the distance matrix and then by correlating the matrix of residuals to a binary matrix with "0" for pairs of individuals of the same taxon and "1" for heterospecific pairs. Significance of correlations was tested by permuting data in matrices 5000 times (partial Mantel test) in FSTAT (Goudet, 1995). We restricted this test to the two pairs of taxa for which it was necessary (see Section 3).

3. Results

Fig. 3 shows the Maximum Likelihood (ML) tree of ITS sequences obtained from 145 individuals (Genbank accession numbers KC207253 to KC207402). We note that the limited level of sequence variation in this phylogeny limits the impact of the chosen model of nucleotide evolution (HKY with gamma) on the generated topology and contributes to the low bootstrap support of most clades. The Bayesian consensus tree is shown as a Supplementary figure (Fig. S1). Maximum Likelihood and Bayesian inference produced compatible phylogenies with respect to the well-supported clades. An initial dichotomy groups *B. dewevrei* with *B. nigritana* and *B. fistulosa* with *B. solida* respectively, with relatively good support. Within these two clades, individuals of each species do not form monophyletic groups. *Barteria nigritana* and *B. dewevrei* are admixed throughout most of the upper clade in Fig. 3 and share many ITS sequences. Individuals of *Barteria fistulosa* and *B. solida* are found in distinct, albeit poorly supported, groups in several parts of the lower clade. In this clade, some geographical grouping can be noted, as individuals from Gabon are paraphyletic with respect to samples from Cameroon. Variation at chloroplast sequences (four markers, see Table 2, Genbank accession numbers KC207127 to KC207252) obtained on a subsample of the four taxa was extremely low, with at most two polymorphic positions separating the same taxa pairs at the ITS marker.

Fig. 4 shows the microsatellite phylogeny grouping the 11-locus genotype of all individuals compared with Nei's minimum distance (Saitou and Nei, 1987). The microsatellite phylogeny could not be rooted because our microsatellites failed to amplify in the other genera of Passifloraceae we tested, including two genera of the tribe Paropsieae. Notably, all individuals from different taxa of *Barteria* constitute different clusters outlined on the figure, with the sole exception of a few possible hybrids of intermediate genotype. The grouping of the two pairs of taxa by ITS sequences (Fig. 3) is also found at microsatellite loci. These highly variable markers further differentiate genotypes of *B. nigritana* and *B. dewevrei*. Individuals from different geographic regions within taxa form very distinct clusters, such that, except for *B. nigritana*, the recognised species of *Barteria* do not appear monophyletic. Among individuals of *B. nigritana* and *B. dewevrei*, the logarithm of spatial distance explained 35.4% of the variance in genetic differentiation (Rousset's a_r) while membership in different taxa explained a further 39%.

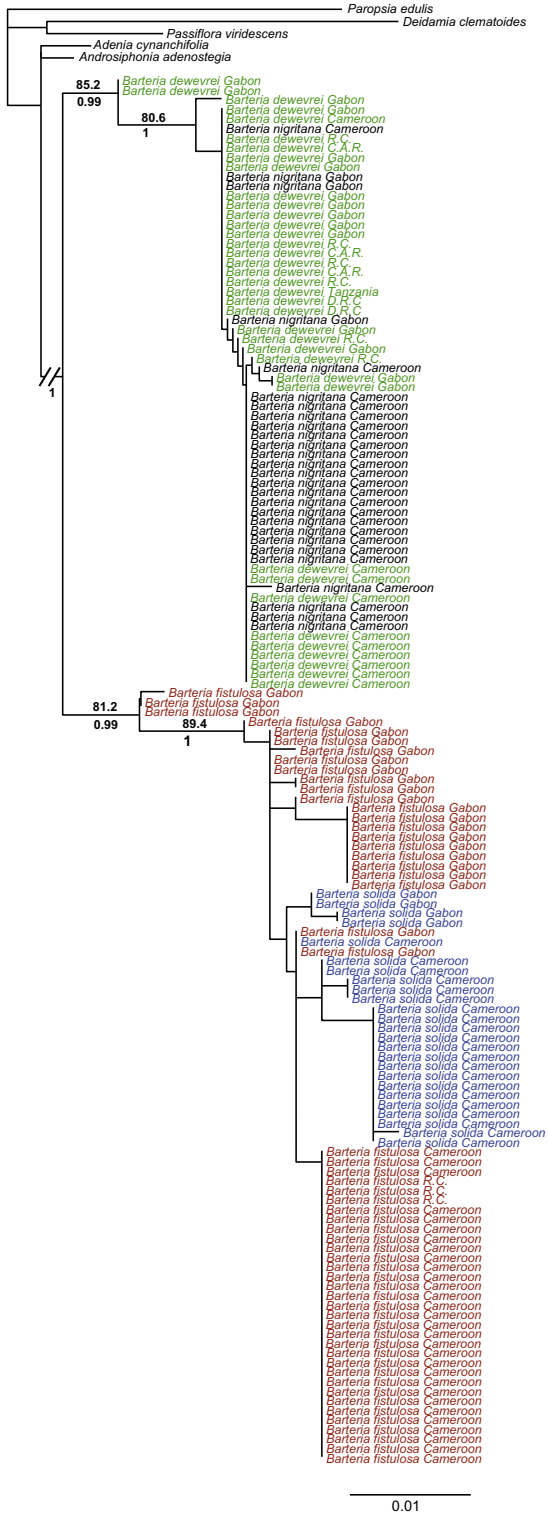

Fig. 3. Maximum likelihood tree of ITS sequences (based on sequences of 521 aligned nucleotides) obtained from the four species of *Barteria* (145 individuals) and outgroups. *Barteria* samples are denoted by species names in different colours and by country of origin (abbreviations: R.C.: Republic of the Congo, C.A.R: Central African Republic, D.R.C.: Democratic Republic of the Congo). Numbers above branches indicate the percentage of bootstrap replicates that support the corresponding clades (only values above 75 are shown) and numbers below branches represent Bayesian posterior probabilities for these clades (showing only values above 0.98). The scale bar does not apply to branches leading to individuals not belonging to *Barteria* because they have been shortened to fit in the figure.

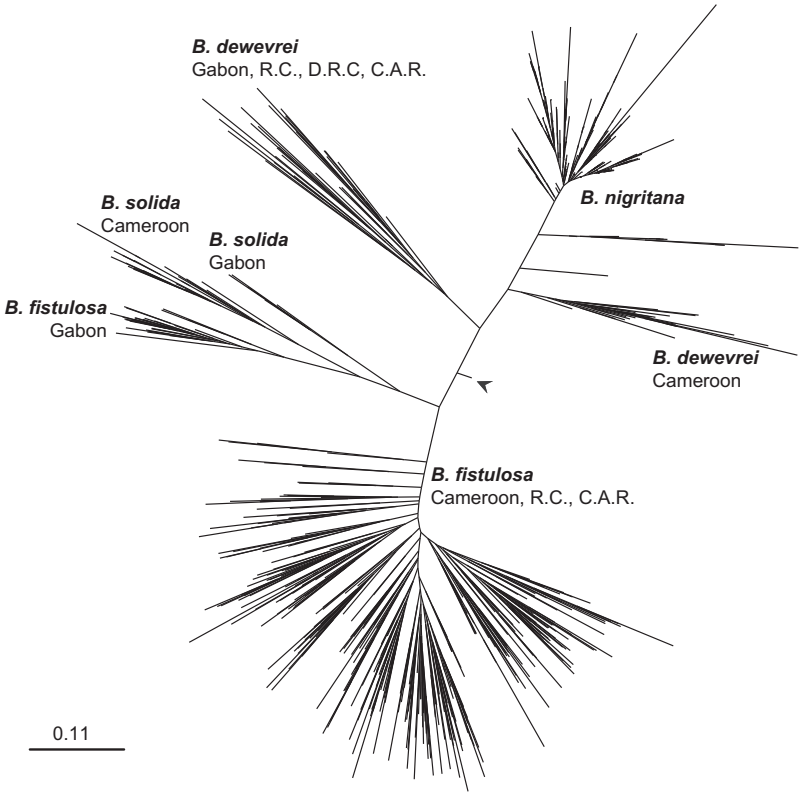

Fig. 4. Neighbour-Joining tree connecting 696 individuals (11-locus microsatellite genotypes) of four *Barteria* species, based on Nei's minimum distance. The origins of individuals composing the main groups are indicated with the same country abbreviations as in Fig. 3. The arrow points to a likely hybrid individual.

For *B. fistulosa* and *B. solida*, the percentages of variance explained were respectively 11.4% and 27.9%. All correlations were highly significant (none of the 5000 permutations produced stronger correlation), showing that membership in different taxa explained genetic differentiation better than mere distance between samples.

We used the seven genotypic clusters delineated in Fig. 4 to build population-based trees using two measures of genetic distance (Fig. 5), in order to decipher and statistically gauge their evolutionary relationships. Topologies grouping *B. nigritana* and *B. dewevrei* are consistent across trees (Figs. 4 and 5) and present relatively good statistical support. Populations of *B. solida* and *B. fistulosa* are also grouped in each tree with varying, poorly supported topologies.

4. Discussion

4.1. Generic and specific status in Barteria

The phylogeny based on ITS sequence data is consistent with monophyly of *Barteria*, an hypothesis that requires further testing by including the genus *Smeathmannia*, which appears to be the closest relative to *Barteria* (Tokuoka, 2012). Our broad sampling across the genus *Barteria* supported taxonomic decisions made in the most recent taxonomic revision (Breteler, 1999). For example, the phylogeny based on microsatellite loci showed that samples of *Barteria* from the type locality of one of the syonyms of *B. nigritana*, *B. braunii* (Batanga, Cameroon), fitted within the clade composed of individuals of *B. nigritana*. Similarly, the sample collected in the same district as the neotype specimen of *B. stuhlmannii* was included among *B. dewevrei*. Sleumer (1974) considered *fistulosa* as a sub-species of *B. nigritana* but Breteler (1999) treated them as two species. The genetic distance between the two taxa clearly establishes that *B. fistulosa* and *B. nigritana* are two separate species.

The microsatellite phylogeny of individuals (Fig. 4) separates genotypes belonging to different taxa into distinct genetic clusters, suggesting that gene flow between these groups is restricted or absent. Gene flow between *B. fistulosa* and *B. dewevrei* must be limited by strong or complete reproductive isolation, since these two taxa form clearly distinct genetic clusters at both ITS sequences and microsatellite markers, although they frequently grow at the same sites in Cameroon and in Gabon. The other two taxa of *Barteria*, i.e. *B. nigritana* and *B. solida*, have colonised distinct habitats and each only rarely grows in sympatry with any other *Barteria*. They may be isolated from related taxa by eco-geographic barriers (geographic isolation resulting from distance between habitats). Because genetic differentiation between individuals of different taxa is not explained by sampling distance alone, additional barriers to gene flow must exist. These barriers may be only historical, representing traces of some past range fragmentation that may in the future vanish through hybridisation. However, for a given pair of taxa of *Barteria*, populations collected in the same region (countries noted in Fig. 4) are not genetically closer than geographically distant populations. For instance, Gabonese *B. solida* are not genetically closer to Gabonese *B. fistulosa* than they are to Cameroonian *B. fistulosa* (Fig. 4). Observations thus indicate the absence of effective gene flow and high levels of reproductive isolation between all taxa of *Barteria*. In three of them, *B. dewevrei*, *B. fistulosa* and *B. solida*, microsatellite data also show deep divergences between geographically separate populations within each of the two countries, Cameroon and Gabon. In the last two species, the geographical differentiation is even visible at ITS sequences (Fig. 3). It is unclear at this stage whether such genetic differentiation only results from gaps in sampling, or whether barriers to gene flow are/were involved. For convenience, we shall refer to each of the four described taxa of *Barteria* as a "species", even though each may not have a unique origin and may actually encompass cryptic geographical species.

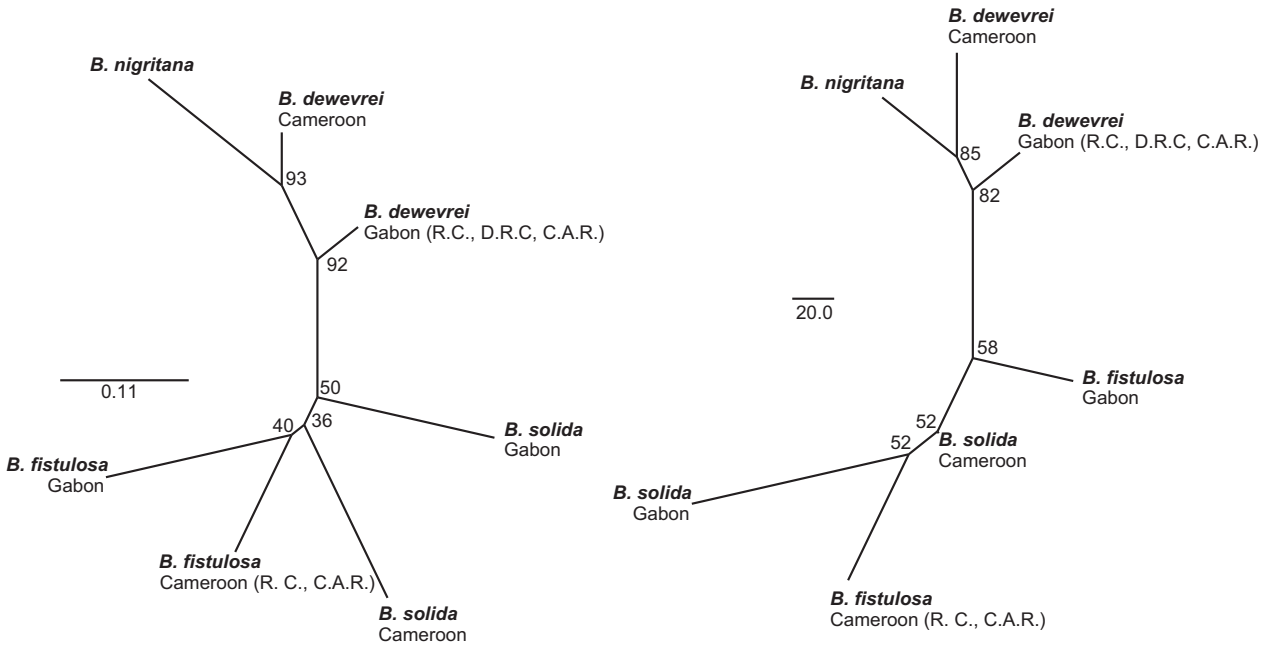

Fig. 5. Neighbour-joining trees connecting populations of four *Barteria* species, based on groups delineated in Fig. 4. Left: tree based on Nei's minimum distance. Right: tree based on the average square distance using information on microsatellite allele length. Numbers near branches indicate the percentage of bootstrap replicates (random draws of loci) that support the corresponding grouping. Country abbreviations as in Fig. 3.

Barteria species do not appear monophyletic based on ITS sequence data (Fig. 3). In terms of number of generations, species of *Barteria* thus appear to have diverged recently from their respective sister groups in comparison to their effective population sizes at the analysed loci (Avise, 2001), a result that is consistent with their overall morphological similarity. Despite their presumed recent divergence, species of *Barteria* show marked differences in respect to symbiotic traits and provide insight into the evolution of myrmecophytic traits. The small number of taxa of *Barteria* prevents statistical inference on ancestral states and on the probability of homoplasy of myrmecophytic traits. However, based on the limited genetic divergence among taxa, this probability seems low, assuming these traits have high heritability (limited plasticity). In the great majority of myrmecophytes, production of domatia has been shown to be inherited, and these structures are produced in the absence of ants (Beattie, 1985; Beccari, 1884–86; Jolivet, 1996). In fact, induction of domatia-like structures in the presence of ants is a highly exceptional phenomenon (Blüthgen and Wesenberg, 2001; Edwards et al., 2009). In *Barteria*, several observations suggest that some crucial myrmecophytic traits, in particular the size and shape of domatia, are heritable. Domatia are produced even in the absence of ants and can, in rare cases, harbour ant species usually associated with other types of domatia (Breteler, 1999; D. McKey, R. Blatrix, C. Djiéto-Lordon, personal observations). In a few instances (e.g., near Mamfé [South-West Region] and at Nkolo [South Region] in Cameroon), individuals of *B. nigritana* were found among trees of *B. fistulosa* and still presented their typical domatia and ants. As domatia shape is likely to have high heritability and taxa of *Barteria* appear to have diverged only recently, the evolution of myrmecophytism in the genus *Barteria* (developed in the following sections) can be discussed with an *a priori* limited risk of homoplasy in domatia shape.

4.2. Initial divergence between species pairs

The rooted ITS phylogeny shows an initial divergence of two pairs of species: *B. dewevrei* and *B. nigritana* versus *B. fistulosa* and *B. solida*. The genetic distance at highly variable microsatellite loci also rapidly reaches saturation and may not reflect increasing divergence between species pairs. However, we note that 6 of the 17 microsatellites initially developed for *B. fistulosa* failed to amplify in both *B. dewevrei* and *B. nigritana* (Molecular Ecology Resources Primer Development Consortium et al., 2012), while all markers could be genotyped in *B. solida*. Amplification failures likely result from mutations at microsatellite flanking regions, which are much less frequent than mutations within the microsatellite themselves (Weber and Wong, 1993). This observation supports the hypothesis of significantly greater genetic divergence between the two pairs of species than between species within each pair. We therefore consider the ancestral divergence indicated by the ITS phylogeny as real.

Interestingly, this initial divergence led to two species of similar phenotype. *Barteria fistulosa* and western *B. dewevrei* both present long, wide domatia that can harbour the large *Barteria*-specific ant species of the genus *Tetraponera*. This phenotype would thus constitute the ancestral state of all sampled *Barteria* populations, should the monophyly of *Barteria* be confirmed after considering relationships with its close relative *Smeathmannia* (Tokuoka, 2012).

4.3. Relationships between B. fistulosa and B. solida

Phylogenies group *B. fistulosa* and *B. solida*, but show different, poorly supported topologies that fail to clarify the relationships between the two taxa. The paraphyly of Gabonese *B. fistulosa* in respect to *B. solida* and *B. fistulosa* from other countries at the ITS gene (Fig. 3) does not appear in the microsatellite tree (Fig. 4), which groups conspecific individuals according to their source regions. Possibly, patterns of historical species paraphyly are being erased by local gene flow, to which multilocus microsatellite genotypes are highly sensitive.

These two morphological taxa present extreme phenotypes: *B. solida* completely lacks domatia and is essentially restricted to widely scattered patches of submontane forest, whereas *B. fistulosa* has long, wide domatia, lives in symbiosis with *Tetraponera*, and is common in lowland forest throughout the region. Our results imply profound evolutionary transitions in habitat and in myrmeco-

phytism. Our aforementioned scenario of ancestral symbiosis with *Tetraponera* would imply a loss (or several losses) of myrmecophytism in *B. solida*. Such loss would be consistent with the adaptation of *B. solida* to higher-elevation habitats that are unfavourable to ants, and with the fact that ITS lineages of *B. solida* are embedded within the diversity of *B. fistulosa*.

4.4. Relationships between B. dewevrei and B. nigritana

ITS-based phylogeny (Fig. 3) showed a large polytomy in the clade composed of *B. dewevrei* and *B. nigritana*, with specimens of both species interwoven. In contrast, microsatellite markers are much more variable, and accordingly, separate the two species in the phylogeny. Microsatellite-based phylogenies consistently show the paraphyly of *B. dewevrei* with respect to *B. nigritana*, with well-supported topologies (Figs. 4 and 5). Genotypes of *Barteria nigritana* constitute a sister group of Cameroonian *B. dewevrei*. This topology may reflect recent ancestry or genetic convergence due to local interspecific gene flow. Genetic convergence is not supported by the few Gabonese *B. nigritana* samples, which appear genetically closer to Cameroonian *B. dewevrei* than to Gabonese *B. dewevrei* sampled at much closer distances. Derivation of *B. nigritana* from a population of *B. dewevrei* is also consistent with its lower diversity at microsatellite markers (Table S1), and constitutes a more likely scenario.

Under this scenario, parsimony posits the phenotype of *B. nigritana*, with horizontal branches swollen only at their basal part, as a derived state. The candidate ancestral state found in *B. dewevrei* corresponds to domatia that extend over the whole length of the branch and vary from narrow to wide. Within *B. dewevrei*, the width of domatia does not seem to be correlated with genetic variation at microsatellite loci, providing no additional information to infer the ancestral phenotype of this species. In the western part of the distribution of *B. dewevrei*, only the phenotype with the widest domatia harbouring *Tetraponera* is present. Because *B. nigritana* is related to Cameroonian *B. dewevrei* and is restricted to the western, coastal part of the region, this species is most likely derived from a population of *B. dewevrei* presenting wide and long domatia. Such a scenario implies a shift from obligate symbiosis with *Tetraponera* to mutualism with less specific "opportunistic" ants of various genera (Djiéto-Lordon et al., 2004). This transition in myrmecophytism would have co-occurred with an ecological shift adapting *B. nigritana* to more open coastal habitats.

4.5. Phylogenetic value of morphological characters

The most relevant morphological characters used to differentiate recognised taxa of *Barteria* are: (i) the shape of the apex of floral bracts, (ii) the number of flowers per inflorescence, (iii) the shape of the fruits, and (iv) the shape of domatia. This last character appears to be of less value than proposed by Breteler (1999) in differentiating the species, as discussed above. For instance, although *B. fistulosa* and *B. dewevrei* belong to clades that are clearly differentiated, they can have similar domatia and host the same specialised ants. In contrast, domatia restricted to the basal part of the lateral branches is an autapomorphy of *B. nigritana*. Both fruit shape and number of flowers per inflorescence follow the same pattern of variation between species: *B. fistulosa* has ellipsoid fruits and numerous flowers per inflorescence, whereas the three other taxa have subglobose fruits and few flowers per inflorescence. The distribution of these two characters is thus in contradiction with the main separation between the two species pairs identified by molecular data in our study. Interestingly, shape of floral bracts is rounded in both *B. fistulosa* and *B. solida*, and acute in both *B. dewevrei* and *B. nigritana*. This character follows the initial divergence of the species pairs, and thus reflects the molecular data better than the other characters discussed here.

4.6. Implications for the evolution of myrmecophytism

Myrmecophytes have appeared many times in the course of evolution (Davidson and McKey, 1993) but only very few constitute evolutionary radiations of any size. This pattern may reflect frequent extinction of specialised myrmecophytes (the "specialisation as evolutionary dead end" hypothesis, Althoff et al., 2012; Tripp and Manos, 2008) and/or reduction or loss of mutualistic traits in the course of evolution (Janzen, 1974). As in *Macaranga* (Blattner et al., 2001; Davies et al., 2001), *Neonauclea* (Razafimandimbison et al., 2005) and *Tococa* (Michelangeli, 2005) our results corroborate the latter hypothesis, suggesting dramatic reductions and loss of a myrmecophytic trait in the course of species evolution. In particular, non-myrmecophytism (as seen in *B. solida*) does not appear a likely ancestral state for the sampled populations of *Barteria*, even though it must be, at a deeper evolutionary scale, because *Barteria* is the only genus of the tribe Paropsieae to bear domatia (Tokuoka, 2012).

Barteria encompasses few species but is successful in an evolutionary sense, since its two myrmecophytic species are very abundant throughout Lower Guinea and the Congo basin. Possibly, mutualism between *Barteria* and *Tetraponera* enabled *B. fistulosa* and *B. dewevrei* to colonise a large core area, from which some descendants dispersed to habitats that favoured reduced investment in myrmecophytic traits (*B. nigritana*, *B. dewevrei* with the narrowest domatia) or even their loss in derived taxa (*B. solida*), resulting in the pattern we see today. This proposed evolutionary scenario is still tentative, but provides working hypotheses for future studies. The discrepancies between myrmecophytic traits and phylogenetic grouping should help to identify the loci controlling these traits, via genetic association studies and population genome scans (Storz, 2005). Detailed study of these loci should provide more definitive insights into the evolution of myrmecophytism.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.11.006.

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Plant-ants use symbiotic fungi as a food source: new insight into the nutritional ecology of ant–plant interactions

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Usually studied as pairwise interactions, mutualisms often involve networks of interacting species. Numerous tropical arboreal ants are specialist inhabitants of myrmecophytes (plants bearing domatia, i.e. hollow structures specialized to host ants) and are thought to rely almost exclusively on resources derived from the host plant. Recent studies, following up on century-old reports, have shown that fungi of the ascomycete order Chaetothyriales live in symbiosis with plant-ants within domatia. We tested the hypothesis that ants use domatia-inhabiting fungi as food in three ant–plant symbioses: *Petalomyrmex phylax*/*Leonardoxa africana*, *Tetraponera aethiops*/*Barteria fistulosa* and *Pseudomyrmex penetrator*/*Tachigali* sp. Labelling domatia fungal patches in the field with either a fluorescent dye or ^{15}N showed that larvae ingested domatia fungi. Furthermore, when the natural fungal patch was replaced with a piece of a ^{15}N-labelled pure culture of either of two Chaetothyriales strains isolated from *T. aethiops* colonies, these fungi were also consumed. These two fungi often co-occur in the same ant colony. Interestingly, *T. aethiops* workers and larvae ingested preferentially one of the two strains. Our results add a new piece in the puzzle of the nutritional ecology of plant-ants.

Keywords: symbiosis; nutritional ecology; ant–plant–fungus interaction; myrmecophyte

1. INTRODUCTION

Ants and plants are among the most dominant taxa in tropical ecosystems, and the ecological importance of their mutualistic interactions is brought to light by two considerations: these interactions structure food webs [1] and the mutualistic benefits exchanged can convey ecological advantages to the partners [2]. Yet the nutritional ecology of these interactions is still poorly understood [3]. In particular, trophic relationships with micro-organisms are just beginning to be investigated [4].

Opportunistic interactions between ants and plants are often mutualistic, and have repeatedly given rise to specialized symbiotic mutualisms between the so-called myrmecophytes (also called 'ant-plants'), which provide symbiotic ants with nesting cavities (specialized hollow structures, called domatia) in addition to food rewards, and specialist 'plant-ants' [5]. Plant-ants often not only protect their hosts against herbivores and pathogens, as well as against competing plants [6–8], they also confer nutritional benefits to their host plants (reviewed by Rico-Gray & Oliveira [9]).

A new, potential source of complexity in the trophic structure of ant–myrmecophyte associations is added by the recent finding that many ant–plant symbioses include long-ignored fungal partners growing within domatia [10]. Patches of fungi growing in domatia were reported as early as the beginning of the twentieth century [11–13], but have attracted little attention, being considered as pests or as commensals [11,13,14]. The difficulty of identifying fungi accurately long discouraged their study. Recent molecular investigations showed that the fungi occupying domatia in a very diverse set of myrmecophytes from throughout the tropics all belong to the ascomycete order Chaetothyriales, and that each seems to be somewhat specific to a particular ant–plant symbiosis [10,15]. Moreover, recent characterization of trophic fluxes among the three partners showed that plant-ants provide their symbiotic fungi with nutrients, probably through the accumulation of waste products [16]. However, the role of fungi in these tripartite symbioses is not yet clear. The trophic relationships between plant-ants

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and their symbiotic fungi may prove particularly important in helping us better understand the nutritional ecology of ant–plant symbioses.

The symbiotic nature of the relationship between domatia-inhabiting fungi and plant-ants, and the peculiar behaviour of ants towards fungal patches [10,16], led us to consider that these interactions could be new cases of agriculture by insects. In this context, we tested the hypothesis that plant-ants consume the symbiotic fungi growing within domatia of their host plants. We experimentally labelled the fungal patch directly in the field either with calcofluor white stain, a fluorescent dye that binds to fungal cell walls, or with ^{15}N, a stable isotope of nitrogen that is very rare in nature. We then looked for the label in ants.

2. MATERIAL AND METHODS

(a) *Study species and sites*

To assess the generality of our findings, we conducted the same experiments on three phylogenetically independent ant–plant symbioses on two continents (see electronic supplementary material, figure S1). In all of these, ants nest in swollen, hollow stems of the host tree.

Petalomyrmex phylax Snelling (Formicinae) is a small ant (worker body length of 2–3 mm) that obligatorily inhabits the understorey myrmecophytic tree *Leonardoxa africana* (Baill.) Aubrév. subsp. *africana* (Fabaceae, Caesalpinioideae). The distribution range of this system is restricted to coastal rainforests of Cameroon [17]. *Tetraponera aethiops* Smith (Pseudomyrmecinae) is a large ant (worker body length approx. 1 cm) that obligatorily inhabits the pioneering tree *Barteria fistulosa* Mast. (Passifloraceae). This system is widespread in central Africa [18]. Fieldwork on these two ant–plant systems was conducted in Cameroon, near the village of Nkolo (3°13.278′ N, 10°14.888′ E). *Pseudomyrmex penetrator* Smith (Pseudomyrmecinae) is a medium-sized ant (worker body length approx. 0.6 cm) that obligatorily inhabits myrmecophytic species of the genus *Tachigali* Aubl. (Fabaceae, Caesalpinioideae). This ant and its host trees are widely distributed in the eastern and central Amazon basin and adjacent Guianas [19]. Fieldwork on this species was conducted in French Guiana, near Kourou (Montagne des Singes; 5°04.443′ N, 52°41.958′ W). The species of *Tachigali* in this site could not be identified with certainty, because we found no flowering individuals. Identifying species of *Tachigali* is difficult in the absence of fertile specimens [20].

In adult ants, a sizeable subspherical pouch called the infrabuccal pocket is located just beneath the tongue. This pouch filters out and compacts the solid debris resulting from cleaning and feeding activities (adult ants can swallow only liquids) in the form of an ovoid pellet that is from time to time regurgitated and discarded. However, workers in the subfamily Pseudomyrmecinae (to which belong *T. aethiops* and *Ps. penetrator*) routinely feed their larvae with infrabuccal pellets. The larvae in this subfamily have a special pocket-like structure below the head, called the trophothylax, that the workers fill with infrabuccal pellets [21,22]. *Petalomyrmex phylax* larvae (subfamily Formicinae) do not have a trophothylax.

Two strains of fungi were previously identified in domatia of *B. fistulosa* occupied by *T. aethiops* [10]. Both strains are classified as members of the Chaetothyriales based on morphological and molecular evidence, but they are too divergent from named species to be confidently assigned to a described genus. These two strains were recently made available as pure cultures [15]. They will be called Y1 and Y9 hereafter and correspond, respectively, to the same species as strains CTeY6 and CTeY7 in the study of Voglmayr *et al.* [15]. Strains detected in fungal patches from *Pe. phylax*/*L. africana* and *Ps. penetrator*/*Tachigali* ant–plant symbioses were different [15]. In this study, we used only pure cultures of the two strains from domatia of *B. fistulosa* occupied by *T. aethiops*.

(b) *Labelling the natural fungal patch with calcofluor*

Experimental labelling of the fungal patch with calcofluor was performed on ten colonies of *Pe. phylax*, ten of *T. aethiops* and three of *Ps. penetrator*. For each colony, a single occupied domatium was cut off the tree. The domatium was carefully cut longitudinally, and the ants (adults and brood) placed in a separate plastic container. The fungal patch received 30–70 µl of pure calcofluor (Fluka, Buchs, Switzerland), depending on its size, so that the whole patch was in contact with the dyeing liquid. After 20 min (1 h for colonies of *Ps. penetrator*, which were tested first; we later established that 20 min was a sufficiently long period) the fungal patch was rinsed five times with water, and the excess of water absorbed with paper. The domatium was reassembled using tape and placed in the plastic container with the ants. The ants moved spontaneously into their domatium within 24 h and were provided ad libitum with diluted multiflora honey from a commercial source. Domatia were frozen once they had been transported back to the laboratory (i.e. 6 days after labelling for *Pe. phylax* and *T. aethiops*, and 11 days for *Ps. penetrator*). For each domatium, we investigated fluorescence under UV light in hyphae of the fungal patch, plant cells of the inner surface of the domatium, gut of larvae, hyphae in workers' infrabuccal pellets and hyphae in the content of larval trophothylaces (the last two categories were not investigated for *Pe. phylax*, whose larvae have no trophothylax and are not fed with infrabuccal pellets). The fungal patch and domatium inner surface were scraped gently with the tip of a scalpel blade, and the collected material was placed in a drop of distilled water between microscope slides. Gut of larvae, workers' infrabuccal pellets and contents of larval trophothylaces were dissected in distilled water and squashed between slides. A microspectrofluorometer (Jobin-Yvon, Longjumeau, France) equipped with an Olympus BX 60 microscope (Olympus, Tokyo, Japan) was used to obtain the emission fluorescence spectrum of a selected area of 4 µm diameter from each item. This surface is smaller than the section of a hypha of the focal fungi. Using a xenon lamp and monochromators, UV light of wavelength of 365.5–368.5 nm was produced to excite the sample. The resultant fluorescence was detected with a charge-coupled device (CCD) camera, and the fluorescence emission spectra were produced by the SpectraMax software package (Jobin-Yvon). In one of the ten *Pe. phylax* colonies, fluorescence was investigated visually, but no measure was performed, which explains why the sample size is ten when accounting for the presence/absence of fluorescence, and nine when measure of wavelength is considered. In this colony, no fluorescence similar to that of the fungal patch was detected in larvae.

As a negative control, we collected a single occupied domatium from each of ten colonies of *Pe. phylax*, ten of *T. aethiops* and one of *Ps. penetrator*. Ants were provided ad libitum with diluted multiflora honey from a commercial

source. Domatia were frozen once transported back to the laboratory, and fluorescence under UV light was investigated following the same procedure as described earlier.

(c) *Labelling the natural fungal patch with ^{15}N*

Experimental enrichment of the fungal patch with ^{15}N was performed on ten colonies of *Pe. phylax*, nine of *T. aethiops* and nine of *Ps. penetrator*. For each colony, two occupied domatia were cut off the tree. One of the two received no treatment (negative control). The other was carefully cut longitudinally and the ants (adults and brood) placed in a separate plastic container. The fungal patch received 30–70 µl of an aqueous solution containing 3 mg ml^{-1} glycine enriched in ^{15}N (98% of molecules marked). After 1 h, the fungal patch was rinsed five times with water, and the excess of water absorbed with paper. The domatium was reassembled using tape and placed in the plastic container with the ants. The ants moved spontaneously into their domatium within 24 h and were provided ad libitum with diluted multiflora honey from a commercial source. Workers, larvae and the fungal patch were collected 4 days later from each domatium and immediately dried under silica gel. Isotopic abundances were measured with an elemental analyser (N) connected to an isotopic mass spectrometer (Finnigan Delta S, Finnigan MAT, Bremen, Germany) at the Service Central d'Analyse of the CNRS (SCA, Solaize, France).

(d) *Replacement of the natural fungal patch with pure cultures of the domatia fungi enriched in ^{15}N*

For the purpose of the experiment, pure cultures of fungal strains Y1 and Y9 were grown in Petri dishes on a 2 per cent malt extract agar medium containing 0.75 mg ml^{-1} glycine enriched in ^{15}N (98% of molecules marked). Both strains can occur in either black yeast or hyphal stage. At the time of the experiment, strains were in the black yeast stage and were thus in the form of a thin crust on top of the culture medium. The experiment was conducted on ten colonies of *Pe. phylax* and ten of *T. aethiops*. However, it could not be performed on *Ps. penetrator*, because fieldwork in French Guiana occurred before pure cultures were obtained. For each colony, two occupied domatia were cut off the tree. Each domatium was carefully cut longitudinally, and the ants (adults and brood) were placed in a separate plastic container. The fungal patch was removed with a scalpel and replaced by an equivalent surface of pure culture of one strain. Only a thin, superficial layer of the fungal culture was used. We are confident that culture medium was not introduced into domatia, because we did not alter the basal part of the fungal culture (which is in contact with culture medium). Each colony received both strains, one in each domatium (these were kept separate). Each domatium was reassembled using tape and placed in the plastic container with the corresponding ants. The ants moved spontaneously into the domatium within 24 h and were provided ad libitum with diluted multiflora honey from a commercial source. Workers and brood were collected 4 days later from each domatium and immediately dried under silica gel. For *Pe. phylax*, all workers from each colony were pooled and treated as a single sample (one measure of δ^{15}N per colony), and larvae were treated the same way, but for *T. aethiops*, we measured δ^{15}N in three individual workers and larvae in each colony. These values were averaged, and the means used as a single value for each colony. For *T. aethiops*, the head of each worker was removed before measurement of δ^{15}N in order to exclude any contribution from hyphae in infrabuccal pellets, and test more specifically for ingestion of fungal material into the gut of workers in this species.

Negative controls were the same as in the previous experiment, so we used tests for independent samples to compare experimental and control values. Isotopic abundances were measured as in the previous experiment.

Statistical analyses were performed using R v. 2.13.1 [23].

3. RESULTS

(a) *Ant larvae ingest hyphae from domatia fungal patches: evidence from calcofluor staining*

Fungal hyphae exposed to calcofluor within domatia showed a strong bluish fluorescence under UV light, whereas fungal hyphae from control domatia emitted no fluorescence (figure 1*a*,*b*). Both wavelength (λ_{max}) and intensity of the most intense peak measured on each spectrum differed significantly between the labelled and control hyphae (figure 2; Mann–Whitney tests, *Pe. phylax*, for both λ_{max} and intensity: $z = 3.7$, $p = 0.00024$; *T. aethiops*, for both λ_{max} and intensity: $z = 3.8$, $p = 0.00016$; no test performed for *Ps. penetrator* because of small sample size). In fact, spectra for control hyphae were similar to those obtained with only water between slides (data not shown). This shows that calcofluor readily stained hyphae in blue, and that the fungal patch did not emit any spontaneous fluorescence that could interfere with the detection of experimental labelling.

Plant cells from the inner surface of domatia showed a very faint yellowish fluorescence under UV light, and were not labelled bluish by calcofluor (figure 1*a*,*b*). Both wavelength and intensity of the most intense peak measured on each spectrum differed significantly between the cells from the inner surface and hyphae in labelled domatia (figure 2; Wilcoxon tests, *Pe. phylax*, for both λ_{max} and intensity: $z = 2.7$, $p = 0.0077$; *T. aethiops*, for both λ_{max} and intensity: $z = 2.8$, $p = 0.0051$; no test performed for *Ps. penetrator* because of small sample size). This shows that the faint fluorescence of plant cells did not interfere with the detection of experimental labelling.

A bluish fluorescence similar to that emitted by labelled fungal patches was detected under UV light in the gut of a few larvae from six of ten colonies of *Pe. phylax*, five of ten colonies of *T. aethiops* and two of three colonies of *Ps. penetrator* (see electronic supplementary material, table S1; figures 1 and 2). Fluorescent parts of larval gut were less conspicuous for *Pe. phylax* than for the two other species, and labelling was thus much more difficult to detect in this species. The nature of the fluorescent material was impossible to determine (figure 1*d*,*e*). Wavelength was not significantly different between labelled fungal hyphae in the patch and fluorescent parts of the gut of larvae (figure 2; each larva was considered an independent sample; Mann–Whitney tests, *Pe. phylax*, $z = 1.6$, $p = 0.10$; for *T. aethiops*, $z = 1.7$, $p = 0.09$; no test performed for *Ps. penetrator* because of small sample size; for sample sizes, see electronic supplementary material, table S1). For *T. aethiops*, hyphae with fluorescence similar to that emitted by labelled fungal patches were observed in the content of trophothylaces from three of six larvae investigated (three colonies), and in all

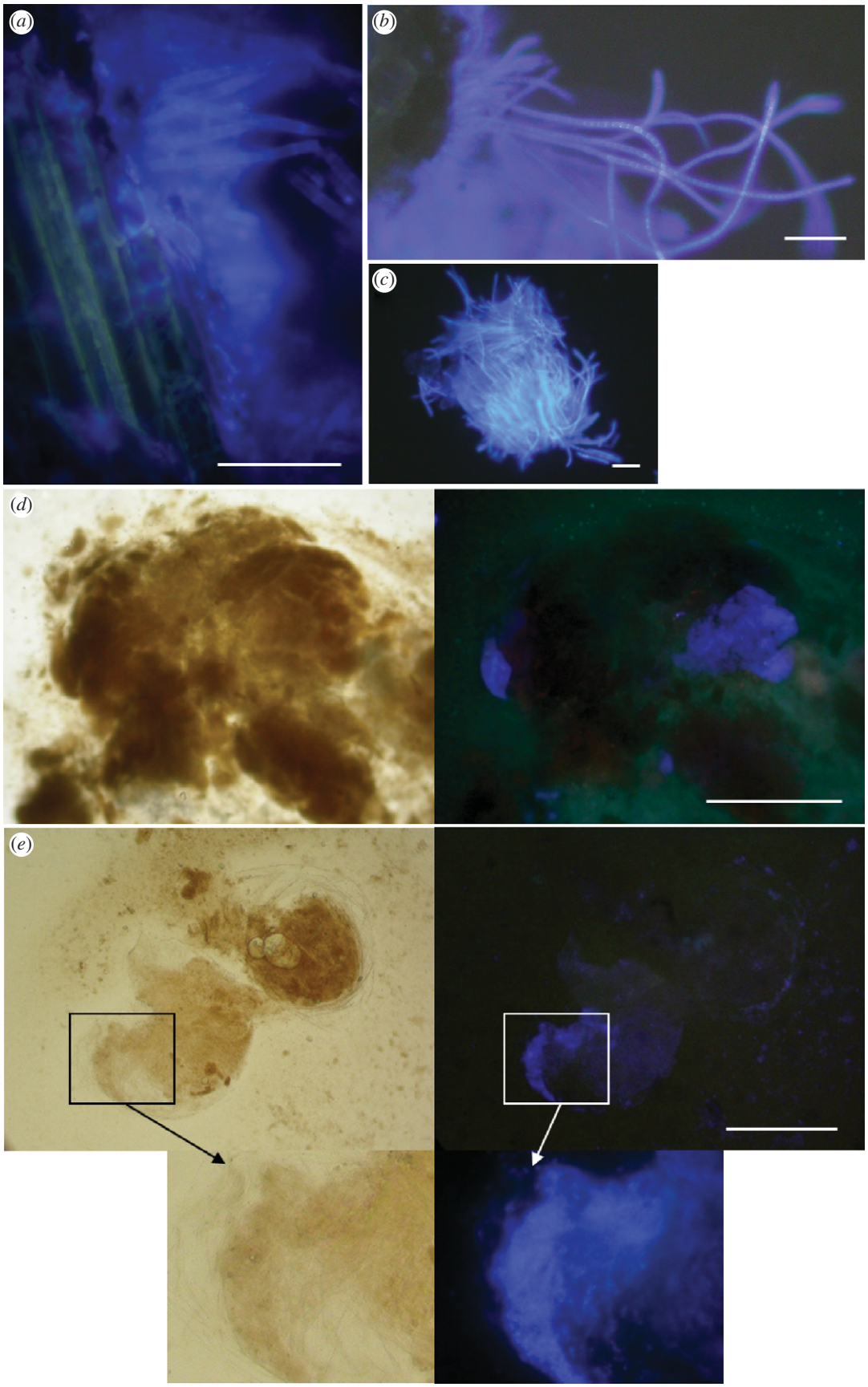

Figure 1. Microscopic observation of fluorescence under UV light after labelling of fungal patches with calcofluor. (*a*) Bluish hyphae from fungal patch in a domatium of *Tachigali* sp. occupied by the ant *Pseudomyrmex penetrator*. Note that the plant cells emit a faint yellowish fluorescence distinct from that emitted by the fungal patch. (*b*) Same as panel (*a*), for the ant–plant system *Tetraponera aethiops*/*Barteria fistulosa*. (*c*) Cluster of bluish hyphae from an infrabuccal pellet of a *Tetraponera aethiops* worker. (*d*) Part of the gut of a large *Tetraponera aethiops* larva emitting, under UV light (right panel), a bluish fluorescence similar to that of the fungal patch; left panel shows same under white light. (*e*) Same as panel (*d*), for a small *Pseudomyrmex penetrator* larva. Scale bars: (*a–c*) 50 μm, (*d*,*e*) 0.5 mm.

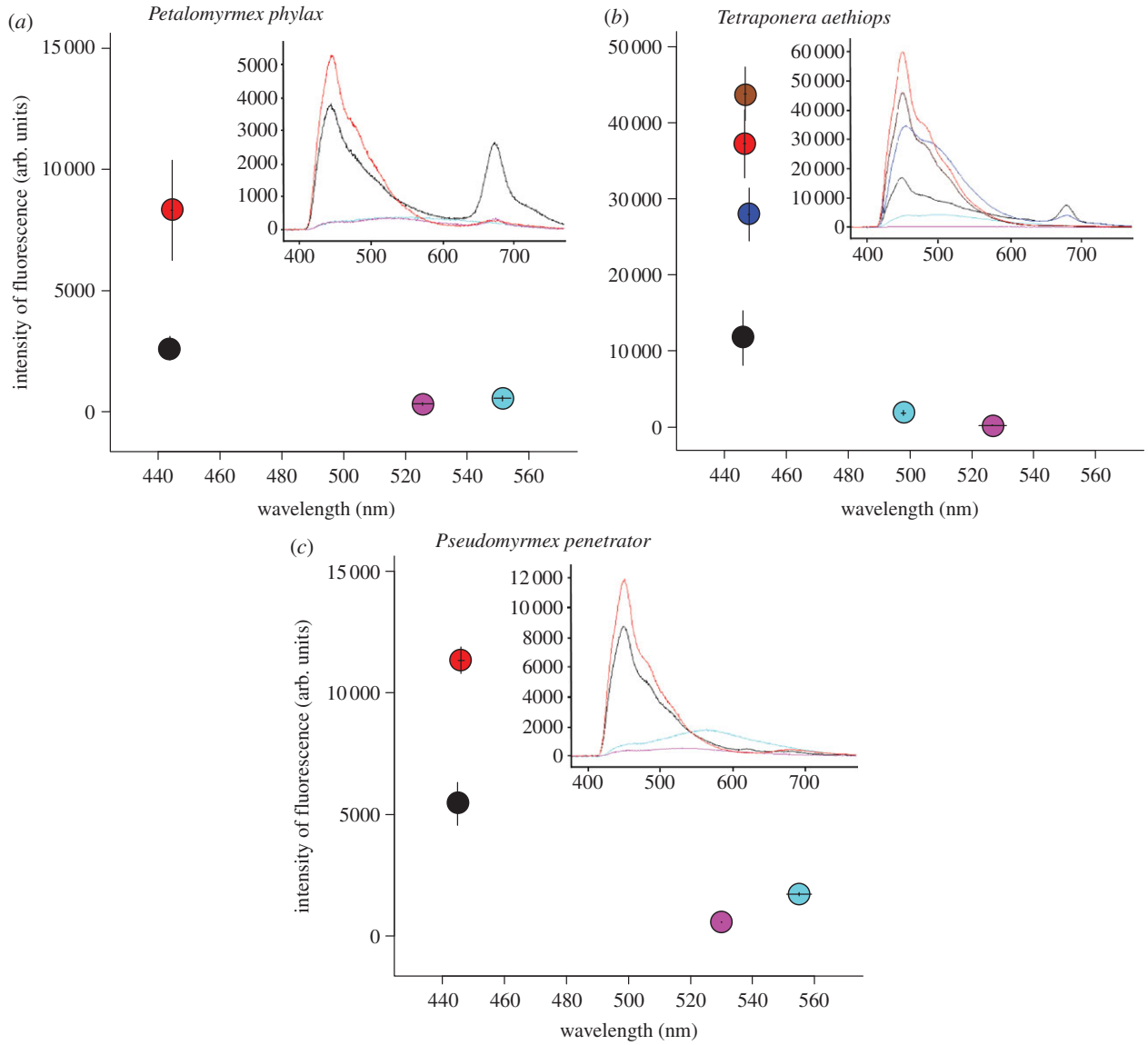

Figure 2. Wavelength and intensity of the most intense fluorescence obtained by microspectrofluorometry under excitation at 365.5–368.5 nm for hyphae of the fungal patch labelled with calcofluor (red), hyphae of the fungal patch in control domatia (pink), plant cell from the inner wall of a domatium in which the fungal patch was labelled with calcofluor (light blue), part of larval gut showing bluish fluorescence (black), hyphae in infrabuccal pellet of workers (and one winged female; dark blue) and hyphae in larval trophothylax (brown), for the three study systems. Means ± s.e. The inserts within each graph show examples of fluorescence emission spectra for each of the above items.

the infrabuccal pellets investigated (24 from 10 colonies; figures 1*c* and 2). One of those infrabuccal pellets was from a winged female, the others from workers. Fluorescent hyphae in infrabuccal pellets and trophothylaces were found to be either isolated or in clusters (as in figure 1*c*).

For control colonies, no bluish fluorescence was detected in any of the investigated larvae: 104 larvae from ten colonies of *Pe. phylax* (8–16 larvae per colony), 140 larvae from ten colonies of *T. aethiops* (10–35 larvae per colony) and 10 larvae from one colony of *Ps. penetrator*. Similarly, no bluish fluorescence was detected in the infrabuccal pellets of 23 workers or in the content of trophothylaces of 29 larvae from ten colonies of *T. aethiops* (two to three infrabuccal pellets, and two to six trophothylaces per colony).

Raw data are available as electronic supplementary material, table S2.

(b) *Ants ingest hyphae from domatia fungal patches: evidence from the ^{15}N pulse-chase experiment*

Fungal patches exposed to ^{15}N-glycine had much higher δ^{15}N values than those from control domatia for both *Pe. phylax* and *T. aethiops* colonies (figure 3; Wilcoxon tests for paired samples, $v = 0$, $p = 0.031$). Regarding *Ps. penetrator*, fungal patches from only two experimental domatia were analysed, and showed δ^{15}N values as high as in the two other systems. This confirms that the labelling procedure was efficient in the three systems.

For *Pe. phylax* and *Ps. penetrator*, values of δ^{15}N were significantly higher for workers and larvae from colonies that had their fungal patch labelled than for control colonies (figure 3; Wilcoxon tests for paired samples, $v = 0$, $p < 0.01$). For *T. aethiops*, the difference was low but significant for workers ($v = 5$, $p = 0.039$), whereas it was not significant for larvae ($v = 12$, $p = 0.25$).

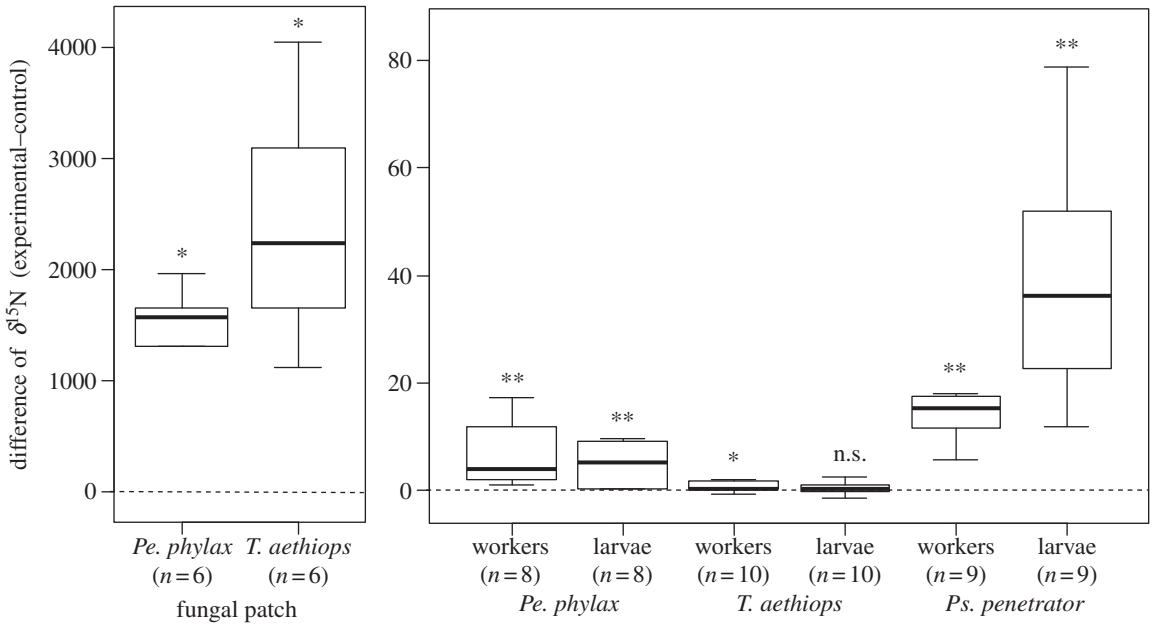

Figure 3. Difference of δ^{15}N between colonies that had their fungal patch exposed to ^{15}N-glycine and control colonies. Horizontal lines represent median, boxes represent first and third quartiles, and whiskers represent first and ninth deciles. $*p < 0.05$, $**p < 0.01$, n.s. not significant.

Raw data are available as electronic supplementary material, table S3.

(c) *Ants ingest hyphae from pure strains of domatia fungi: evidence from the fungal patch replacement experiment*

Petalomyrmex phylax workers and larvae from colonies that received pieces of labelled pure cultures of either fungal strain had δ^{15}N values higher than control colonies (see electronic supplementary material, figure S2; Wilcoxon tests for independent samples, for workers, strain Y1: $w = 10$, $p = 0.0062$, strain Y9: $w = 12$, $p = 0.012$; for larvae, strain Y1: $w = 15$, $p = 0.055$, strain Y9: $w = 0$, $p = 0.00067$). By contrast, *T. aethiops* workers and larvae had δ^{15}N values higher than control colonies only when they received strain Y1 (see electronic supplementary material, figure S2; for workers, strain Y1: $w = 0$, $p = 0.000022$, strain Y9: $w = 23$, $p = 0.079$; for larvae, strain Y1: $w = 17$, $p = 0.022$, strain Y9: $w = 34$, $p = 0.40$).

Raw data are available as electronic supplementary material, table S4.

4. DISCUSSION

Our results clearly demonstrate that the three plant-ants we studied ingest fungi from the patches growing within domatia. After labelling the fungal patch with a fluorescent dye, we found the same fluorescence in the gut of larvae of the three species as in hyphae of the fungal patch. This strongly suggests that the larvae consumed hyphae from domatia fungi, because we could not detect such fluorescence in any part of the ant-plant except the labelled fungal patch. However, a low proportion of larvae showed the fluorescence: 10 per cent for *Pe. phylax* and *T. aethiops*, and 50 per cent for *Ps. penetrator* (see electronic supplementary material, table S1). Experimental colonies of this last species were exposed for 11 days to the labelled fungal patch instead of 6 days as in the two other species, strongly suggesting that duration of the experiment was too short for an accurate assessment of fungal ingestion by larvae. As pseudomyrmecine ants feed their larvae with the content of their infrabuccal pocket [22], which is used to store debris from cleaning and feeding activities, we also checked the content of infrabuccal pellets and of trophothylaces (infrabuccal pellets packed in a special pouch of the larva) for labelled hyphae in one of our study models (*T. aethiops*). The presence of labelled hyphae in both structures confirmed that hyphae from domatia fungal patches were transferred to larvae by workers. Although all *T. aethiops* workers examined had infrabuccal pellets containing labelled hyphae, most larvae had none in their trophothylax, showing that they were not fed on a daily basis. This suggests again that the labelling experiment should have been conducted over a longer period of time to estimate accurately the extent to which larvae are fed with domatia fungi.

Labelling domatia fungal patches with ^{15}N also showed that larvae of *Pe. phylax* and *Ps. penetrator* consume domatia fungi. We could not detect any increase in δ^{15}N in larvae of *T. aethiops*. As discussed earlier, larvae of this species are not fed on a daily basis, and it is possible that the larvae we used for δ^{15}N had not been fed in the course of the experiment, especially as exposure to labelled fungi lasted only for 4 days. Workers of *T. aethiops* are much larger than those of the two other species; in consequence, fewer larvae were used for isotopic analysis. We thus had a higher chance of including larvae fed with labelled fungi in the two other species, which would explain why we detected an increase in δ^{15}N in larvae of these species over such a short exposure time. The ^{15}N-labelling experiment also showed that workers of the three species had collected domatia fungi. However, worker heads were included in ^{15}N analyses, so that an increase in δ^{15}N may be explained by the presence of hyphae from fungal patches in the infrabuccal pocket as the result of cleaning activities.

Replacing the natural fungal patch with a piece of pure culture of domatia fungal strains labelled with ^{15}N

showed that larvae and workers ingested the introduced fungus. Unlike in the previous experiment, heads of *T. aethiops* were removed before isotopic analysis, so we are confident that the increase in ^{15}N in workers resulted from true ingestion of the fungus, rather than from the simple presence in the infrabuccal pocket, owing to cleaning activities, of fungal material that would eventually be discarded. The two fungal strains introduced were isolated from patches in *B. fistulosa* domatia occupied by *T. aethiops*, and tested on both *P. phylax* and *T. aethiops*. *Petalomyrmex phylax* apparently did not show a consistent difference in its consumption of the two strains. However, consumption by larvae and workers of *T. aethiops* was detected only with strain Y1. The two strains were initially isolated from a single fungal patch, meaning that they co-occurred in a single host plant and colony. Our results suggest that *T. aethiops* workers are able to discriminate between the two strains and prefer one to the other, a phenomenon that should be further investigated.

Pseudomyrmecine ants feed their larvae with the contents of the infrabuccal pocket [22], which contains debris from cleaning activities. Ingestion of hyphae from domatia fungal patches could thus be a mere by-product of such activities. However, although *Pe. phylax* workers do not feed their larvae with infrabuccal pellets, larvae nevertheless ingested fungal hyphae, suggesting active feeding on domatia fungi in this species at least. Behavioural observations [10], specificity of fungal strains with plant-ant species [15] and trophic flow from ants to domatia fungi [16] also suggest tight interaction between domatia fungi and plant-ants. The use of these fungi as a food source thus probably explains why ants maintain them within domatia. Whatever the mechanism behind ingestion of domatia fungi (either passive or active), our results demonstrate that they are part of the diet of plant-ants. Whether the ants depend on this resource still remains to be determined. Defossez *et al.* [16] fed *Pe. phylax* ants with ^{15}N and showed that N was transferred to the fungal patch and the host plant within 10 days. After almost 2 years, $\delta^{15}N$ values were still very high in the system and similar for the three partners, indicating nitrogen recycling. Our results add a new piece of information about the trophic web of this symbiosis. Not only do the ants feed the fungal patch, they also feed on it.

Our study clearly shows that plant-ants have access to food sources that were unsuspected prior to this study. The geographical and taxonomic diversity of the three systems studied, and the fact that fungi seem to be associated with most ant–plant symbioses [11–13,15,24–26], suggest that ingestion of domatia-inhabiting fungi by plant-ants may be a widespread phenomenon. The main obstacle to understanding the nutritional ecology of ant–plant interactions is the lack of knowledge on the qualitative and quantitative aspects of the food sources available to ants. The composition of extrafloral nectar and food bodies is known only in a relatively small subset of plants, and the role of other potential food sources, such as the one evidenced in this study, remains virtually unexplored. Systematic survey of inputs in ant nutrition and detailed characterization of the role of microsymbionts are challenges for future research that must be surmounted if we are to understand the evolutionary diversification of ants and how they contribute to the functioning of tropical ecosystems.

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RESEARCH ARTICLE

The Evolution of Communication in Two Ant-Plant Mutualisms

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Abstract Myrmecophytes are plants that provide nesting sites and food to ants that protect them against herbivores. Plant signals function to synchronize ant patrolling with the probability of herbivory. We compared the communication signals in two symbioses involving ant and plant pairs that are closely related. The two plants emitted the same volatile compounds upon damage. These compounds are simple molecules common in the plant kingdom. Electroantennography revealed that the two symbiotic ants, as well as several other ant species, were able to perceive these compounds. However, workers of one species responded only to hexanal, while those of the other species responded mostly to methyl salicylate. The two signals involved in the focal symbioses are 'cheap' (low metabolic cost), which is consistent with theoretical predictions for the evolution of signalling between partners with convergent interests. They are also not specific, which is expected between plants and broad-spectrum predators such as ants. The fact that different signals are used in the two sister symbioses suggests different mechanisms underlying similar adaptations in the evolution of communication.

Keywords Symbiosis · Coevolution · Volatile organic compound · Myrmecophyte · *Leonardoxa*

Introduction

Plants produce secondary compounds that can act either as direct or indirect defences. Direct defences involve compounds that are repulsive or not palatable to herbivores, but herbivores can evolve counter-adaptations to those compounds. Reciprocal adaptation of plants and herbivores is considered to be responsible for the increase in the diversity of secondary compounds produced by plants over evolutionary time (Vermeij 1994; Becerra et al. 2009). Herbivore-induced plant volatiles are known to attract enemies of herbivores, and constitute one of the best documented cases of indirect defence (Turlings et al. 1990; Turlings and Wäckers 2004; Heil 2008). Many parasites and parasitoids of herbivores are highly specialized and their attraction to herbivore-induced plant volatiles can only be favoured by selection if the volatiles emitted by the plant are herbivore-specific and if they respond selectively to those compounds. The specificity of both plant volatiles and responses of herbivore enemies has been largely documented and seems to be widespread (Takabayashi et al. 2006). This suggests that indirect defence may also select for a large diversity of secondary compounds. However, some predators of herbivores are generalists and plants would thus benefit from attracting them. This is particularly the case of ants, many species of which prey on a wide variety of arthropods. For many plant species, attraction of ants involves the production of extrafloral nectar, which in

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most cases represents an unspecific signal (and reward). However, attraction of ants by plant volatiles has been relatively poorly investigated. Because of the lack of specificity of these predators, we may expect plants to evolve non-specific and cheap (low metabolic cost) signals in this case.

Myrmecophytes are plants that provide nesting sites (specialized hollow structures called domatia) and food (e.g. extrafloral nectar) to the ants. In return, ants protect the host plant against herbivores, pathogens, and competing vegetation (Letourneau 1998; Suarez et al. 1998; Rosumek et al. 2009). Some ant species are considered parasites of mutualisms because they use plant resources, do not protect the plant and exclude the mutualist (Janzen 1975; Yu and Pierce 1998; Gaume and McKey 1999). Damaged parts of host plants attract the mutualist ants (Rico-Gray and Oliveira 2007), but response of parasite species is unknown. The compounds released by the damaged plant allow synchronization of ant patrolling with herbivore activity. Such communication has been shown in numerous ant-plant symbioses (Blatrix and Mayer 2010). However, the compounds constituting the signal have been identified in only one ant-plant symbiosis (Schatz et al. 2009).

We conducted a comparative analysis of the evolution of the chemical communication signals emitted by two sister myrmecophytes, that induce patrolling by their respective mutualist ants. To determine whether the nature of the signal was constrained (1) by plant metabolic capacities and (2) by ant sensory capacities, we first identified the compounds released upon artificial damage by the two focal myrmecophytes and by their closest relative. Then we tested the ability of each mutualist ant, as well as eight other ant species of various ecologies and taxonomic origins, to perceive the compounds of the two focal plants, using electroantennography. We then conducted behavioural tests in the field to determine which plant compound(s) was (were) used as signal(s) in the two focal ant-plant symbioses. The communication signal in one of the focal symbioses had already been identified in a previous study (Schatz et al. 2009) by presenting pure synthetic versions of the compounds at the entrance of domatia, but we conducted new tests closer to the natural situation, i.e. by depositing compounds on the leaflets to measure ant attraction. These experiments allowed comparison with the results obtained for the other focal symbiosis.

Materials and Methods

Study Species and Field Sites

Leonardoxa africana (Baill.) Aubrév. (Fabaceae, Caesalpinioideae) is a plant species comprised of four subspecies endemic to Atlantic rain forests of Central Africa (McKey 2000). Our study systems were the two allopatric myrmecophyte subspecies *Leonardoxa africana africana* McKey and *L. a. letouzeyi* McKey. They both produce foliar nectar and caulinary domatia. They are occupied respectively by the mutualist ants *Petalomyrmex phylax* Snelling and *Aphomomyrmex afer* Emery (Fig. 1). Both ant genera are monotypic and the two ants are sister species (Chenuil and McKey 1996). These ants are small but colonies can be very populous. They are particularly effective against small chewing and sap-sucking insects, which are the dominant herbivores on *Leonardoxa* plants (Gaume et al. 1997; Gaume and McKey 1998). Some individuals of *L. a. africana* are colonized by the ant *Cataulacus mckeyi* Snelling, considered a parasite of the mutualism (Gaume and McKey 1999). Young *L. a. letouzeyi* trees can be occupied by opportunistic ants belonging to several genera (*Crematogaster, Axinidris, Monomorium, Cataulacus*). *Leonardoxa africana gracilicaulis* McKey lacks domatia and is thus not a myrmecophyte. Non-resident ants opportunistically visit its foliar nectaries. It is considered to be the closest to the putative common ancestor of *Leonardoxa* (Brouat et al. 2004). *Leonardoxa africana africana* and *L. a. gracilicaulis* co-exist in a few sites in a narrow band but remain distinct (Léotard et al. 2008) (Fig. 1e).

All field sites are in Cameroon. *L. a. letouzeyi* and its associated ants were studied in Korup National Park (05°0′30″N, 08°51′36″E). Out of more than 300 individuals found in this population, only 16 were suitable for study (i.e., big enough to host the mutualist ant species and with branches low enough to allow behavioural experiments from the ground). *Leonardoxa africana africana* and its associated ants were studied near the villages of Nkolo (03°13′19″N, 10°14′58″E) and Ebodjé (02°34′05″N, 09°50′37″E) (20 individuals in each site). Samples from *L. a. gracilicaulis* (ten trees) were also collected near Nkolo.

Study of Foliar Compounds

Volatile organic compounds emitted by leaves of *L. a. africana* and *L. a. letouzeyi* after artificial damage were identified by Schatz et al. (2009). To determine their occurrence and quantify their abundance in the three studied subspecies (*africana*, *letouzeyi* and *gracilicaulis*), single leaflets (15, 8 and 20 respectively) collected from four trees of each subspecies were soaked in 30 ml of hexane for 30 min. Leaflets were mature and intact, young and intact, or mature and artificially damaged (three types of damage were performed by cutting triangular pieces of lamina of linear distances of 2, 4 and 12 cm). No differences in compound quantities could be detected among trees and among types of leaflets within subspecies. This was possibly due to the fact that soaking leaflets in hexane

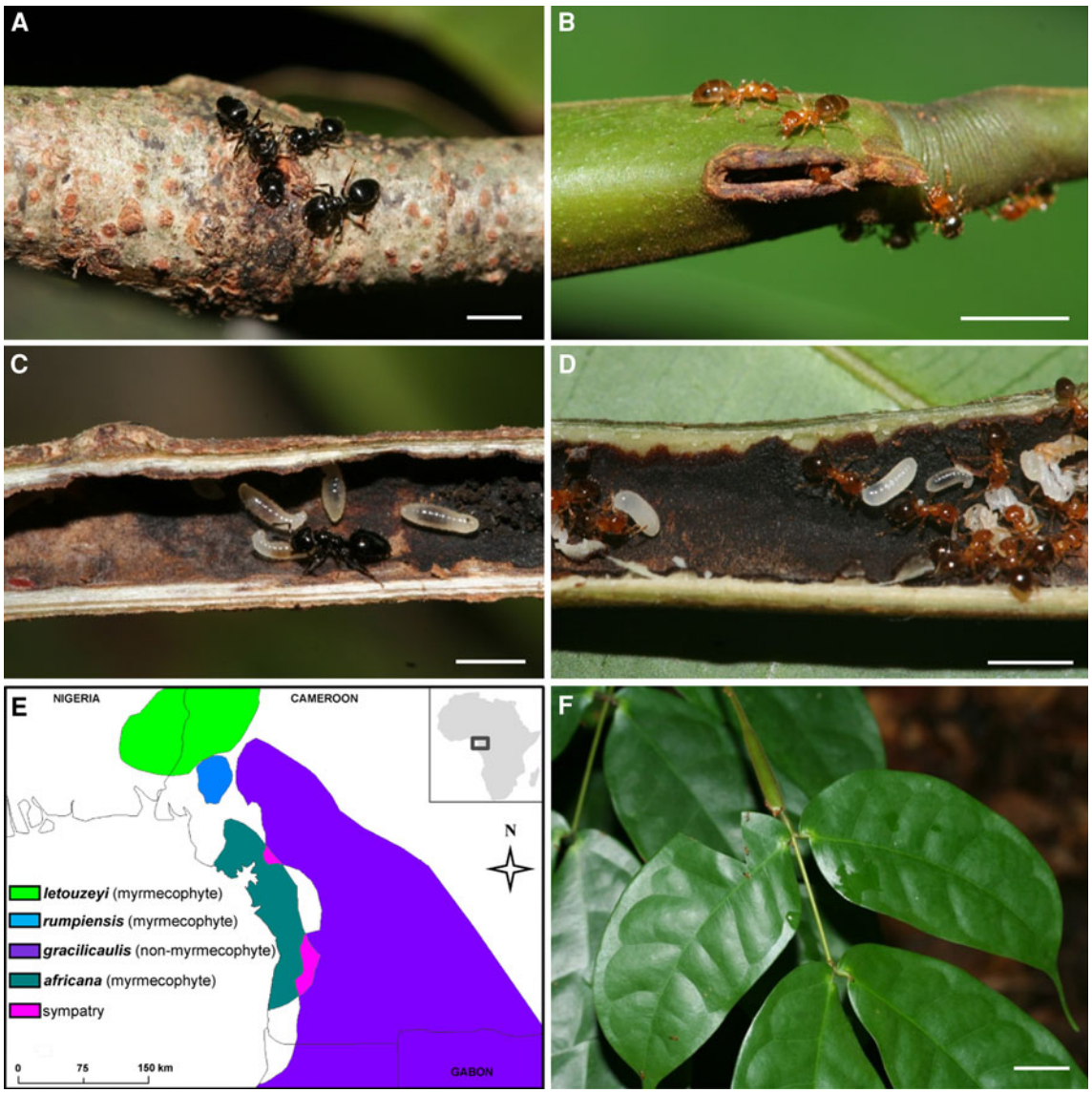

Fig. 1 Worker ants at the entrance of a domatium of the host plants (**a**, **b**) and domatia cut longitudinally showing ants inside (**c**, **d**) for the symbioses between the ant *Aphomomyrmex afer* and the plant *Leonardoxa africana letouzeyi* (**a**, **c**) and between the ant *Petalomyrmex phylax* and the plant *L. a. africana* (**b**, **d**). **f** A distal domatium of *L. a. africana* with a compound leaf showing artificial damage and patrolling ants on one of the basal leaflets (*left*). Scale bar is 3 mm for **a** to **d**, and 2 cm for **f**. **e** Distribution map of subspecies of the *Leonardoxa africana*. *Leonardoxa africana rumpiensis* was not studied. Adapted from Léotard (2007)

induced the release of stress compounds independently of the initial state of the leaflet. We thus pooled data from the different types of leaflets to compare amounts of compounds among subspecies. The control (one in each sampling site) consisted of a vial containing 30 ml of hexane left open for 30 min. Samples were concentrated by evaporating them under nitrogen flow until they reached a volume of 0.1 ml. We added 2.9 µg of nonane to each sample, as an internal standard allowing quantification of the compounds. An aliquot of 1 µl of each extract was analyzed by Gas Chromatography/Mass Spectrometry using electronic impact ionization mode on a Varian Saturn 2000 ion trap spectrometer, interfaced with a Varian CP-3800 gas chromatograph (Palo Alto, CA, USA). The Varian CP-3800 was equipped with a split-splitless injector (200°C) and a CPSil-8CBLow bleed MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness, Chrompack®), with helium as carrier gas. The chromatograph oven was programmed as follow: 50°C for 3 min, 50 to 100°C at 3°C min^{-1}, 100 to 140°C at 2.7°C min^{-1}, 140 to 180°C at 2.4°C min^{-1}. Mass spectra were recorded in electronic impact at 70 eV. Leaf volatile compounds were identified by comparison with the NIST 98 and Adams (2007) libraries and with pure synthetic

standards (Fluka, Buchs, Switzerland). A second lane was used to obtain a chromatogram for quantitative analysis. Integration of the internal standard peak allowed determining the output $O = Nn/An$ for each chromatogram (Nn: nonane quantity present in the sample; An: area of the corresponding nonane peak). The amount of each compound contained in each sample was estimated as $N_c = O*A_c$ (A_c: area of the peak for the compound). The quantity of the compound found in the corresponding control was subtracted from N_c to separate quantities due to external pollution from quantities actually contained in the leaves. Quantities were compared among subspecies.

Electroantennography

To test the sensory capacities of ants we recorded antennal electrical response to volatile compounds with a Syntech electroantennograph device (Hilversum, Netherlands). Electroantennogram recordings were made using glass electrodes filled with saline solution. The head of the tested living ant was excised and placed on the indifferent electrode, with the antennae protruding. The tip of an antenna was inserted into the recording electrode. A metal tube delivered a purified and humidified air stream flowing continuously (22.6 ml s^{-1}) over the preparation. Each stimulus was prepared less than 2 min before being tested by depositing 0.4 µl of the pure compound to be tested (same volume as in the behavioural tests, see below) on a rectangle of filter paper (2 cm^2) placed in a glass Pasteur pipette. The tip of the pipette was connected to the metal tube and the stimulus was directed to the antenna by blowing an air pulse (11.3 ml s^{-1} for 0.5 s) through the pipette. When several compounds were successively delivered on the same antenna, stimuli were released at 2-min intervals to avoid receptor saturation.

In a first series of tests we recorded antennal responses of *P. phylax* (24 workers from 7 colonies), *A. afer* (25 workers from 4 colonies) and *C. mckeyi* (24 workers from 2 colonies) to pure synthetic versions of methyl salicylate, (E)-2-hexenol, hexanal, P-cymene and R-α-phellandrene. The first three compounds are the only ones detected in volatile emission from wounded leaves of focal subspecies (Schatz et al. 2009). We will call them hereafter "candidate compounds". The last two were found as traces in solvent extracts. A mixture of the three candidate compounds (80% methyl salicylate, 15% (E)-2-hexenol and 5% hexanal), referred to hereafter as "the mixture", was also tested on antennae. The proportions used in the mixture were those observed by Schatz et al. (2009) in volatile emissions of leaves. We assessed antennal response to these five compounds of eight additional ant species from various subfamilies and habitats: *Crematogaster* sp1 and sp2 (Myrmicinae, tree-dwelling, collected in Korup National Park as opportunistic inhabitants of *L. a. letouzeyi*, 10 workers from one colony of each species), *Axinidris bidens* Shattuck (Dolichoderinae, tree-dwelling, collected in Korup National Park as an opportunistic inhabitant of *L. a. letouzeyi*, 12 workers from four colonies), *Polyrhachis laboriosa* Smith and *Oecophylla longinoda* Latreille (both Formicinae, tree-dwelling, collected near Nkolo, 10 workers from one colony of each species), *Plagiolepis pygmaea* Latreille and *Camponotus aethiops* Latreille (both Formicinae, ground-dwelling, collected near Montpellier, France, 10 workers from five and four colonies of each species respectively). Each worker was tested for all compounds and the mixture in a random order. Each series of stimuli started and ended with a control stimulus consisting of an air flow through a glass Pasteur pipette containing a filter paper. The control stimulus triggered a small, but appreciable EAG response, as in other studies (Sheridan et al. 1996; D'Ettorre et al. 2004; Lopez-Riquelme et al. 2006). This may be due to the presence of mechanoreceptors on the antennae (Vaitkeviciene et al. 1994).

A second series of tests was conducted on *P. phylax*, *A. afer*, *C. mckeyi* and *A. bidens*, aiming to assess the response threshold to each candidate compound. Because only few *C. mckeyi* workers were available, we could only test methyl salicylate on this species (six workers tested). For the other species, 10 workers were tested for each compound. Six quantities were tested on each antenna in the following order: 0.4 nl, 0.8 nl, 4 nl, 8 nl, 36 nl and 0.4 µl. We prepared corresponding solutions of increasing concentrations, so that each quantity to test was contained in 0.4 µl of the corresponding solution. We used dichloromethane as solvent. Each series of stimuli started and ended with a control stimulus consisting of a filter paper loaded with 0.4 µl of dichloromethane.

Intensity of depolarization generally decreases with time. We assumed response decay was linear. The initial and final control stimuli allowed estimation of the rate of decay, and thus calculation for each stimulus of the putative amplitude of depolarization that a control stimulus would trigger at the same time (D_{tn}):

$$D_{tn} = D_{ti} + [(D_{tf} - D_{ti})/(T_f - T_i)] * (T_c - T_i)$$

where D_{ti} is the depolarization observed in response to the initial control; D_{tf} is the depolarization observed in response to the final control; T_i is the time lag between recording start and pulse of the initial control stimulus; T_f is the time lag between recording start and pulse of the final control stimulus; and T_c is the time lag between recording start and pulse of the focal stimulus. Antennal response to a stimulus was tested by comparing the amplitude of depolarization induced by the stimulus (D_c) with the corresponding D_{tn}.

Behavioural Assays

Ant behavioural response to candidate compounds, their mixture, and artificial leaf damage was assessed through the paired-leaves (here, paired-leaflets) protocol commonly used in the study of plant protection by symbiotic ants (Grangier et al. 2008). In *Leonardoxa*, a paripinnately compound leaf is associated with each domatium. We thus used, for each colony (i.e. for each tree), a pair of basal leaflets near an occupied domatium (Fig. 1f), one leaflet being used as control and the other for the experimental treatment. Control and experimental treatments were assigned randomly to the leaflets. After the application of the treatment and once a worker was observed on one of the focal leaflets, we recorded the number of workers on each leaflet every minute for 20 min. Tests were conducted during the period of peak ant activity (~11h00–15h00). Artificial damage on the experimental leaflet consisted in cutting a triangular piece of lamina with scissors (linear distance: 4 cm). The control leaflet was slightly shaken to mimic the disturbance caused by the scissors. The response to this artificial damage was tested in 16 colonies of *A. afer*, and 40 colonies of *P. phylax* ants. Colonies with the highest response to artificial damage (eight colonies of *A. afer* and 10 of *P. phylax*) were then used to test the response to candidate compounds and their mixture following a similar protocol on the same leaflet pairs used previously. A circular piece of adhesive tape (0.2 cm^2) was applied on each leaflet and 0.4 µl of pure compound or mixture was deposited on the experimental one. This volume of compound is the lowest that we could measure with repeatable accuracy in the field. Moreover, we used pure compounds to avoid possible confounding effects of solvent on ant behaviour. Each leaflet pair was tested successively for each candidate compound and their mixture, in a random order, and after intervals between treatments of at least 24 h. We calculated, for each test, the mean number of workers on the control and on the experimental leaflet (over the 20 min.). These values were compared with a Wilcoxon test for paired samples to determine which treatments had a significant effect on the ant number. In some cases, the difference between these two means was calculated to allow comparison between types of treatments (we used Wilcoxon tests for paired samples because the same colonies were used for the different types of treatments).

Near the village of Nkolo, where *L. a. africana* and *L. a. gracilicaulis* occur in sympatry, we compared the reaction of *P. phylax* ants (12 colonies) to artificially damaged mature leaflets of their host plant (*L. a. africana*) and to artificially damaged and intact mature leaflets of the non-myrmecophytic *L. a. gracilicaulis*, using the protocol described in Schatz et al. (2009). We did not test the reaction of *A. afer* to damaged leaflets of *L. a. gracilicaulis* because the two plants have never been found in sympatry. Leaflets were held at 1 cm from the entrance of a domatium for 3 min. We noted the time elapsed before the first worker exited and recorded the number of ants seen exiting from this domatium for the duration of the test. If no ant exited, the latency was thus 180 s. We applied all three treatments (*africana* damaged, *gracilicaulis* damaged, *gracilicaulis* intact) to each domatium in a random order and after intervals of at least 24 h. Tests were conducted during day time in periods of inactivity (before 11h00 and after 15h00) to ensure that few workers (if any) exited solely to forage for nectar.

All results were statistically analyzed using R 2.9.2 (R core development team). Raw data generated in this study have been deposited in the Dryad database (www.datadryad.org) under accession number. doi:10.5061/dryad.b779h.

Results

Foliar Compounds

All the foliar extracts analyzed contained the same compounds: dihydro-citronellol (traces), cyclopropane-1-carboxylic acid (traces), (E)-2-hexenal, (E)-2-hexenol (traces), hexanal, methyl salicylate, p-cymene (traces) and R-α-phellandrene (traces). Relative amounts of (E)-2-hexenal, hexanal and methyl salicylate appeared to differ among the three species considered. It is noteworthy that we identified substantial amounts of (E)-2-hexenal and only traces of (E)-2-hexenol in the solvent extracts, whereas Schatz et al. (2009) identified no (E)-2-hexenal and substantial amounts of (E)-2-hexenol in the volatile fraction of artificially damaged leaves. We could not quantify hexanal consistently because of partial overlap with the solvent peak. Amounts of methyl salicylate differed among subspecies (Kruskal–Wallis test, $H = 13.6$, $P < 0.001$). Indeed, methyl salicylate was more abundant in *L. a. africana* leaflets ($n = 15$) than in those of *L. a. letouzeyi* ($n = 20$) (Mann–Whitney test: $Z = 3.17$, $P < 0.01$) and *L. a. gracilicaulis* ($n = 8$) ($Z = 2.58$, $P < 0.01$) (Fig. 2). Differences were still significant after sequential Bonferroni correction (Holm 1979). Amounts of (E)-2-hexenal were not different among subspecies (Kruskal–Wallis test, $H = 0.015$, $P = 0.99$).

Antennal Responses

Each candidate compound and the mixture were detected by the antennae of workers of all ant species tested. However, capacity for detecting p-cymene and R-α-phellandrene

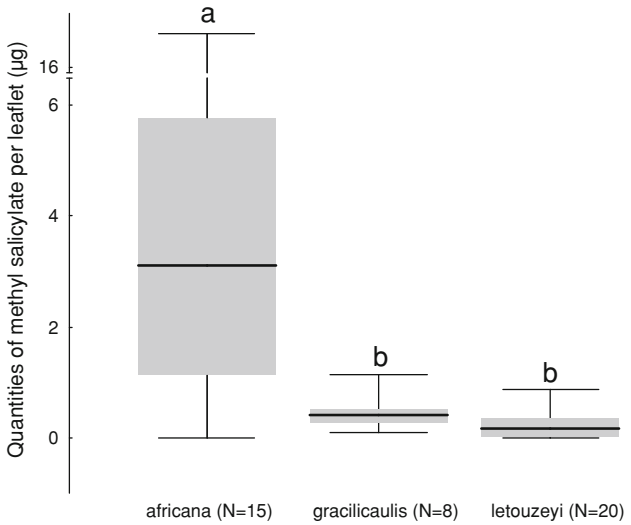

Fig. 2 Amount of methyl salicylate in leaflets of the three focal plant subspecies. Horizontal line, boxes and whiskers represent respectively median, 1st and 3rd quartiles, and 1st and 9th deciles. *Different letters* indicate significant differences (Mann–Whitney tests, $P < 0.05$, after sequential Bonferroni correction)

differed among species (Table 1 and Fig. S1 in Electronic Supplementary Material), showing that some compounds may not be detected and thus, that the responses to candidate compounds were not mere responses to the presence of any compound. Detection thresholds of the candidate compounds were determined for *A. afer*, *P. phylax*, *C. mckeyi*, and *A. bidens* by testing, for each quantity, the significance of depolarization relative to the corresponding putative control (D_{tn}, see methods). All dose–response curves showed a regular increase of depolarization with increase of compound quantity (Fig. S2). Table 2 shows response thresholds for each species for each compound. The amount of methyl salicylate used in behavioural tests (0.4 µl = 470 µg) was higher than the amount contained in an entire leaflet (see Fig. 2). However, the dose–response curves with the three candidate compounds show that antennal perception is not saturated, nor is it inhibited by quantities as high as those used in the behavioural tests. The amounts used in behavioural tests are thus compatible with ant physiological capacities.

Table 2 Perception thresholds of antennae for pure compounds determined from dose–response curves

	Methyl salicylate	Hexanal	(E)-2-hexenol
Aphomomyrmex afer	0.4 nl*	4 nl*	4 nl**
Petalomyrmex phylax	0.4 nl*	36 nl*	4 nl**
Cataulacus mckeyi	0.8 nl*	NA	NA
Axinidris bidens	0.4 nl**	0.8 nl**	4 nl**

NA data not available. Wilcoxon tests comparing the amplitude of depolarization induced by the stimulus with the amplitude of the corresponding control: * $P < 0.05$, ** $P < 0.01$. *P*-values for other amounts are displayed in Fig. S2

Behavioural Response

More *P. phylax* and *A. afer* workers were present on artificially damaged than on control leaflets of their host species, *L. a. africana* and *L. a. letouzeyi* respectively (Fig. 1f and 3; Wilcoxon test: *A. afer*: $n = 16$, $Z = 3.0$, $P < 0.01$, *P. phylax*: $n = 40$, $Z = 4.8$, $P < 0.001$).

Among the candidate compounds, hexanal was the only one to elicit more patrolling by *A. afer* than on the control (Fig. 4a; Wilcoxon test: $n = 8$, $Z = 2.2$, $P < 0.05$). Ant reaction to hexanal was lower than that induced by artificial damage (Wilcoxon test: $n = 8$, $Z = 2.1$, $P < 0.05$). Ant reaction to the mixture could not be tested statistically because only three colonies were tested.

By contrast, each of the three compounds, and their mixture, elicited significant patrolling by *P. phylax* workers (Fig. 4b; $n = 10$, Wilcoxon tests, $P < 0.05$ in all cases). Responses differed significantly among treatments

Table 1 Perception of compounds by the antennae of several ant species measured with the method of electroantennography

	P-cymene	(E)-2-hexenol	Hexanal	Mixture	Methyl salicylate	R-α-phellandrene
Petalomyrmex phylax	0.45	*0.00013*	*0.000018*	*0.000018*	*0.000018*	0.21
Aphomomyrmex afer	*0.00015*	*0.000012*	*0.000012*	*0.000012*	*0.000012*	*0.000072*
Cataulacus mckeyi	0.084	*0.000018*	*0.00044*	*0.000018*	*0.000021*	*0.015*
Axinidris bidens	*0.013*	*0.0022*	*0.0022*	*0.0022*	*0.0022*	*0.0022*
Crematogaster sp1	0.8	*0.0077*	*0.0051*	*0.0051*	*0.0051*	0.26
Crematogaster sp2	0.059	*0.0069*	*0.0051*	*0.0051*	*0.0051*	*0.022*
Camponotus aethiops	*0.0051*	*0.0051*	*0.0051*	*0.0051*	*0.0051*	*0.0051*
Oecophylla longinoda	*0.0093*	*0.0051*	*0.0051*	*0.0051*	*0.0051*	*0.0093*
Plagiolepis pygmaea	0.059	*0.0051*	*0.0051*	*0.0051*	*0.0051*	0.074
Polyrhachis laboriosa	*0.037*	*0.0051*	*0.0051*	*0.0051*	*0.0051*	*0.0069*

Values given are *P*-values of Wilcoxon tests comparing the depolarization measured for the compound and that for the corresponding putative control. Significant antennal responses ($P < 0.05$) are highlighted in italics

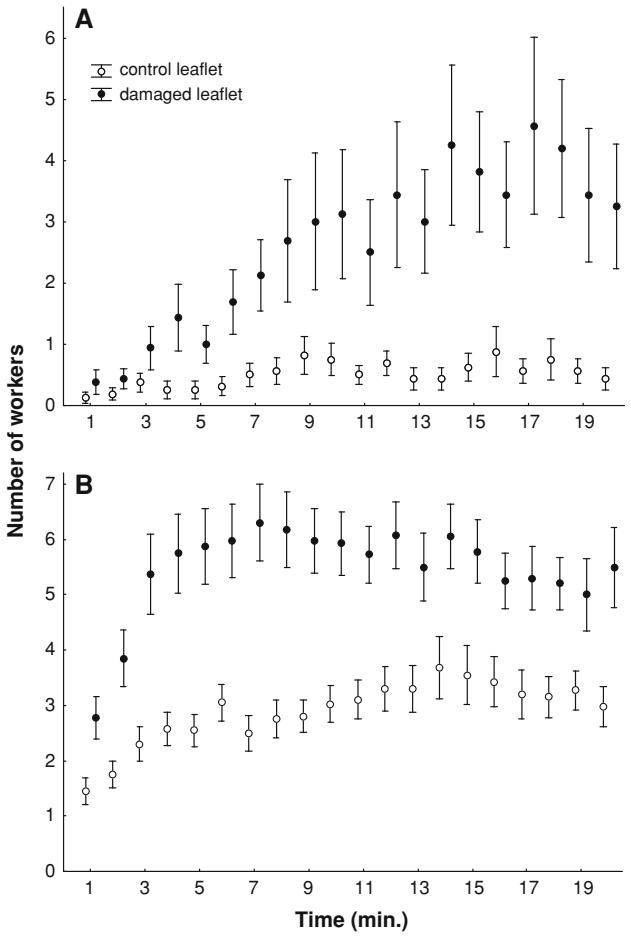

Fig. 3 Change over time of the mean (±SE) number of workers of (**a**) *Aphomomyrmex afer* ($n = 16$) and (**b**) *Petalomyrmex phylax* ($n = 40$) patrolling artificially damaged leaflet (*filled circles*) and undamaged control leaflet (*open circles*) of their host plant, respectively *Leonardoxa africana letouzeyi* and *L. a. africana*

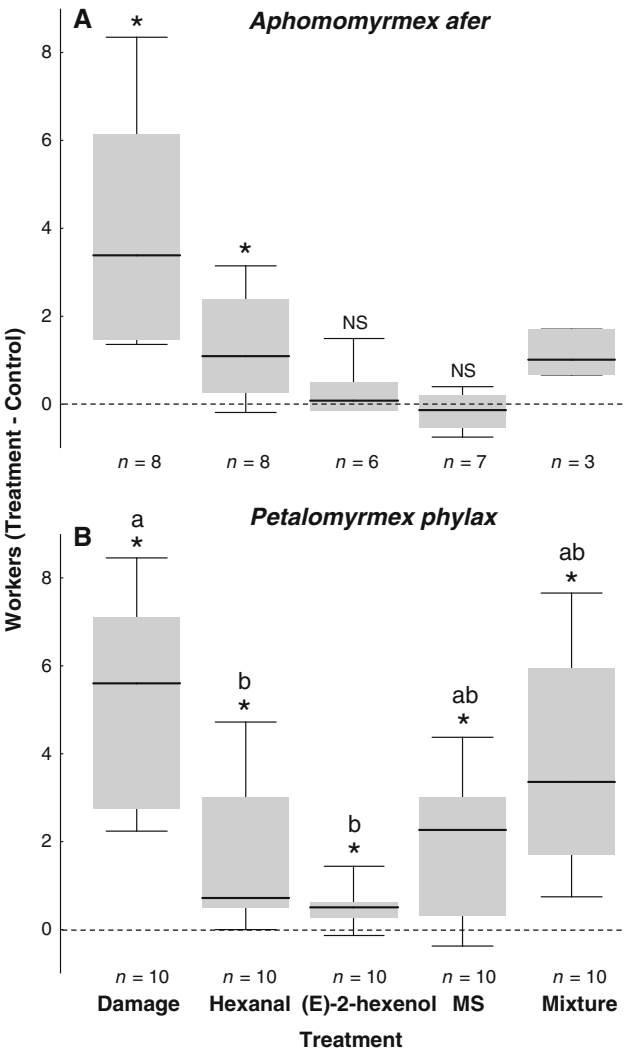

Fig. 4 Number of patrolling workers of (**a**) *Aphomomyrmex afer* and (**b**) *Petalomyrmex phylax* induced by various treatments (artificial damage of the leaflet, deposition of pure compounds or their mixture). The number of induced workers corresponds to the difference of patrolling workers between a control and an experiment leaflet. For "Damage" treatment, only the colonies also tested for other treatments are represented (see Fig. 3 for the whole data set for damage treatment). *MS* methyl salicylate. *Horizontal line, boxes* and *whiskers* represent respectively median, 1st and 3rd quartiles, and 1st and 9th deciles. *Number of workers significantly different from zero (Wilcoxon tests, $P < 0.05$), *NS* not significant. Test for the treatment with mixture on *A. afer* was not performed because of the low sample size. Different letters indicate significant differences between treatments (Wilcoxon tests, $P < 0.05$, after sequential Bonferroni correction)

(Friedman test, $n = 10$, $F = 21.4$, $P < 0.001$). Products inducing the strongest behavioural response were methyl salicylate and the mixture (Fig. 4b). We tested differences between all pairs of treatments (10 Wilcoxon tests) and applied the sequential Bonferroni correction. Responses to artificial damage, methyl salicylate and the mixture did not differ significantly. However, responses induced by hexanal and (E)-2-hexenol were significantly lower than that induced by artificial damage, but not different than that induced by methyl salicylate and the mixture. Thus, methyl salicylate appears to be the main component of the signal, but hexanal and, to a lesser extent, (E)-2-hexenol also probably contribute to ant response.

To test the effect of artificially damaged leaflets of *L. a. gracilicaulis* (the non-myrmecophyte) on *P. phylax* (inhabitant of *L. a. africana*) we presented leaflets at the entrance of domatia, counted the number of ants exiting and recorded the time elapsed (latency) before the first ant exited. *P. phylax* reacted differently to leaflets of *L. a. africana* (damaged) and *L. a. gracilicaulis* (intact or damaged) (Friedman tests, $n = 12$, latency: $F = 12.0$, $P < 0.01$; number of exiting workers: $F = 12.6$, $P < 0.01$). Artificially damaged leaflets of *L. a. africana* induced a stronger ant response than did artificially damaged leaflets of *L. a. gracilicaulis* (Wilcoxon tests: shorter latency: $Z = 2.3$, $P < 0.05$; more exiting workers: $Z = 2.1$,

$P < 0.05$). Responses to intact and artificially damaged leaflets of *L. a. gracilicaulis* did not differ significantly (latency: $Z = 1.0$, $P = 0.31$; exiting workers: $Z = 1.1$, $P = 0.26$), but tended to be more pronounced with artificially damaged leaflets.

Discussion

Our results showed that the two focal ant-plant symbioses have evolved communication through different processes. Behavioural assays revealed that the communication signal was different between the two systems: *P. phylax* responded most strongly to methyl salicylate whereas *A. afer* responded only to hexanal. In our study we used artificial damage on leaves as a proxy for herbivore damage. For some plant species, compound emission is known to differ depending on the type of damage, as well as on the stage of the herbivore (Turlings and Wäckers 2004). This is due to components of insect oral secretions that function as elicitors for some specific plant compounds. Herbivore-specific plant signals can be used by parasitoids to detect their insect host. Attraction can only be favoured by selection in parasitoids (or learned by them) if the information conveyed by the signal is reliable, which probably explains why herbivore-specific signalling has evolved in plants. In contrast with parasitoids, ant symbionts of plants have an interest in chasing any type of herbivore because the plant per se is a valuable resource (nest and food), and ants are broad-spectrum predators. Though we have not tested for variability of damage-induced signal according to the type of damage in our study models, it seems reasonable to assume that such variability is low and that artificial damage is a good proxy for herbivory. Good evidence for this assumption is our result that ants were attracted by compounds released upon artificial damage. Methyl salicylate and hexanal are commonly expressed by plants upon stress and leaf damage (Arimura et al. 2009; Dicke 2009). They usually play a defensive role either in within- and between-plant signalling activating pathogen resistance (Shulaev et al. 1997; Park et al. 2007) or by attracting predators and parasitoids of herbivores (Turlings et al. 1990; Arimura et al. 2009). Hexanal is also known to be attractive to phytophagous insects (Bruce et al. 2005) and pollinators (Raguso and Light 1998). These two compounds are very common in the plant kingdom and could thus hardly be used for signalling specific herbivores. In contrast with plants signalling specific herbivores to parasites or parasitoids (Takabayashi et al. 2006), our results strongly suggest that plant signalling damage to broad-spectrum predators such as ants involves unspecific signals.

Identification of foliar compounds revealed that the three investigated subspecies of *Leonardoxa*, including the non-myrmecophytic one, expressed the same compounds, and in particular the two used as communication signals, i.e. methyl salicylate and hexanal. Moreover, among the 80 or more genera in the tribe Detariae (Fabaceae, Caesalpinioideae), only two include myrmecophytes (Davidson and McKey 1993), *Leonardoxa* and *Humboldtia*, which are not closely related (Bruneau et al. 2001). This strongly suggests that myrmecophytism is a derived state in *L. a. africana* and *L. a. letouzeyi*, but that the expression of methyl salicylate and hexanal, and thus the biochemical pathways for their production, predates the origin of these symbioses. The signals involved in symbiosis are cheap, because they do not require the evolution of new biochemical pathways and their structure is rather simple and does not include limiting elements (only C, O and H). Communication theory predicts that honest signals are costly when partners have diverging interest, but that signals can be cheap when interests converge (Maynard-Smith 1991). This is the case in our focal symbioses: the plant benefits from being protected against herbivores and the ant benefits from protecting the plant because it provides food and nesting space.

Electroantennography revealed a striking result: all ant species tested were able to perceive the volatile compounds emitted by *Leonardoxa*, i.e. methyl salicylate, (E)-2-hexenol and hexanal. The ant species tested belonged to three subfamilies: Formicinae (to which belong *P. phylax* and *A. afer*), Dolichoderinae and Myrmicinae. The ants tested included both tropical and temperate and both tree-dwelling and ground-nesting species. Their taxonomic and ecological diversity suggests that the perception of these compounds is a capacity that is not restricted to symbionts of *Leonardoxa* but is instead widespread in ants. Thus, the perception of these compounds predates the evolution of symbiosis with plants in the lineage formed by *A. afer* and *P. phylax*, suggesting low cost for host plant signal perception. Ants may be predisposed to detect compounds produced by plants because they use a great diversity of compounds in their social activities, such as nestmate discrimination, alarm, recruitment and trail pheromones (Blatrix and Mayer 2010), and many of these compounds are also produced by plants (Arimura et al. 2009). It is noteworthy that the ant *C. mckeyi*, the parasite of the mutualism between *L. a. africana* and *P. phylax,* perceives the compounds emitted by the host plant, but does not respond to them behaviourally. This ant does not protect its host plant (Gaume and McKey 1999), nor does it respond to artificially damaged leaves (Schatz et al. 2009). Thus, the only level of specialisation that differs between the parasite *C. mckeyi* and the mutualist *P. phylax* is the behavioural response to plant compounds, and not the capacity to perceive them.

The use of different signals in communication is particularly surprising in the focal systems because they

involve two pairs of partners that are closely related: the plants *L. a. africana* and *L. a. letouzeyi* are considered as subspecies (McKey 2000) or incipient species, and the ants *P. phylax* and *A. afer*, although they belong to two different genera (both monotypic), are sister species (Chenuil and McKey 1996). Moreover, our data revealed that species of both pairs share common physiological features related to communication: both plants emitted the same compounds and both ants were able to perceive all those compounds. Two scenarios may be invoked to explain the use of different signals in the two symbioses. First, as the focal systems are exclusively allopatric, specific ecological or environmental constraints may apply in each distribution area, driving evolution of different signals in each symbiosis. However, the two systems occur in very similar environments: they grow in the understory of lowland tropical rain forest, near streams or swampy areas, and the centres of their respective distributional ranges are only about 200 km distant. Second, stochasticity may have played a role at some point in the evolution of communication, and led to the selection of different signals. Even if the outcome of adaptive selection can be somewhat predictable, different mechanisms may underlie functionally similar adaptations (Gould and Lewontin 1979; Huey et al. 2000).

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New Phytologist

Ant-plants and fungi: a new threeway symbiosis

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Summary

• Symbioses between plants and fungi, fungi and ants, and ants and plants all play important roles in ecosystems. Symbioses involving all three partners appear to be rare. Here, we describe a novel tripartite symbiosis in which ants and a fungus inhabit domatia of an ant-plant, and present evidence that such interactions are widespread.

• We investigated 139 individuals of the African ant-plant *Leonardoxa africana* for occurrence of fungus. Behaviour of mutualist ants toward the fungus within domatia was observed using a video camera fitted with an endoscope. Fungi were identified by sequencing a fragment of their ribosomal DNA.

• Fungi were always present in domatia occupied by mutualist ants but never in domatia occupied by opportunistic or parasitic ants. Ants appear to favour the propagation, removal and maintenance of the fungus. Similar fungi were associated with other ant-plants in Cameroon. All belong to the ascomycete order Chaetothyriales; those from *L. africana* formed a monophyletic clade.

• These new plant–ant–fungus associations seem to be specific, as demonstrated within *Leonardoxa* and as suggested by fungal phyletic identities. Such tripartite associations are widespread in African ant-plants but have long been overlooked. Taking fungal partners into account will greatly enhance our understanding of symbiotic ant–plant mutualisms.

Introduction

As the primary source of carbon, plants are involved in a great diversity of interactions with other organisms. Among these, symbioses with mycorrhizal fungi (Smith & Read, 1997) or endophytic fungi (Arnold & Lutzoni, 2007) are particularly common and have a major structuring role in many ecosystems (van der Heijden & Sanders, 2002), while myrmecophytes (i.e. plants symbiotically associated with ants; Davidson & McKey, 1993) are common and play an important role in tropical ecosystems (Davidson *et al.*, 2003; McKey *et al.*, 2005). Ants and fungi are themselves linked by mutualistic associations. Symbiosis between ants and fungi has evolved at least once, in the myrmicine tribe Attini that cultivates fungal mycelia in their nests, and whose > 200 species include some of major importance in tropical ecosystems (Mueller *et al.*, 2001). However, only one example of a tripartite plant–ant–fungus symbiotic mutualism has been recorded: *Allomerus decemarticulatus* ants in the myrmecophyte *Hirtella physophora* use mycelia of as yet unidentified fungi, as well as trichomes of the host plant, to construct a trap on the plant's twigs for capturing large insects (Dejean *et al.*, 2005). In this paper, we describe a very different plant–ant–fungus interaction of a type that seems to be quite widespread in myrmecophytes. We identify the fungi involved and provide arguments that the interaction is a tripartite symbiosis.

Myrmecophytes are plants that provide symbiotic ants with nesting cavities (specialized hollow structures termed domatia) and food. In return, the ants protect their host plant against herbivores. The presence of fungi inside domatia of myrmecophytes was first described in *Myrmecodia* and *Hydnophytum* from Java (Miehe, 1911). According to Huxley (1978), fungi are 'widespread in many species of ant-plants in Papua New Guinea'. Bailey (1920) reported similar fungi in domatia from

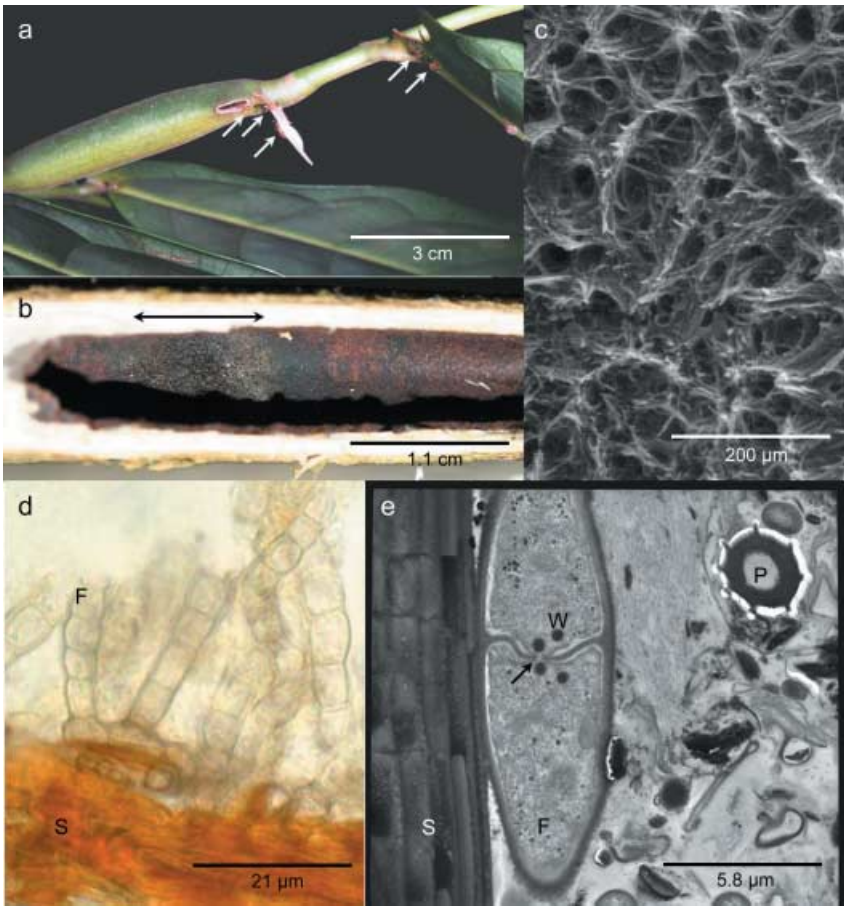

Fig. 1 The fungus growing in domatia of the ant-plants *Leonardoxa africana africana* and *Leonardoxa africana letouzeyi*. (a) External view of a domatium of *L. a. africana* and its ant *Petalomyrmex phylax* (workers indicated by arrows). (b) Domatium of *L. a. africana* cut longitudinally, showing the black fungal patch (under the double arrow). (c) Organisation of mycelia in a *L. a. africana* domatium (ESEM). (d) Interface between the plant *L. a. africana* (S, sclerenchymatous tissue of the inner wall of the domatium) and the fungal hyphae (F) (optical microscopy, visible light, without staining). (e) Transmission electron microscopy view of septum and septal pore (arrow) in fungal hyphae (F) from a *L. a. letouzeyi* domatium, showing Woronin bodies (W) typical of ascomycetes, plant sclerenchyma (S) and various wastes (including a pollen grain, P).

various African, American and Asian myrmecophytes belonging to a large array of families. 'Fungus gardens' in *Barteria fistulosa* were also noted by Janzen (1972) but were not further investigated (D. Janzen, pers. comm.). However, despite these reports over the past 90 yr of the widespread presence of fungi in ant-plant domatia, no work has been done on their functional role. Furthermore, there have been few attempts to identify the fungi involved. Using *in vitro* isolation, Huxley (1978) identified *Arthrocladium* spp. in domatia of *Myrmecodia* and *Hydnophytum*. However, isolation is sensitive to contamination, thus raising doubts that the symbiotic fungus was cultivated. Molecular tools, which have allowed great advances in fungal identification (Bidartondo & Gardes, 2005), have never been applied to these fungi.

Despite the potential ecological importance of the phenomenon in terms of the functioning of these symbioses and their apparent prevalence, no work to date has elucidated the nature of these associations. Here, we report on a similar fungus in domatia of African myrmecophyte genera from three different families (Fabaceae, Passifloraceae and Lamiaceae). We focus on two subspecies of *Leonardoxa africana* (Fabaceae: Caesalpinioideae), for which data from a comparative analysis allow inferences about the nature of the interaction. Our aims were: to characterize the occurrence and the distribution of domatia-inhabiting fungi across the distribution area of *L. africana*, and within single plants, as a function of the ant species occupying the plant; to detect and describe specific behaviours of ants towards the fungus; and to obtain preliminary identification of the fungi using morphological and molecular characters.

Materials and Methods

Sampled species

Leonardoxa africana (Baill.) Aubrév. has a restricted range in Lower Guinea (i.e. in coastal forests from Nigeria through Cameroon and Equatorial Guinea to Gabon). It is composed of four subspecies varying in traits of their association with ants (McKey, 2000). Biological studies of these taxa, regarded as in incipient stages of speciation (McKey, 2000) confirm that by some species definitions (biological, phylogenetic) they can be considered as full species (Léotard *et al.*, 2008). *Leonardoxa africana gracilicaulis* McKey has foliar nectaries that attract opportunistic ants, but lacks domatia. The three other taxa are true myrmecophytes. Each domatium is a hollow cavity within the twig comprising from one to three internodes (Fig. 1a). Each mature plant includes up to 2000 domatia, each unconnected

to any other domatium. Ants must bore an entrance hole through the wall of each domatium to access the cavity for nesting. The hole is chewed in a specialized part of the internode, the prostoma, that is devoid of the subepidermal layer of sclerenchyma that otherwise encloses the domatium and that bears little xylem tissue (Brouat *et al.*, 2001). Of the three myrmecophytes, *Leonardoxa africana rumpiensis* McKey has been relatively little studied. This taxon is endemic to a small, remote area of submontane forest; its interactions with diverse and unusual arboricolous ants are poorly understood and it was not investigated in this study. The second myrmecophyte, *Leonardoxa africana letouzeyi* McKey, is inhabited by diverse ant species in the sapling stage, but mature plants are occupied mostly by the mutualist ant *Aphomomyrmex afer* (Formicinae). The third and most specialized myrmecophyte, *Leonardoxa africana africana*, is associated with its host-specific mutualist ant *Petalomyrmex phylax* (also Formicinae), and the sister species of *A. afer* (Chenuil & McKey, 1996; Lapolla *et al.*, 2006), both constituting monotypic genera, at all stages from seedling to mature plant (Fig. 1a). However, a small but variable proportion of *L. a. africana* plants are occupied by the ant *Cataulacus mckeyi* (Myrmicinae), a specific parasite of the mutualism that does not protect the plant but feeds on its foliar nectar and prevents occupation by the mutualist *P. phylax* (McKey, 1984; Gaume & McKey, 1999). Moreover, a few domatia of plants inhabited by *P. phylax* are occasionally occupied by various opportunistic ants.

Samples of *Leonardoxa* were collected between 2000 and 2008 over the entire known species range for *L. a. africana* and from two distant sites within the range of *L. a. letouzeyi*. All samples were preserved in alcohol. Samples from 2000 to 2006 were collected for other purposes and stored in a refrigerated room, and then retrospectively checked for the presence of fungi. A total of 1185 domatia from 92 *L. a. africana* trees, 116 from 46 *L. a. letouzeyi* trees, and one domatium from an F_1 hybrid between *L. a. africana* and *L. a. gracilicaulis* were opened. Inner walls of domatia were checked under a binocular microscope for the presence of fungal mats. For nine *L. a. africana* trees out of the 91 examined, all the domatia were opened and the distribution of the fungus throughout the whole plant was investigated. Finally, five other ant-plants from Cameroon were also examined for domatium-occupying fungi: three individuals of *Barteria fistulosa* Mast. (Passifloraceae), one of *Calpocalyx cauliflorus* Hoyle (Fabaceae: Mimosoideae), three of *Kaetia hispida* (Rubiacea), one of *Vitex thyrsiflora* Baker and one of *Vitex grandifolia* Gürke (the two latter are Lamiaceae formerly placed in Verbenaceae).

Microscopy

The plant–fungus interface and the morphological structure of fungi in domatia of *L. a. africana* and *L. a. letouzeyi* were characterized. Fresh samples were used for optical microscopy and environmental scanning electron microscopy (ESEM). For transmission electron microscopy (TEM), samples were preserved by quick fixation in 2.5% (v : v) glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature and then overnight at 4°C. After rinsing them three times with the fixing cacodylate buffer, samples were dehydrated in an ascending series of ethanol solution to 100%, incubated in two changes of absolute acetone and infiltrated in Epon-Araldite resin. The resin was polymerized for 24 h at 60°C. Embedded samples were processed for ultramicrotomy: semi-thin sections of 0.5 μm were stained with 1% toluidine blue and ultra-thin (70 nm) sections were counterstained with uranyl acetate and lead citrate. Analyses were carried out under a Philips CM10 transmission electron microscope.

Microcinematography

Behaviour of *P. phylax* ants within domatia of *L. a. africana* was recorded in January and February 2008 near the village of NkolloBondé, Cameroon (3°13′N, 10°15′E). A video camera was used equipped with a 2.7 mm diameter endoscope (Bipol, Bezannes, France) fitted into holes that were drilled into domatium walls 2 d before observation. In the period between drilling and observation, holes were closed with a piece of *L. africana* leaf taped around the domatium. The endoscope was fitted into the hole 30 min before recording ant behaviour to allow ants to resume normal behaviour following this disturbance. Light was provided by a red light-emitting diode through an optical fibre included in the endoscope. The infrared filter of the video camera was removed to enhance red light perception.

Molecular identification of fungi

Samples of fungi were collected at NkolloBondé in February 2008 from domatia of four *L. a. africana*, two *B. fistulosa* and one *K. hispida*, occupied respectively by *P. phylax*, by *Tetraponera aethiops* (Pseudomyrmecinae) and by *Crematogaster* sp. (Myrmicinae). Each sample consisted of a whole fungus patch scraped from a domatium. Samples were stored in AP1 buffer of the DNeasy Plant Kit (Qiagen, Courtaboeuf, France), until laboratory analysis. DNA was extracted from each sample with the DNeasy Plant Kit, following the protocol for fungal tissue and adding 2 g polyvinyl polypyrrolidone at the initial step to eliminate phenolic compounds. DNA amplification of fungal internal transcribed spacer (ITS) of ribosomal DNA was performed by polymerase chain reaction (PCR) using the primers ITS1F (3′-TCCGTAGGTGAACCTGCGG-5′, specific for fungi) and ITS4 (3′-TCCTCCGCTTATT GATATGC-5′, universal for eukaryotes). An initial denaturing step at 94°C for 4 min was followed by 35 cycles of 30 s at 94°C, 1 min at 53°C, 1 min at 72°C and a final 10-min extension step at 72°C. The PCR products were sequenced by Agowa (Berlin, Germany) from both strands using the PCR

Table 1 Occurrence of fungi in domatia of the ant-plants *Leonardoxa africana africana*, *Leonardoxa africana letouzeyi* and an *africana* × *gracilicaulis* F_1 hybrid

Plant sp.	Ant species preponderant on plant	Number of plants	Ant sp. in domatia	Number of domatia	Number of domatia with fungus
L. a. africana	*Petalomyrmex phylax*	53	*P. phylax*	961	916
			P. phylax founding queen	1	0
			Axinidris	5	0
			No ant	82	16
	Cataulacus mckeyi	26	*C. mckeyi*	108	0
			C. mckeyi founding queen	2	0
			No ant	5	0
	?[a]	12	*Axinidris*	1	0
			Crematogaster	1	0
			Monomorium	1	0
			Tapinoma	2	0
			No ant	6	0
			Domatia closed	10	0
Hybrid	*Axinidris*	1	*Axinidris*	1	0
L. a. letouzeyi	*Aphomomyrmex afer*	29	*A. afer*	72	67
			No ant	1	0
			Closed domatia	1	0
	?[a]	17	*A. afer* founding queen	1	0
			Axinidris	4	0
			Cataulacus	10	0
			Crematogaster[b]	16	1
			Technomyrmex	1	0
			No ant	10	0

Plants are distinguished according to the main inhabiting (= preponderant) ant species and domatia are classified according to their occupancy state.

[a]The nature of the occupancy of the plant was not checked.

[b]One domatium occupied by *Crematogaster* sp. contained some fungal filaments, which differed in aspect from the fungus in domatia occupied by *A. afer*.

primers and then edited and aligned using SEQUENCHER 4.5 for MacOsX (Gene Codes Corporation, Ann Arbor, MI, USA). In order to find the closest relatives, sequences were blasted against GenBank using the algorithm BLASTN (NCBI: http://ncbi.nlm.nih.gov/BLAST/in_250). Our attempt to amplify fungal DNA from ethanol-preserved samples ($n = 20$) failed. Samples preserved in AP1 yielded good quality sequences that are all reported here.

Results

Occurrence and distribution of fungi

Results from the investigation of domatia are given in Table 1. The domatia of hybrids of *L. a. africana* and *L. a. gracilicaulis* are usually not suitable for hosting ants but one was found to be colonized by a colony of an *Axinidris* sp. (Dolichoderinae), with no fungus. A fungus patch occurred in 95% of the *L. a. africana* domatia occupied by the mutualist ant *P. phylax* ($n = 961$), but never in domatia occupied by the parasitic ant *C. mckeyi* ($n = 108$). This difference in fungus distribution was highly significant (χ^2 statistic with Yates correction = 96, $P < 10^{-5}$). Fungi were found in 93% of the *L. a. letouzeyi* domatia occupied by the mutualist ant *A. afer* ($n = 72$) and in only 3.2% (one domatium) of the 31 domatia occupied by other ant species (χ^2 statistic with Yates correction = 20, $P < 10^{-5}$). This domatium occupied by *Crematogaster* sp. contained filaments of an unidentified fungus, the aspect of which was quite different from those in domatia occupied by *A. afer*.

Nine *L. a. africana* plants occupied by *P. phylax* were investigated in detail, and found to contain a total of 854 domatia. Among 767 domatia occupied by the mutualist ant, five occupied by the ant *Axinidris* sp. and 85 unoccupied domatia, 734 (> 95%), none (0%) and 16 (< 19%) respectively contained fungi (see Table 1). In any case, the fact that entrance holes of these domatia were open indicates that they had been occupied fairly recently, because entrance holes close as a consequence of stem growth whenever ants do not actively maintain the opening. Fungi were never seen in unopened domatia. Some opened, unoccupied domatia on ant-occupied plants can thus contain fungus, but it is likely that these had been recently or temporarily deserted. It is noteworthy that, in all cases, only a single fungus patch per domatium was seen.

Table 2 Molecular identification of the fungi found in domatia of *Leonardoxa africana africana*, *Barteria fistulosa* and *K. hispida*

Host sp.	Sample no.	GenBank accession no.	Best taxonomically informative BLAST (with e-value)[a]	% similarity with other sequences[b]					
				La1	La2	La3	Bt1	Bt2	Cs1
L. a. africana	La1	EU856528	EU139157 *Capronia* sp. (7e-112)	–	**99.3**	**99.5**	67.8	68.3	71.0
	La2	EU856529	EU139157 *Capronia* sp. (4e-134)	**99.3**	–	**99.5**	68.7	69.1	72.3
	La3	EU856530	EU139156 *Capronia* sp. (1e-134)	**99.5**	**99.5**	–	67.7	68.1	72.3
B. fistulosa	Bt1	EU856531	EU139150 *Capronia* sp. (9e-136)	67.8	68.7	67.7	–	**94.5**	67.4
	Bt2	EU856532	DQ914667 *Cladophialophora* sp. (2e-132)	68.3	69.1	68.1	**94.5**	–	69.1
K. hispida	Cs1	EU856533	AJ507323 *Phaeococcomyces chersonesos* (6e-177)	71.0	72.3	72.3	67.4	69.1	–

[a]The BLAST expected value represents the number of sequence matches expected at random (the smaller the value, the better the match between our sample sequences and those in the NCBI database); all reported species belong to Chaetothyriales.
[b]Values over 90% are in bold type.

Fungus patches of similar aspect were found in four other myrmecophytes: *B. fistulosa* occupied by *Tetraponera aethiops* ($n = 3$), *K. hispida* occupied by *Crematogaster* sp. ($n = 3$), *V. grandifolia* occupied by *A. afer* ($n = 1$), and *V. thyrsiflora* occupied by *Tetraponera tessmanni* ($n = 1$). In a fifth myrmecophyte, *C. cauliflorus* occupied by *Atopomyrmex calpocalycola* (Myrmicinae) ($n = 1$), the fungus covered almost the whole inner surface of the hollow twig collected.

Morphological characterization of the fungus from *L. a. africana* and *L. a. letouzeyi*

Each fungal patch had the aspect of a small, disc-shaped mat, thin and regular in thickness, covering the inner wall of the domatium (Fig. 1b). Patches were constituted of prostrate filaments running along the internal surface of the domatium wall with short branches erected perpendicularly to the surface (Fig. 1c,d). Transmission electron microscopy confirmed the hyphal nature of the filaments. Filaments were regularly septate, with a central pore at each septum and Woronin bodies (i.e. specialized peroxisomes typical for ascomycetes; Fig. 1e). No hyphae were detected extending within the sclerenchymatous tissue of the plant lining the inner wall of domatia (Fig. 1d,e), suggesting that the fungus did not grow endophytically. Numerous bacterial cells were also seen.

Ant behaviour

Video recordings of *P. phylax* behaviour within domatia of *L. a. africana* revealed four types of interactions between ants and fungal patches. (1) The ants were often seen defecating and depositing detritus on the fungus (see the Supporting Information, Video S1). (2) Worker ants were observed chewing the hyphae; this was behaviour specific to the ant–fungus interaction (Video S2). (3) On a few occasions a worker chopped a piece of fungus and transported it in its mandibles out of the field of view of the camera (Video S3). (4) A fourth interaction was noted after we unintentionally disturbed ants in some domatia. Before recording ant behaviour, various ways to fit the endoscope within domatia were tried, by drilling a hole in the wall and positioning the endoscope tip with precision. These domatia were disturbed more than the others, in which only one hole was drilled and quickly covered by a piece of leaf taped around the domatium. These domatia, in which ants were more intensively disturbed, were deserted by the ants a few days later, and the fungal patch also disappeared. No dead hyphae were ever detected in these domatia, whereas hyphae were present at the time of intrusion. This, in addition to the capacity of ants to chop pieces of fungus, strongly suggests that the ants can entirely remove the fungal patch.

Molecular identification of fungi

The ITS sequences were directly obtained from PCR products in all samples investigated from *L. a. africana*, *B. fistulosa* and *K. hispida*: although we cannot exclude that other fungi were present, this suggested that the corresponding fungus dominated the patch. BLAST analysis revealed that all fungi sequenced were ascomycetes, congruently with TEM data, and belonged to the order Chaetothyriales (Table 2). However, no close relatives were found in GenBank, and the ITS sequences did not furnish enough informative positions to build a well-supported tree (Fig. S1). However, samples clustered according to their host species. Moreover, for fungi from the two species from which two or more sequences were obtained, *L. a. africana* and *B. fistulosa*, there was greater sequence similarity between different samples from the same host species (> 94%) than between host species (< 70%), suggesting that a different set of related, but different Chaetothyriales clades were associated with each plant species.

Discussion

This is the first study to provide clues on the nature of the relationships between ant–myrmecophyte symbioses and fungi growing within myrmecophyte domatia, and the first to establish the taxonomic identity of myrmecophyte-associated fungi using molecular tools. Our results suggest fungi are symbiotic with the plant *L. africana* and its associated ants. The arguments leading to these conclusions are detailed hereafter.

Occurrence of a fungus is constant

The first striking fact is the regular occurrence of the fungus in domatia of *L. a. letouzeyi* and *L. a. africana* occupied by mutualist ants. Several plants were sampled from various populations in different years, showing with confidence that the occurrence of these fungi is constant in these taxa. Moreover, nearly all domatia investigated contained the fungus and plants that were entirely dissected had few ant-occupied domatia without fungus. In all domatia where fungi occurred, a single patch was recorded per domatium.

Ants are responsible for, or at least facilitate, the spread of the fungus

Observations in *L. a. africana* plants that were entirely dissected allowed comparison of the occurrence of the fungus as a function of the presence or absence of the mutualist ant at the scale of individual domatia. In each plant, the fungus was present in nearly all domatia occupied by *P. phylax*, but in only 19% of unoccupied but open domatia, indicating that this particular ant had a specific role in the spread and development of the fungus within the growing plant. Moreover, no fungus was detected in unopened domatia, and young, only recently occupied domatia often had no detectable patch or only a small patch of fungus, suggesting that inoculation comes from the outside by way of ants, and not from within plant tissues (in which the fungus was never seen).

The fungus occurs only in association with ants that are mutualists of the plant

In both *L. a. africana* and *L. a. letouzeyi* we were able to compare plants that were occupied by the mutualist ants and plants occupied by other species. No fungus was found in domatia of plants occupied by *C. mckeyi*, the parasite of the *L. a. Africana–P. phylax* mutualism. The *L. a. letouzeyi* plants contained fungus when occupied by *A. afer*, the mutualist ant, but not when occupied by one of the various opportunistic ant species that inhabit juvenile trees. Thus, only mutualist ants have a role in the occurrence of the fungus, which seems tightly linked to the ant–plant mutualism. Moreover, *C. mckeyi* usually colonizes *L. a. africana* after an initial colony of *P. phylax* has perished, owing to, for example, a major disturbance (Debout, 2003), which means that most trees occupied by *C. mckeyi* had fungi previously. Thus, the mutualist ant is needed not only for establishment of the fungus but also for its persistence, at least in the long term.

Fungal sequences are consistent with host specificity

Under the dissecting microscope, all fungal patches had a similar aspect and TEM showed they are ascomycetes. Internal transcribed spacer sequencing provided only one sequence per sample. However, we cannot exclude the presence of other undetected fungi. Molecular identifications yielded very similar sequences in the four samples from *L. a. africana*, suggesting a single clade, related to, but slightly different from, those identified in *B. fistulosa* and *K. hispida*. Given the small sample size for molecular identification, additional samples should be studied to test whether these particular fungal clades are as widely distributed in their hosts, but our preliminary molecular results are consistent with a specific association of the fungus with the ant–plant mutualism. The order Chaetothyriales, to which the fungi identified belong, is poorly studied from the ecological point of view. It includes species with various lifestyles, such as epiphytes and endophytes with weakly pathogenic to mutualist effects on plants, as well as saprophytes; the family Herpotrichiellaceae also includes some pathogens of animal skin (Geiser *et al.*, 2006). A species is occasionally found in association with *Lasius* ants of the subgenera *Dendrolasius* and *Chtonolasius*, which are known to use and tend fungi as a structural component of their nests (Schlick-Steiner *et al.*, 2008). However, these ants are more regularly associated with fungal species from other orders. Which of these ecologies characterized the ancestors of the fungi detected here is unknown. Although a plant-associated ecology seems more likely, an animal-pathogenic ecology could have conferred a predisposition to digest fragments of cuticles of ants and other invertebrates, such as those we found among hyphae.

The ants display specific behaviours toward the fungus

This study is the first to provide behavioural observation of ants within domatia of a myrmecophyte with minimal disturbance. Video recordings within the domatia of *L. a. africana* showed the ant *P. phylax* chewing the fungus. The function of this behaviour remains to be elucidated but at least shows that this ant displays a specific behaviour toward the fungus, suggesting a tight relationship between the two organisms. Moreover, we have direct and indirect evidence that the ants are capable of removing the fungal patch: first, a video recording showed a worker cutting off a part of the patch with her mandibles; second, disturbed domatia were deserted not only by the ants, but also by the fungus, in a few days. The disappearance of the fungus was not the result of its quick death, because we could maintain fungal patches intact

for several days in isolated domatia from which ants had been removed manually. These observations were made in domatia that had been artificially disturbed and should thus be interpreted with caution. However, there was no obvious indication that ants were alarmed, or otherwise disturbed, at the time the recordings were made.

Observing the behaviours of this ant, which are reminiscent of pruning activities known in fungus-farming ants (Bass & Cherrett, 1996), it is tempting to speculate that they contribute to the characteristic shape and form of the fungal colonies. Fungal patches are very regular in shape and thickness, with no hyphae protruding out of the mat; in addition, only a single patch occurs per domatium. This distinctive and highly regular form and distribution, which is unexpected for a fungal mycelium, was at first more reminiscent of patches of some type of plant hairs with determinate development. The tending activities of ants probably have a strong impact on fungal morphogenesis. Similarly, the striking absence of reproductive structures (e.g. asexual spores) of the fungus, if confirmed, would make it strongly dependent on ants for dissemination, as again is the case for the fungal mutualists of farming ants (Mueller *et al.*, 2001).

Outline and perspectives

All these arguments point to the conclusion that these associations are symbiotic. The next step will be to focus on the nature of the fungal partner and its role, which remains poorly documented here. Is the fungus mutualistic, parasitic or commensal to the ant and/or the plant? The investigation of costs and benefits between the three partners, through experimental exclusion or demonstration of nutrient flux, for example, will be necessary to understand the relationships in these systems. We observed that the fungus patch was often associated with various remains that apparently constituted refuse piles of the ant colony, including pieces of arthropod cuticle in which we recognized parts of the mutualist ants. As tropical soils are usually characterized by low availability of nitrogen and phosphorus (Newbery *et al.*, 1986), the fungus could be a nutrient recycler decreasing the nitrogen and phosphorus cost of the symbiosis for the plant, but this hypothesis remains to be tested.

Historical records (Miehe, 1911; Bailey, 1920; Janzen, 1972; Huxley, 1978), together with our study, show that occurrence of fungi in myrmecophyte domatia is a widespread phenomenon, occurring in tropical Asia, Africa and the Americas, and involving at least 15 myrmecophytic plant genera. Taking into account this additional partner in ant–plant symbioses will lead to a reinterpretation of these associations and should have a strong impact on our understanding of the functioning of ant–plant relationships. In particular, study of the poorly understood nutritional functioning of ant–plant symbioses (Davidson, 1997; Davidson *et al.*, 2003; Cook & Davidson, 2006) could gain new impetus from this discovery.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Consensus tree (from 100 phylograms) for the Chaetothyriales, including fungus samples from domatia of the myrmecophytes *Leonardoxa africana africana*, *Barteria fistulosa* and *K. hispida*.

Video S1 Video recording of ants depositing detritus and defecating on the fungus. The red circle indicates the fungal patch.

Video S2 Video recording of an ant chewing the fungus.

Video S3 Video recording of an ant removing a piece of the fungus.

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Coevolution between slave-making ants and their hosts: host specificity and geographical variation

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Abstract

We explored the impact of a slave-making ant, *Protomognathus americanus*, on two of its hosts, *Leptothorax longispinosus* and *L. ambiguus*. We showed that, on average, slave-maker colonies conduct raids on 2.7 *L. longispinosus* and 1.4 *L. ambiguus* nests in a single year. The more common host, *L. longispinosus*, survives raiding and colony-founding events in a third of the cases, but the less common host rarely survives attacks from the slave-makers. We compare our results, collected in Vermont, to a study conducted in New York where the slave-maker pressure is much stronger. Our results suggest that in Vermont the slave-maker has a sparing strategy when raiding *L. longispinosus*, but not when raiding *L. ambiguus*. Thus coevolution between slave-making ants and their hosts shows host specificity and geographical variation.

Keywords: coevolution, Formicidae, *Leptothorax*, microsatellites, *Protomognathus*, social parasitism

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Introduction

Insect societies represent unique targets for parasites to exploit (Schmid-Hempel 1998), and evolutionary opportunities for exploitation by parasites of social species culminate in the social parasites, which exploit societal structure itself (Hölldobler & Wilson 1990). Recently, the concept of host–parasite coevolution via an arms race (Thompson 1994) has been successfully invoked to explore reciprocal selection in slave-making ants and their hosts (Foitzik *et al*. 2001; Foitzik & Herbers 2001a,b; Hare & Alloway 2001). These socially parasitic species start a colony when a slave-making ant queen invades a host nest; typically she kills or drives off its resident queen and subjugates host workers to become her slaves. Then, she lays eggs that are reared by the slaves to adulthood. The resulting slave-maker workers are unable to care for the brood or even to feed themselves (Wheeler 1910; Wesson 1939; Stuart & Alloway 1985); rather, they perform raids on nearby hosts to replenish the colony with slaves (Buschinger 1986). Throughout their lives, the slave-makers exploit the host work force to forage, nurse the brood, and perform other routine ant tasks. However, there is counter-selection on hosts to resist invasion and raiding from the parasite (Foitzik *et al*. 2001, 2003; Hare & Alloway 2001). In fact, intruding queens and raiding parties are not always successful, and resistance by host colonies sometimes prevents enslavement (Foitzik *et al*. 2001). This gives evidence that host species exert reciprocal selection pressure on the slave-maker.

Protomognathus (= *Harpagoxenus*) *americanus* (Emery) is an obligate slave-making ant in temperate deciduous forests of eastern North America where it enslaves three formicoxenine host species of the genus *Leptothorax* (*L. longispinosus*, *L. ambiguus* and *L. curvispinosus*). During colony foundation, the *P. americanus* queen kills or drives off all adult residents (queen and workers) in a host nest and settles down to await emergence of new slave workers from the remaining pupae. Both colony founding and raiding activity are potentially harmful to the host population, but the relative risks of the two sources of parasite pressure are not well understood. Colonies of the slave-maker are polydomous (Alloway *et al*. 1982; Alloway & Del Rio Pesado 1983), i.e. they occupy several nest sites at a time. The secondary nests typically arise during a raid, when the slave-makers stay in the raided nest instead of going back to their colony (Wesson 1939).

Interactions of *P. americanus* with *L. longispinosus* vary across locations, mediated by behavioural and ecological shifts of both host and parasite (Herbers & Stuart 1998;

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Foitzik *et al*. 2001; Foitzik & Herbers 2001a,b; Herbers & Foitzik 2002). Here we follow up on an intensive study of the host–parasite relationship in one location, replicating methodologies used by Foitzik & Herbers (2001a,b) in a second location. By using spatially explicit genetic and demographic data, we can estimate the impact of the slave-maker on its hosts there and can make valid comparisons between sites to explore coevolutionary dynamics between the parasite and its hosts. Our study therefore aims to test the hypothesis that different locations show different outcomes of the host–parasite interaction. According to the concept of the geographical mosaic of coevolution (Thompson 1999), the location near Albany, New York (NY), studied by Foitzik & Herbers (2001a,b) can be considered as a 'hot spot' (a spot where both parasite strategy and host defence evolve rapidly because of a strong reciprocal selection), because the parasite exerts a strong selective pressure on its primary host *L. longispinosus*, and the host has an effective defence strategy (Foitzik *et al*. 2001). By contrast, we suspected a site in Colchester Vermont (VT) might represent a cold spot (a spot showing a weak reciprocal selection) for coevolution between *P. americanus* and *L. longispinosus* for two reasons. First, the host nest density has strongly declined over the past 20 years, but that decline could not be linked to slave-maker pressure (Herbers & Foitzik 2002). Second, two host species occur at this location, and host switching could dilute the impact of the slave-maker on *L. longispinosus*. The ecological background to our focal species in the VT site differs substantially from that in NY, leading us to predict that coevolution between *P. americanus* and *L. longispinosus* would be weak, i.e. VT is a coevolutionary cold spot.

Materials and methods

Study system

We collected nests of the slave-maker *Protomognathus americanus* and its hosts in Vermont, at Niquette Bay State Park (formerly Mallett's Bay State Park, Chittenden County). There two host species occur, *Leptothorax longispinosus* and *L. ambiguus* (Herbers 1989), with the former being more abundant. These ants nest in small, preformed cavities and forage through the leaf litter as scavengers. We excavated nine plots, each 6 m × 6 m, in September 2001, after the raiding season had concluded. Plots were about 50 m away from each other. We mapped and collected every nest of both hosts and slave-makers; they were then counted and frozen for subsequent genetic analysis. When a slave-maker nest was found less than 1 m from the edge of the plot, we collected every nest (hosts and slave-makers) within a further distance of 1 m from the edge to have a greater chance of collecting potential survivors of raids and colony subunits.

Table 1 Variation in four microsatellite loci in the slave-maker *Protomognathus americanus* and its two host species *Leptothorax longispinosus* and *L. ambiguus*, from a population in Vermont

Locus	No. of alleles	Frequency of most common allele	H_E
P. americanus (11 nests, 31 individuals)			
LXAGT1	6	0.565	0.620
L18	8	0.274	0.813
L5	11	0.210	0.864
Myrt3	11	0.290	0.835
L. longispinosus (33 nests, 535 individuals)			
LXAGT1	20	0.193	0.906
L18	17	0.144	0.899
L5	16	0.295	0.846
Myrt3	8	0.260	0.816
L. ambiguus (9 nests, 123 individuals)			
LXAGT1	16	0.291	0.843
L18	16	0.228	0.871
L5	11	0.234	0.838
Myrt3	12	0.209	0.872

H_E, expected heterozygosity.

Genetic analysis

Genetic analyses were performed on individuals from slave-maker nests and nearby hosts' nests in our plots. Number of individuals genotyped per colony was proportional to colony size. For colony sizes up to 10 we genotyped all the individuals; for colony sizes 11–20 we genotyped 10 individuals; and so on, up to 30 individuals in the largest nest. Total numbers of nests and individuals genotyped are summarized in Table 1. DNA was extracted by grinding individual ants in cell lysis solution and incubating at 65 °C for 1 h. Ammonium acetate was added and the sample was centrifuged. The DNA from the supernatant was precipitated with isopropanol, dried, and then resuspended in 100 μL 1× TE.

We amplified by polymerase chain reaction (PCR) four microsatellite loci: LXAGT1 (Bourke *et al*. 1997), Myrt-3 (Evans 1993), L-5 and L-18 (Foitzik *et al*. 1997). All these loci showed enough variation in the slave-maker and its two hosts to ask detailed questions about slave origin and family structure (Table 1). Protocols and visualization methods are described in Foitzik & Herbers (2001a).

We analysed the genetic data by inspection and Mendelian inference to reach conclusions about family structure, polydomy, host survival and number of family groups represented in slave pools (Foitzik & Herbers 2001a,b). We estimated heterozygosities and relatedness coefficients (and their jackknifed standard error, SE, over loci) with the program RLAT 4.2, weighting individuals equally (Queller & Goodnight 1989).

Table 2 Content of nine 36-m² plots excavated in Vermont, and colony structure of the slave-maker, *Protomognathus americanus*

Plot	*P. americanus* nests (total)	Unidomous colonies	Polydomous colonies	Founding queens	*L. longispinosus* nests	*L. ambiguus* nests
I	0	0	0	0	12	0
II	0	0	0	0	13	0
III	1	0	0	1	20	0
IV	4	1	0	3	3	4
V	2	0	1	0	15	5
VI	0	0	0	0	1	4
VII	2	0	1	0	4	0
VIII	2	0	1	0	3	0
IX	0	0	0	0	2	5

Table 3 Demography of nests and colonies of *Protomognathus americanus* in our sample

Nest	Slave-maker queen(s)	Slave-maker workers	*L. longispinosus* slaves	*L. ambiguus* slaves
Newly founded colonies				
III	1	0	4	0
IV	1	0	3	0
IV	1	0	12	0
IV	1	0	74	0
Mature nests/colonies				
IV	0	2	14	0
V	1	14	170	7
V	0	3	115	11
VII	0	2	16	16
VII	0	4	38	16
VIII	0	1	15	0
VIII	0	4	62	0

We collected four newly founded colonies and seven mature colonies. The mature nests were organized into four colonies, three containing two nests each.

Results

Colony structure of the slave-maker, Protomognathus americanus

From the nine plots excavated, five contained slave-makers (Table 2). Across the five plots, we collected four newly founded slave-maker colonies (containing a slave-maker queen and slaves but no slave-maker workers) and seven mature slave-maker nests. These nests were located in plots of variable host density (Table 2).

Newly founded slave-maker colonies had a few slaves and small broods (Table 3); the slaves were always *Leptothorax longispinosus*. Mature slave-maker nests were also small, with a few workers and a few dozen slaves (Table 3). The largest nest had 14 workers and 177 slaves of both host species. Most of the mature nests were queenless (Table 3), which reflected either polydomous colony architecture or orphaning events. To distinguish, we superimposed worker genotypes onto maps to examine whether neighbouring nests contained sets of sister workers (Fig. 1). Inspection of the genetic data showed that the seven mature nests belonged to four colonies (Table 2), only one containing a queen. Three polydomous colonies contained two nests each, located between 0.40 m and 5.52 m apart (average distance: 2.38 ± 1.59 m); the final nest was its own monodomous colony. In three of our four colonies, workers were full-sisters, and in one colony, two unrelated matrilines were detected ($r = -0.03 \pm 0.05$). On average, slave-maker worker relatedness within the colony was 0.65 ± 0.14.

In plot IV, the three founding queens were not related to each other ($r = -0.14 \pm 0.02$), nor were they relatives of workers in the nearby mature nest in that plot (for queen 1: $r = 0.05 \pm 0.07$; for queen 2: $r = -0.08 \pm 0.07$; for queen 3: $r = 0.11 \pm 0.08$). Thus our limited data gave no signal of population viscosity caused by limited queen dispersal.

Colony structure of the primary host, L. longispinosus

We found free-living *L. longispinosus* hosts in every plot (Table 2) at an average nest density of 0.23 ± 0.06 nest/m². Neither nest density, queen number, numbers of larvae,

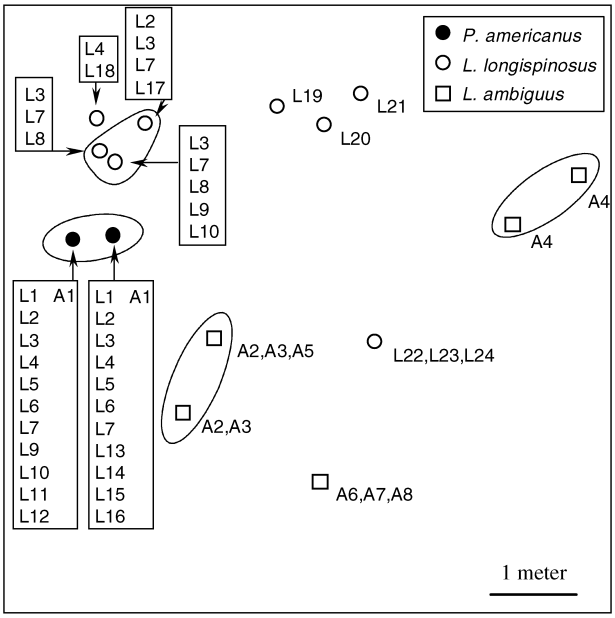

Fig. 1 Distribution of our focal species on one plot, illustrating polydomous colony structure, host nest survival, and slave-sharing. Ellipsoids surround members of polydomous colonies; this plot contained one polydomous colony of the slave-maker, one of the host *Leptothorax longispinosus*, and two of the host *L. ambiguus*, as well as unidomous colonies of both host species. The boxes give multilocus genotypes of the *Leptothorax* workers; each entry denotes a line of full-sisters, and 'L' refers to *L. longispinosus* 'A' to *L. ambiguus*. Thus the slave-maker nests each contained slaves of both species with seven lines of full-sisters of *L. longispinosus*, and one line of full-sisters of *L. ambiguus* slaves; in addition each of the two slave-maker nests contained four lines of full-sisters of *L. longispinosus*.

nor worker number differed between hosts nests collected on plots with or without slave-makers (Table 4; χ^2 test and Mann–Whitney *U*-tests, respectively: $U = 6$, $P > 0.1$; $\chi^2 = 3.2$, $P > 0.1$; $U = 555$, $P > 0.1$; $U = 539$, $P > 0.1$).

We restricted our genetic analysis of hosts to those on plots containing slave-makers to facilitate comparison with their counterparts in the enslaved condition (Foitzik & Herbers 2001b). Our genetic analysis of 345 workers and 21 queens from 22 free-living *L. longispinosus* nests yielded two polydomous colonies, each containing three nests. Of those 22 nests, eight comprised simple families (genotypes consistent with a single queen mated once), while 14 (64%) contained more than one set of sisters. All, however, had genotype arrays consistent with extended families, having workers parented by sisters, mother–daughters, or aunt–niece combinations; we found evidence of unrelated queens contributing jointly to the worker pool in only two nests. Both queenless and queenright nests of this host species contained multiple sets of sisters and similar frequencies of simple vs. complex families (*G*-test, $P > 0.05$) Average worker relatedness in host nests was less than the three-quarters expected under Hamilton's (1964) theory of kin selection ($r = 0.63 \pm 0.03$, one-tailed *t*-test $P < 0.001$) and the average nest included 2.05 lines of full-sisters (Table 5). Taken together, these data show that queen turnover was common in the host, and supercedure typically involved a close relative rather than a stranger.

Table 4 Demography of nests of the host species, *Leptothorax longispinosus* and *L. ambiguus*, collected in Vermont

Plots	With slave-makers	Without slave-makers
L. longispinosus nests	42	28
Queenless	4 (9.5%)	7 (25%)
Monogynous	27 (64.3%)	16 (57.1%)
Polygynous	11 (26.2%)	5 (17.9%)
No. of larvae (mean ± SE)	69 ± 7	65 ± 10
No. of workers (mean ± SE)	44 ± 5	42 ± 7
L. ambiguus nests	9	9
Queenless	2 (22%)	4 (44.5%)
Monogynous	2 (22%)	4 (44.5%)
Polygynous	5 (56%)	1 (11%)
No. of larvae (mean ± SE)	66 ± 18	49 ± 11
No. of workers (mean ± SE)	33 ± 9	47 ± 8

Colony structure of the secondary host, L. ambiguus

We found *L. ambiguus* in four plots (Table 2), and nests did not differ in queen number, number of larvae, or worker

Table 5 Relatedness estimates and number of lines of full-sisters of host workers in free-living nests and in slave pools of the slave-maker nests

	Worker relatedness	No. of lines	No. of nests	No. of workers genotyped
L. longispinosus				
Free-living nests	0.63 ± 0.03	2.05 ± 0.23	22	345
Slaves in mature nests	0.26 ± 0.06	6.00 ± 1.38	7	128
Slaves in foundations	0.53 ± 0.19	2.50 ± 0.65	4	41
L. ambiguus				
Free-living nests	0.68 ± 0.03	2.00 ± 0.45	5	69
Slaves in mature nests	0.48 ± 0.15	2.75 ± 1.18	4	38

Values in the first two columns are mean ± SE.

number depending on whether there were slave-makers nearby (Table 4; χ^2 test and Mann–Whitney *U*-tests, respectively: $\chi^2 = 4$, $P > 0.1$; $U = 33$, $P > 0.1$; $U = 27$, $P > 0.1$). We genotyped 69 workers and 16 queens from five free-living *L. ambiguus* nests in the one plot that contained slave-makers with *L. ambiguus* slaves (plot V). The five nests belonged to three colonies, two of which were polydomous. Three nests (60%) contained more than one line of full-sister workers; a fourth with a simple family (i.e. generated by a single queen mated once) was queenless. In only one was the resident queen mother of all the workers. Average worker relatedness was 0.68 ± 0.03, reflecting two lines of full-sisters on average (Table 5). The queens in complex colonies were sisters of most workers, implying that queen supercedure involved close relatives in this species as well.

Impact of the social parasites

We can gain considerable insight into slave-maker behaviour by inspecting slave genotypes (Foitzik & Herbers 2001b). We therefore genotyped 169 *L. longispinosus* and 38 *L. ambiguus* slaves from 11 and four slave-maker nests, respectively. The relatedness estimates and number of lines of full-sisters of slave pools for both host species are given in Table 5.

Newly founded colonies were particularly interesting. These all had slaves only of *L. longispinosus*, and relatedness among these slaves was similar to conspecifics in unenslaved host nests (Table 5; cf $r = 0.53 \pm 0.19$ to $r = 0.63 \pm 0.03$, Mann–Whitney *U*-test, $U = 42.5$, $P > 0.1$). Newly founded colonies typically consisted of the remnants of a single host nest, except one newly founded colony that contained very low slave relatedness ($r = 0.06 \pm 0.05$).We looked for surviving relatives of the enslaved workers in the neighbourhood of the one newly founded colony, interpreting any multilocus genotypes shared between slaves and nearby host nests as a reflection of recent invasion event during which some of the hosts escaped and survived. Two of the newly founded slave-maker colonies contained slaves that had no surviving relatives in the neighbourhood, but for the other two newly founded slave-maker colonies we identified relatives nearby. In one case, the surviving relatives, including the queen and workers, were nesting in the same stick just a few centimetres away from the slave-maker. These data imply that host nests can survive invasion by a slave-maker queen. An alternative explanation is that only one nest of extended polydomous host colonies had been invaded. While our data do not allow us to distinguish these possibilities, they do show unequivocally that enslaved workers can retain inclusive fitness through their free-living relatives.

Mature slave-maker nests contained both host species as slaves, and we looked for evidence of the two hosts suffering differential pressure. Comparing mean relatedness of free-living *L. longispinosus* workers (0.63) to mean relatedness of slaves in mature nests (0.26) suggested that a slave-maker nest contained on average the results of raiding 2.4 *L. longispinosus* nests. Comparing the number of lines of full-sisters between these two groups (Table 5) yielded a similar estimate of 2.9 raids (assuming equal representation of all lines); below we use a composite figure of 2.7 raids on *L. longispinosus* per slave-maker nest per year. Mean relatedness of free-living *L. ambiguus* workers (0.68) compared to mean relatedness of slaves (0.48) suggests that the slave-maker nests exploiting the secondary host raided an average of 1.4 nests of this species. The ratio of numbers of lines of full-sisters (Table 5) also yielded an estimate of 1.4 raids per year on *L. ambiguus*. The weighted average of slave-makers exploiting just *L. longispinosus* and those exploiting both hosts indicates that a mature slave-maker nest on average conducted up to 3.7 raids every year.

Comparisons of slave genotypes to conspecifics in nearby host nests (Fig. 1) yielded three slave-maker nests for which *L. longispinosus* slaves had no surviving relatives in the neighbourhood. For the four others, we identified sisters of *L. longispinosus* slaves in five free-living nests. Given that each slave-maker nest engaged in 2.7 raids on this host, these data imply that a *L. longispinosus* host nest had a 26.5% chance of surviving a raid. By contrast, four slave-maker nests contained *L. ambiguus* slaves, but those slaves had no relatives in the neighbourhood. Thus of six raids on this host, none left survivors; this is particularly evident in Plot VII, which contained slaves but no free-living nests of *L. ambiguus* (Table 2).

We examined slave genotypes in the different subunits of our three polydomous colonies, and consistently found sister slaves in both subunits. Just as consistently, the two subunits also included unique slave genotypes (Fig. 1). On average, 60% of the slaves (*L. longispinosus* and *L. ambiguus*) were shared among subunits. Thus, subunits of polydomous colonies traded slaves frequently, but also conducted independent raids.

Finally we can compute for each host the probabilities of being invaded by a slave-maker queen and of being raided by slave-maker workers (Foitzik & Herbers 2001b). For *L. longispinosus* (73 free-living nests) these probabilities are 5.5% (because an invading queen only affects one host nest) and 25.9% (because a slave-maker nest raids on average 2.7 times in a single year). The chances of surviving a raid (26.5%) mean that a *L. longispinosus* host nest has mortality risks of 19% per year. Mortality from raids and founding queens together reduce life expectancy of a *L. longispinosus* nest to about 5 years, even though *Leptothorax* (*Myrafant*) queens are known to live 10–15 years (Plateaux 1986).

In our sample, no *L. ambiguus* workers were found in the slave pools of founding queens; for this host, the primary

risk came from raiding parties. With 18 free-living *L. ambiguus* nests and four slave-maker nests raiding on average 1.4 *L. ambiguus* host nests each, every *L. ambiguus* nest has a 31% chance of being raided in a single year. Since we never found survivors of those raids, we estimate life expectancy of a *L. ambiguus* nest to be less than 4 years.

Discussion

Our study in Vermont was patterned on one conducted by Foitzik & Herbers (2001a,b) in New York because ecological (Herbers & Foitzik 2002) and behavioural (Foitzik *et al*. 2001) differences between sites strongly suggested the slave-maker–host interactions followed a coevolutionary trajectory. Our respective studies represent snapshots of the interaction that can be interpreted in the context of a larger database (Herbers & Foitzik 2002).

Colony structure of the slave-maker was similar in our study to that documented for New York: nests are patchily distributed in the forest, and colonies can be polydomous. Queenless nests are common, resulting not just from colony subdivision but also from orphaning. Queen turnover usually involves a close relative, which maintains high relatedness among workers. One interesting difference is that in New York the subunits of polydomous colonies always had separate slave pools (Foitzik & Herbers 2001b), whereas in Vermont slaves across colony subunits were a mix of shared and unique families. Thus in Vermont but not New York, slaves move between polydomous colony subunits.

Host colony structure showed some similarities between sites as well. Colonies of *Leptothorax longispinosus* can be polydomous (Herbers 1984 and this study), consisting of simple or extended families, and multiple laying queens are common within the same colony (Herbers & Stuart 1996). In New York the presence of slave-makers has strong consequences for host demography: in neighbourhoods containing slave-makers, *L. longispinosus* colonies are smaller with fewer queens than their larger polygynous counterparts in areas without slave-makers (Foitzik & Herbers 2001b; Herbers & Foitzik 2002). We found no such signature of slave-maker presence in this host species' demography in Vermont, however; host nests had similar numbers of queens and workers in neighbourhoods with or without slave-makers. This result echoes a larger analysis of long-term data (Herbers & Foitzik 2002) that showed that the slave-maker has strong effects on host demography in New York but not in Vermont. This distinction between the sites is underscored by a long-term increase in slave-maker density in New York but not in Vermont (Herbers & Foitzik 2002). Thus the interaction between the social parasite and its primary host has been temporally stable in Vermont relative to New York.

The more common host, *L. longispinosus* has strongly different relative frequencies in the two sites (Herbers 1989).

Table 6 Estimated values of the impact of the slave-making ant *Protomognathus americanus* on two host species, *Leptothorax longispinosus* and *L. ambiguus*, in two locations, Vermont and New York

	Vermont	New York
P. americanus		
No. of raids per nests per year	3.7	6
L. longispinosus		
Proportion out of all host nests	86%	98%
Probability of being raided	25.9%	56%
Probability of being invaded by a founding queen	5.5%	2.3%
Survivorship of raided nests	26.5%	0%
Nest life expectancy (years)	5	2
L. ambiguus		
Proportion out of all host nests	14%	2%
Probability of being raided	31%	—
Survivorship of raided nests	0%	—
Nest life expectancy (years)	4	—

Data derive from this study and two published papers (Foitzik & Herbers 2001b; Herbers & Foitzik 2002).

Moreover, availability of *L. ambiguus* host nests for raiding is considerably higher in Vermont than in New York (Herbers & Foitzik 2002, see Table 6). We therefore looked for differential impact of the slave-maker on its primary host between sites, as well as between host species within the Vermont site (Table 6).

In New York, raided nests of *L. longispinosus* rarely survived attack (Foitzik & Herbers 2001b), but we found considerable survivorship in Vermont. Similarly, slave-maker nests raid more often in New York. In our study, nests of *L. longispinosus* had a lower risk of being attacked by the slave-maker (raiding parties and founding queens) than in New York. Thus, life expectancy of *L. longispinosus* nests is higher in Vermont than in New York, indicating that this host suffers less from slave-makers in Vermont (Table 6).

Similarly, *L. longispinosus* in Vermont suffers less than its congener, the alternative host *L. ambiguus*. The secondary host had a higher risk of being attacked by raiding parties, and we found no evidence of any survivors; thus life expectancy for *L. ambiguus* is less than 4 years in Vermont (Table 6). Yet, slave-maker queens attempting to found new colonies rarely invade this species' nests, possibly reflecting its low density.

Comparisons between New York and Vermont, as well as comparisons of two hosts in Vermont, can be interpreted in light of the coevolutionary mosaic hypothesis (Thompson 1994, 1999). We have documented clear differences in the strength of selection pressure put on the primary host, and hosts from New York are more aggressive than elsewhere in the face of attacks from the slave-makers (Foitzik

et al. 2001). Clearly, the New York site is a coevolutionary 'hot spot' where the arms race has accelerated for both parasite and host. By contrast, the impact of *Protomognathus americanus* on *L. longispinosus* in Vermont is considerably less severe, perhaps mediated by the presence of a second host species there. The secondary host *L. ambiguus* has a higher per-nest risk of being raided by the slave-maker and a lower survivorship thereafter than the primary host. We can therefore extend the concept of a coevolutionary mosaic to include hot and cold hosts. Because parasite pressure can differ among sympatric host species, geographical comparisons of the interaction between a parasite and one of its hosts must be predicated on a full understanding of evolutionary options. A second intriguing observation from the historical database is that the primary host *L. longispinosus* has been declining in nest density over the past 20 years, an effect that could not be causally linked to pressure from the social parasite (Herbers & Foitzik 2002). In the face of declining host density, there may have been counter-selection for prudence in the slave-maker (Hare & Alloway 2001). Our hypothesis that Vermont slave-makers are prudent parasites can explain why we could find no signature of slave raiding in the population structure of its primary host. The surprising observation that polydomous subunits of slave-maker colonies shared some (but not all) slave pools might reflect good husbandry as well. Conversely, the lack of survivors among the secondary host might reflect a lack of selection for prudence with this host. These ideas concerning prudence must remain conjectures until more work is conducted on additional populations to characterize the outcome of slave-maker–host coevolution.

Our study highlights the need for a comparative approach when trying to elucidate the processes driving coevolution. Variation of interactions over time and space provides the data by which to assess how host and parasite direct each others' evolution (Thompson 1994). Social parasites, especially the avian brood parasites and insect social parasites, are systems of special value (Davies *et al*. 1989), in which that variation can be readily exploited.

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