Impacts of global changes on biological invasions and interspecific hybridization within the Tetramorium caespitum ant species complex

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Impacts of global changes on biological invasions and interspecific hybridization
within the *Tetramorium caespitum* ant species complex

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Avant-Propos / *Foreword*

Cette thèse a bénéficié d’une bourse doctorale de recherche financée par l’école doctorale E2M2 et d’un soutien financier de l'Agence Nationale de la Recherche via le LABEX IMU de l’Université de Lyon (ANR-10-LABX-0088), le programme "Investissements d’Avenir" IDEX Lyon (ANR-11-IDEX-0007) et le projet GEOSUD (ANR-10-EWPX-20). Cette thèse de doctorat porte sur des problématiques globales s’inscrivant dans le prolongement de travaux conduits à l’échelle internationale -- concernant notamment l’espèce invasive *Tetramorium immigrans* -- et nécessitant de fait l'intégration de ces recherches aux niveaux européen et international. Afin d'en faciliter la restitution et d’assurer son accessibilité et sa plus large diffusion, elle est donc rédigée en anglais.

This thesis has benefited from a doctoral research grant funded by the E2M2 doctoral school and from a financial support from the National Research Agency via the LABEX IMU of the University of Lyon (ANR-10-LABX-0088), the " Investissements d’Avenir" IDEX Lyon program (ANR-11-IDEX-0007) and the GEOSUD project (ANR-10-EWPX-20). This thesis deals with global issues and is a continuation of work carried out internationally, particularly concerning the invasive species Tetramorium immigrans*, requiring the integration of this research at European and international levels. To ensure their accessibility by the scientific community and their wide dissemination, this doctoral thesis is written in English to facilitate the restitution of this work.*

Short abstract

Climate change, urbanization, biological invasions and interspecific hybridization are among the greatest current threats to biodiversity; their impacts could increase by the end of the 21st century. The objective of this thesis was to assess their interactive effects in ant species of the *Tetramorium caespitum* complex from the Rhône-Saône valley, France. Using a broad diversity of concepts and tools from biogeography, population genetics, landscape ecology, behavioral biology and chemical ecology, I investigated samples from almost two thousand colonies from five *Tetramorium* species including *T. immigrans* and *T. caespitum* cryptic species. Both climate and urbanization limited the ranges of these species, and urbanization impacted their distributions simultaneously at several spatial scales. The occurrence probabilities of *T. immigrans* depended on the interaction between climatic and urban factors. Four genetically distinct populations of *T. immigrans* resulted of several introductions from external sources, making it likely not native to Southeastern France, at least in the northernmost urban areas. The detection of nuclear DNA backcrosses and mitochondrial-nuclear DNA discordance between *T. immigrans* and *T. caespitum* suggested that hybridization lead to fertile offspring. Differentiated cuticular hydrocarbon profiles and heightened interspecific aggression against heterospecific revealed well-differentiated species recognition cues. These species presented a monogyne/polyandrous mating system, in which hybrid queens but no hybrid fathers contributed to hybrid offspring. So far, studies on interspecific hybridization have only occasionally been concerned with concepts such as global change or biological invasion. These concepts themselves are infrequently studied together, and their interactive effects are totally overlooked in current literature. Taking these interrelationships into account and exploring multiple scales are essential to better understand the processes that generate patterns of genetic exchange. The joint study of polyandry and species discrimination mechanisms within a hybrid zone is unprecedented and offers the opportunity to investigate the selective processes involved in the evolution of interspecific mating. Many other systems could and should be investigated in the light of these results, which strongly suggest an integrated exploration of the many global changes facing biodiversity and human populations today.

Keywords: *Aggressive behavior, Biological invasion, Chemical communication, Climate, Gene flow, Global changes, Interspecific hybridization, Introgression, Landscape genetics, Mating system, Pavement ant, Species distribution,* Tetramorium *sp., Urbanization*

Résumé détaillé

Le changement climatique, l'urbanisation, les invasions biologiques et l'hybridation interspécifique constituent pour la biodiversité l'une des plus grandes menaces actuelles, et leurs impacts pourraient fortement augmenter d'ici la fin du 21ème siècle. Ces problèmes environnementaux sont souvent considérés comme résultant d’accumulations de phénomènes indépendants ayant de multiples causes. Cependant, changements globaux, invasions biologiques et hybridation interspécifique sont étroitement liés et aggravés par l’expansion des activités humaines. Parmi les changements globaux, les changements climatiques et l’urbanisation sont des facteurs déterminants de la répartition des espèces et constituent donc une menace sérieuse pour la persistance des espèces. Les invasions biologiques sont également une cause majeure de préoccupation pour la conservation, notamment du fait de la propension des humains à disperser des espèces non indigènes, un phénomène qui pourrait surpasser la sélection et la dispersion naturelles. Ces impacts de l’homme sur son environnement sont à l’origine de nouvelles possibilités d’hybridation entre des espèces auparavant allopatriques qui se sont déplacées et se sont rencontrées. Ainsi, les changements climatiques, l'urbanisation et les introductions biologiques pourraient agir en interaction et promouvoir de nouveaux échanges génétiques interspécifiques.

En raison de leur organisation sociale, leur comportement de reproduction et leur haplo-diploïdie, les fourmis (famille des formicidés) sont particulièrement sujettes à l’hybridation interspécifique. Les déplacements accidentels de colonies par l’homme, par exemple dans le cadre du commerce international de fleurs en pot, induisent de fréquentes introductions biologiques de ces organismes en dehors de leurs aires de répartition d’origine. Ces évènements d’introduction favorisent l'établissement de nouvelles zones de contact entre espèces, générant des échanges génétiques entre les taxons natifs et introduits. La plupart des espèces de fourmis sont spécialisées pour certains types d’habitats ou de micro-habitats, et leur répartition et leur écologie sont fortement influencées par le climat, notamment les paramètres liés aux températures et aux précipitations. Les patrons d'activité annuels et quotidiens ainsi que l’initiation de la reproduction et de la dispersion dépendent directement de déclencheurs environnementaux, eux-mêmes sous influence des changements climatiques. Les fourmis comptent plusieurs espèces communes dans les villes, mais aussi de nombreuses espèces affectées négativement par l'urbanisation. Ces caractéristiques en font un taxon pertinent pour étudier les relations entre les changements globaux, l'invasion et l'hybridation.

J'ai travaillé sur cinq espèces de fourmis appartenant au genre *Tetramorium*, en m'intéressant plus particulièrement aux espèces cryptiques *T. immigrans* et *T. caespitum*, ayant récemment fait l'objet d'une révision taxonomique. Avant la présente thèse, la biologie, l'histoire et l'écologie de ces espèces étaient très peu connues. Leur distribution à échelle précise en France, l'impact de l'urbanisation sur ces taxons ou leurs systèmes d'accouplement n'avaient par exemple jamais été étudiés. Des études antérieures avaient posé l'hypothèse d'une hybridation entre *T. immigrans* et *T. caespitum* suite à l'observation de deux individus présentant une discordance entre leurs ADN nucléaire et mitochondrial, mais cette hybridation n'avait pas encore été étudiée. *Tetramorium immigrans* est une espèce particulièrement intéressante car invasive en Amérique du Nord, où elle a été introduite dans des villes au 19ème siècle, voire plus tôt. Elle est considérée indigène en Europe où son statut n'a jamais été remis en question.

Mes investigations ont été menées dans les vallées du Rhône et de la Saône (Sud-est de la France, Europe occidentale), une zone particulièrement pertinente pour étudier les problèmes liés aux changements globaux, aux invasions biologiques, à la répartition des espèces et aux échanges génétiques entre espèces. Ces vallées recouvrent un gradient climatique et environnemental le long d'un axe Nord-Sud qui traverse la frontière entre les régions biogéographiques méditerranéennes et continentales. En raison de sa situation intermédiaire entre les refuges glaciaires des péninsules ibériques, italiennes et balkaniques, cette zone correspond également à une importante voie de recolonisation postglaciaire. Les Alpes agissent pour de nombreux organismes comme une barrière de dispersion, générant dans les vallées un réseau de zones de contact conduisant à des zones d'hybridation. La vallée Rhône-Saône est urbanisée depuis l'époque romaine et les processus d'urbanisation sont encore importants aujourd'hui, donnant naissance à de vastes zones urbaines en expansion constante. Cette zone est donc particulièrement adaptée à l'étude des impacts de l'urbanisation. La vallée connaît également une forte croissance de ses activités de transport et de logistique et, par conséquent, elle présente des risques majeurs d'introduction d'organismes exogènes, et donc une forte sensibilité aux risques d'invasions biologiques.

L'objectif de cette thèse était donc d'évaluer les effets relatifs de l'urbanisation et du climat sur les échanges de gènes et les schémas d'hybridation entre les espèces de fourmis du complexe *Tetramorium caespitum* dans la vallée du Rhône-Saône. Pour mener à bien ces travaux, j'ai mobilisé une grande diversité d'outils (tels que des marqueurs moléculaires, les SIG, la technique de chromatographie en phase gazeuse) issus de la biogéographie, de la génétique des populations, de l'écologie du paysage, de la biologie comportementale et de l'écologie chimique. Cette synthèse de trois années de recherche fournit une perspective intégrée sur les réponses de la biodiversité aux changements globaux à travers l'étude des échanges génétiques.

Le **Chapitre 1** présente la thèse et donne un bref aperçu des principaux thèmes développés, à savoir comment les espèces, en particulier les fourmis, réagissent aux changements globaux, et quelles sont les conséquences sur les invasions biologiques et les échanges génétiques interspécifiques.

Le **Chapitre 2** examine les limites de l'aire de répartition le long d'un gradient climatique (la vallée Rhône-Saône) de cinq espèces de *Tetramorium* constituant un groupe d'espèces incluant des taxons cryptiques, en tenant compte des erreurs attribuables à la fois à l'hybridation et à la rareté. Pour cela, près de 1700 colonies appartenant au genre *Tetramorium* ont été échantillonnées et identifiées au niveau de l'espèce en utilisant une approche intégrative basée sur un processus en deux étapes combinant ADN nucléaire (14 marqueurs microsatellites), examen morphologique et séquençage de l'ADN mitochondrial (Cytochrome Oxydase I). Cinq espèces de *Tetramorium* ont été identifiées : *T. forte*, *T. moravicum*, *T. semilaeve*, *T. immigrans* et *T. caespitum*. Les distributions spatiales des trois dernières étaient fortement corrélées aux conditions climatiques. Les limites de distribution de la plupart des espèces correspondaient à la limite biogéographique entre les régions continentales et méditerranéennes, sauf pour *T. immigrans* dont la répartition couvrait les deux régions. *Tetramorium caespitum* a été principalement trouvé au nord de la limite, tandis que *T. semilaeve* et *T. forte* ont été trouvés au sud. *T. moravicum* a été trouvé principalement près de la limite, avec peu d'occurrences dans les sites plus au nord. Ces résultats mettent en évidence le rôle fondamental du climat en tant que facteur limitant les aires de répartition des espèces au niveau d'une limite biogéographique importante. La seconde partie du chapitre 2 porte sur la structuration de la distribution des fourmis du genre *Tetramorium* à différentes échelles spatiales en réponse à l'urbanisation. Sur la base des méthodes de combinaison de modèles (*model averaging*), j'ai étudié environ 1400 sites de nidification appartenant à quatre espèces de *Tetramorium* à quatre échelles spatiales distinctes (du micro-habitat au paysage) dans 19 gradients urbains. J'ai montré que les probabilités d'occurrence de *Tetramorium caespitum* et de *T. immigrans* dépendaient simultanément de l'urbanisation au niveau du paysage et aux échelles locales. En effet, *T. caespitum* évite les microhabitats urbains et les paysages imperméabilisés alors que *T. immigrans* est extrêmement présente dans de tels milieux. Ces impacts d'échelle de l'urbanisation dépendaient des espèces : *Tetramorium moravicum* était associée à des paysages non urbains uniquement, alors qu'aucune préférence n'a été mise en évidence pour *T. semilaeve*, quelles que soient les variables testées. Ces résultats démontrent l'importance de considérer simultanément plusieurs échelles spatiales pour étudier l'impact de l'urbanisation sur la distribution des espèces. Les réponses très contrastées à l'urbanisation de *T. immigrans* et de *T. caespitum* indiquent probablement des processus de partitionnement de niche induits par l'urbanisation.

Le **Chapitre 3** porte sur l'étude de l'influence combinée des changements globaux sur l'expansion de *Tetramorium immigrans*. A partir de l'échantillonnage de 544 individus de *T. immigrans* prélevés dans 16 gradients urbains et génotypés pour 14 marqueurs microsatellites, j'ai combiné l'utilisation de méthodes d'écologie moléculaire (analyses bayésiennes et basées sur la fréquence) et de modèles statistiques pour évaluer l'impact de l'interaction entre le climat et l'urbanisation sur la distribution de cette espèce. Les probabilités d'occurrence de *T. immigrans* dépendaient de l'interaction entre les facteurs climatiques et urbains. Deux groupes génétiques de *T. immigrans* distincts latitudinalement étaient structurés hiérarchiquement en deux sous-groupes, suggérant des histoires de colonisation différentes. De forts effets fondateurs ont indiqué des introductions successives depuis des populations sources externes à la zone d'étude, suivies par une colonisation favorisée par les activités humaines dans les zones urbaines du nord. Bien que l'effet de l'interaction climat-urbanisation sur la distribution des espèces soit souvent négligé dans la littérature, de nombreux taxons peuvent se conformer au modèle de *T. immigrans*. L'étude combinée des changements globaux est donc importante et devrait être envisagée dans les études futures. La fin du chapitre considère le fait que les modèles de distribution observés concordent avec des patrons de distribution similaires dans l'aire de répartition invasive de *T. immigrans*, ce qui rend probable que cette espèce ne soit pas originaire du sud-est de la France, du moins dans les zones urbaines les plus septentrionales.

Le **Chapitre 4** porte sur l'hybridation entre *Tetramorium immigrans* et *T. caespitum* de laquelle résulte une progéniture fertile. Cette introgression a été mise en évidence par la détection de rétrocroisements basée sur 14 microsatellites nucléaires, mais également par plusieurs occurrences de discordance entre les identifications basées sur l'ADN mitochondrial et l'ADN nucléaire. Les résultats étaient cohérents sur le plan spatial, car les individus identifiés comme étant hybrides étaient situés à des latitudes auxquelles les deux espèces parentales coexistent en sympatrie. J'ai ensuite étudié la reconnaissance des espèces et la discrimination entre *Tetramorium immigrans*, *T. caespitum* et les hybrides en comparant leurs profils d'hydrocarbures cuticulaires et en mesurant l'agressivité intra- et interspécifique des ouvrières stériles issues, soit des zones de sympatrie entre les espèces, soit des zones d'allopatrie. L'étude montre que les individus des espèces parentales ainsi que les hybrides présentent des profils d'hydrocarbures cuticulaires différenciés et une agression accrue envers les membres hétérospécifiques, ce qui implique des indices de reconnaissance des espèces bien différenciés. Les hybrides avaient des profils d'hydrocarbures cuticulaires correspondant à un mélange de bouquets parentaux, mais le signal chimique des hybrides présentait certains composés dans des quantités plus élevées que les deux espèces parentales. Les tests comportementaux ont montré que *T. immigrans* était aussi agressif envers les hybrides que contre les hétérospécifiques. L'agression entre les ouvrières hétérospécifiques était plus forte lorsque les protagonistes étaient issus de zones où les espèces parentales sont en sympatrie que des zones d'allopatrie. Ces résultats soulignent que les zones hybrides offrent une occasion unique d'examiner en profondeur les mécanismes de reconnaissance et de discrimination entre les espèces. Dans une troisième étude s'intéressant plus particulièrement aux hybrides, j'ai analysé 15 marqueurs microsatellites sur 15 fourmis par colonie dans 28 colonies pures de *Tetramorium immigrans*, 15 colonies pures de *T. caespitum* et 27 colonies hybrides. Cette étude a révélé que le système de reproduction de ces espèces était un système d'accouplement monogyne/polyandre, avec un taux d'accouplement plus élevé chez *T. caespitum*. J'ai déduit du génotype des ouvrières qu'elles descendaient parfois de reines hybrides, mais jamais de pères hybrides. Ce résultat est conforme à la règle de Haldane étendue aux organismes haplodiploïdes, selon laquelle le sexe haploïde devrait plus souvent être stérile ou non-viable. Dans quatre colonies, l'hybridation et la reproduction multiple ont permis la production simultanée de descendants hybrides et non-hybrides. Bien que rares, ces situations suggéraient que dans les colonies possédant une reine *T. caespitum*, les différents mâles accouplés à la reine contribuent de manière asymétrique à la production de la progéniture, avec plus de descendant issus des mâles hétérospécifiques que des mâles conspécifiques. Une telle étude conjointe de la polyandrie dans une zone hybride est sans précédent et ouvre la possibilité d'étudier les processus sélectifs impliqués dans l'évolution de l'accouplement multiple.

Enfin, le **Chapitre 5** synthétise brièvement les chapitres précédents avant de discuter l'ensemble des résultats obtenus au travers de cette thèse. Cette partie expose notamment le fait que les patrons décrits par les résultats de ces recherches sont plus complexes que supposé initialement. En effet, l'urbanisation, le climat et les invasions biologiques n'ont pas agi séparément et individuellement sur la distribution des espèces étudiées. J'ai montré que l'urbanisation affecte directement les flux de gènes et l'hybridation par des modifications de l'habitat et par la création d'écotones à la périphérie des zones urbaines. De plus, les modifications des échanges génétiques intra- et interspécifiques décrites dans cette thèse ont probablement joué un rôle dans l'histoire de l'invasion de *T. immigrans*, en association avec l'urbanisation et le climat.

Les études sur l'hybridation interspécifique n'ont été qu'occasionnellement liées à des concepts tels que les changements globaux ou les invasions biologiques. Ces concepts eux-mêmes sont peu étudiés de manière concomitante et leurs effets interactifs sont totalement ignorés dans la littérature actuelle. Mes recherches portent donc sur une nouvelle compréhension de la relation entre les invasions biologiques, l'urbanisation et l'hybridation entre les espèces. La prise en compte de ces interrelations et l’exploration de multiples échelles sont essentielles pour mieux comprendre les processus qui génèrent des modèles d’échanges génétiques. L’étude des mécanismes de reconnaissance et l’étude de la polyandrie dans une zone hybride sont sans précédent et ouvrent la possibilité d'étudier les processus sélectifs impliqués dans l'évolution de l'accouplement interspécifique.

Forte de ces résultats, ma thèse ouvre quatre grandes perspectives, qui peuvent être appréhendées sous forme de questions.

(i) D'où vient *Tetramorium immigrans* ? Aujourd'hui, *T. immigrans* n'est connue que dans des localités sporadiques à travers l'Europe. Un échantillonnage uniforme à l'échelle du continent et mobilisant systématiquement une approche basée sur la génétique permettrait de mettre en évidence des modèles de diversité génétique conduisant à la localisation des populations sources probables. En outre, une telle approche devrait confirmer que *T. immigrans* n'est pas originaire du sud-est de la France. Une comparaison spatialement explicite des haplotypes issus des séquences d'ADN mitochondrial pourrait également aider à l’identification des populations sources, et ainsi les zones d'origine de *T. immigrans*.

(ii) Pourquoi *T. immigrans* est-elle un remarquable « exploiteur urbain » ? *Tetramorium immigrans* a pu, par le passé, se répandre progressivement et à une vitesse limitée dans les vallées du Rhône et de la Saône. Cependant, sa propagation pourrait être plus rapide et de plus large envergure dans les prochaines décennies. Une telle accélération est probable en raison des opportunités de plus en plus nombreuses de dispersion par les transports humains, favorisant l’établissement de nouveaux foyers d’invasion bien en avance sur le front de dispersion de l’espèce, faisant rapidement progresser l’invasion. Ces évènements de dispersion s’ajoutent aux effets combinés de l’étalement urbain et du réchauffement climatique, eux aussi susceptibles d’accélérer la propagation de cette espèce. L’exploration des caractéristiques écologiques et fonctionnelles de *T. immigrans* faisant de cette espèce un exploiteur urbain aussi remarquable présente un intérêt primordial pour l’écologie de l’espèce et la compréhension des mécanismes impliqués dans l’exclusion compétitive de *T. caespitum* en dehors des villes. Je suggère donc que d’autres études prennent en compte le rôle de la tolérance de *T. immigrans* à de multiples stress lui conférant la capacité d’être une espèce dominante dans les zones urbaines.

(iii) Comment *T. immigrans* et *T. caespitum* surmontent-elles les obstacles à l'hybridation ? Le chevauchement de phénologie au moment de la reproduction devrait faire l'objet d'un examen plus approfondi dans la zone hybride, car un tel chevauchement pourrait être favorisé par le contexte écologique fourni par l'interaction entre le climat et l'urbanisation. Il est également nécessaire d'étudier si les individus reproducteurs sont capables de discriminer l'identité du partenaire avant et pendant l'accouplement, car la discrimination des hétérospécifiques par les individus reproducteurs peut différer de celle des ouvrières. En effet, les signaux de fertilité pourraient par exemple homogénéiser les profils des reproducteurs et réduire leur capacité à utiliser les signaux distinctifs des espèces. Enfin, l'exploration des rôles joués par les barrières postzygotiques pourrait apporter un nouvel éclairage sur les processus de sélection naturelle et sexuelle déterminant la viabilité et la fertilité des hybrides et de leur progéniture. De telles questions pourraient notamment être abordées à travers la comparaison entre les biais d'insémination (c.-à-d. entre spermatozoïdes hétérospécifiques et conspécifiques) et les biais dans la production de descendants à différents stades de maturité.

(iv) Que nous disent les tendances observées sur l'avenir de la zone hybride ? Le modèle clinal de la zone hybride *T. immigrans* x *caespitum* peut être maintenu par sélection endogène contre des génotypes hybrides (zones de tension), par sélection favorisant différents types parentaux à chaque extrémité d'un gradient environnemental, ou par sélection dans des habitats intermédiaires favorisant les individus d'ascendance mixte (modèle de supériorité hybride). Le modèle de zone de tension prédit que le centre de la zone hybride devrait correspondre à une zone de faible densité de population des espèces parentales. Un tel patron pourrait être confirmé dans le système *T. immigrans* x *caespitum* en étudiant les densités d'espèces pures dans la zone hybride de sorte à comparer ces densités à celles observées dans les zones où les espèces sont allopatriques. En raison de la position intermédiaire des zones hybrides entre les habitats majoritairement urbanisés et les environnements plus naturels, les hybrides pourraient avoir connu des conditions écologiques différentes des espèces parentales dans les paysages agricoles qui entourent les zones urbanisées. Il est donc possible que des génotypes ou phénotypes hybrides spécifiques soient soumis à des processus de sélection relativement importants dans cette zone hybride. Une telle distribution indépendante de la dispersion à travers les paysages pourrait être cohérente avec le modèle de supériorité hybride limitée aux écotones étroits. La possibilité d'une spécialisation pour les habitats agricoles devrait induire des niches écologiques différentes entre les hybrides et les espèces parentales, soit parce que les espèces pures présentent une moins bonne aptitude dans ces habitats, soit que l'aptitude des hybrides est plus élevée dans ces habitats.

Les résultats et les recommandations découlant de ces travaux ne se limitent pas au seul système biologique de *T. immigrans* x *caespitum*. Bien que la zone hybride présentée dans cette étude soit particulièrement adaptée à l’étude des problématiques abordées, il serait pertinent de reproduire l'étude dans d'autres zones d'hybridation entre *T. immigrans* et *T. caespitum* (si de telles zones existent). Plus important encore, ces questions peuvent être exportées vers d'autres systèmes d'hybridation, impliquant d'autres espèces envahissantes, afin d’évaluer la robustesse du cadre théorique et tester la force des interactions entre les changements globaux, l'hybridation et l'invasion chez d’autres taxons. De nombreux autres systèmes pourraient et devraient être étudiés à la lumière de ces conclusions, qui plaident fortement pour une étude plus intégrée des nombreux changements globaux auxquels sont aujourd’hui confrontées la biodiversité et les populations humaines.

Mots - clés : Changements globaux, Climat, Communication chimique, Comportement d’aggressivité, Distribution des espèces, Flux de gènes, Génétique du paysage, Hybridation interspécifique, Introgression, Invasion biologique, Système de reproduction, *Tetramorium sp.*, Urbanisation

Detailed abstract

Climate change, urbanization, biological invasions and interspecific hybridization are among the greatest current threats to biodiversity and their impacts could increase by the end of the 21st century. These environmental issues are often considered an accumulation of independent phenomena with multiple causes. However, these threats are deeply intertwined and aggravated by the expansion of human activities. Among global changes, climate changes and urbanization are major determinants of species' distribution and therefore pose a serious threat to species' persistence. Biological invasions are also a leading cause of conservation concern, because humans as dispersers of nonindigenous species could surpass natural selection and dispersal. These impacts of humans on their environment have created new opportunities for hybridization between previously allopatric species which have moved and met. Climate changes, urbanization and biological introductions could therefore act in interaction to promote interspecific genetic exchanges.

Because of their social organization, reproductive behavior and haplo-diploidy, ants (family Formicidae) are particularly prone to interspecific hybridization. The frequent biological introductions of these organisms, particularly due to the accidental human movements of colonies, for example via the international trade in potted flowers, favor the establishment of novel contact zones between species, leading to genetic exchanges between native and introduced taxa. Most ant species are habitat or microhabitat specialists whose distribution and ecology are strongly influenced by climate (i.e. temperature or precipitation). Annual and daily activity patterns as well as the timing of reproduction and dispersal depends on environmental triggers that climate changes might disrupt. Ants include several species common in cities as well as others negatively impacted by urbanization. These characteristics make it a relevant taxon for studying the relationships between global changes, invasion and hybridization. I worked on five species of *Tetramorium* ants, with a focus on the cryptic species *T. immigrans* and *T. caespitum* which have recently been subject to taxonomic revision. Before the present thesis, little was known on their biology, life history and ecology (e.g., about their fine-scale distributions in France, the impact of urbanization on these taxa, or their mating systems). Previous studies suspected hybridization between *T. immigrans* and *T. caespitum*, based on mitochondrial-nuclear discordance, but this hybridization had not been further investigated. *Tetramorium immigrans* is particularly worth studying because it is an invasive species in North America, where it was introduced in cities in the 19th century or earlier, and considered native in Europe where its status had never been questioned.

My investigations were conducted in the Rhône-Saône valley, which is especially relevant to investigate issues related to global changes, biological invasions, species distribution and genetic exchange between species. This valley spans a steep North-South climatic and environmental gradient across the boundary between the Mediterranean and Continental biogeographical regions in Southeastern France, Western Europe. Due to its intermediate location between glacial refugees of the Iberian, Italian and Balkan peninsulas, it also corresponds to a major postglacial recolonization route. As a consequence, a network of contact zones is spread over this valley as the Alps mountain often act as a dispersal barrier, inducing secondary contact between populations and species and leading to hybridization areas. The Rhône-Saône valley is urbanized since Roman times and urbanization processes are still important today, giving birth to large and constantly expanding urban areas which are particularly suited for the study of the impacts of urbanization. The valley is also experiencing further growth in its transport and logistic activities and, as a consequence, is an area of major risks of biological invasions.

The objective of this thesis was therefore to assess the relative effects of urbanization and climate on gene exchanges and hybridization patterns between ant species of the *Tetramorium caespitum* complex from the Rhône- Saône valley. I therefore used a broad diversity of concepts and tools (e.g. Molecular markers, GIS technology, Gas Chromatography) from biogeography, population genetics, landscape ecology, behavioral biology and chemical ecology. This synthesis of three years of research provides an integrative perspective on the responses of biodiversity to global changes through genetic exchanges.

Chapter 1 introduces the thesis and provides a short review of the main topics of the thesis, i.e. how species, especially ants, respond to global changes and consequences on biological invasions and interspecific genetic exchanges.

Chapter 2 investigates the range limits over a climatic gradient (along the Rhône - Saône valley) of five *Tetramorium* species constituting a group of inconspicuous species including cryptic taxa, taking into account and avoiding errors attributable to both hybridization and rarity. Close to 1700 *Tetramorium* colonies were sampled and identified at species level using an integrative approach based on a two-step process combining nuclear DNA (14 microsatellite markers), morphological examination and mitochondrial DNA cytochrome oxidase I sequencing. Five *Tetramorium* species were identified: *T. forte*, *T. moravicum*, *T. semilaeve*, *T. immigrans*, and *T. caespitum*. The spatial distributions of the last three were strongly correlated to climatic conditions. The limits to the distribution of most species corresponded to the biogeographical boundary between the Continental and

Mediterranean regions, except for *T. immigrans* whose distribution covered both regions. *T. caespitum* was mostly found north of the boundary, while *T. semilaeve* and *T. forte* were found to the South. *T. moravicum* was found mostly close to the boundary, with few locations further north. These results highlight the fundamental role of climate as a factor limiting the species ranges at a well-known biogeographical limit. A second study within Chapter 2 investigates how urbanization structures the distribution of *Tetramorium* ants at different spatial scales. Based on model averaging procedures, I studied ~1400 nesting sites belonging to four *Tetramorium* species at four distinct spatial scales, from urban microhabitat to urban landscape in 19 urban gradients. I showed that the probabilities of occurrences of *Tetramorium caespitum* and *T. immigrans* simultaneously depended on urbanization at the landscape and local scales, with *T. caespitum* avoiding urban microhabitats and impervious landscapes whereas *T. immigrans* favored them. These scaling impacts of urbanization were species-dependent as *T. moravicum* was associated with nonurban landscapes only, and *T. semilaeve* showed no association whatever the tested variables. These results demonstrate the importance of considering several spatial scales simultaneously to study the impact of urbanization on species distribution. The highly contrasted responses to urbanization of *T. immigrans* and *T. caespitum* indicate niche partitioning processes driven by urbanization.

Chapter 3 investigates the combined influence of global changes on the expansion of the pavement ant *Tetramorium immigrans*. Based on 544 *T. immigrans* individuals sampled from 16 urban gradients and genotyped at 14 microsatellite markers, I combined molecular ecology methods (Bayesian and frequency-based analyses) and statistical modelling to evaluate the impact of the interaction between climate and urbanization on the pavement ant distribution patterns. The occurrence probabilities of *T. immigrans* depended on the interaction between climatic and urban factors. Two latitudinally distinct clusters of *T. immigrans* were hierarchically structured in two sub-clusters each, suggesting different colonization histories. Strong founder effects indicated introductions from external sources followed by colonization favoured by human activities in the northern urban areas. Despite the fact that the effect of climate-urbanization interaction on species distribution is mostly overlooked in the literature, many taxa may conform to *T. immigrans* pattern, making the combined study of global changes a necessary challenge for future studies. The end of the Chapter discusses the fact that the observed distribution patterns concurred with similar observations in the invasive range of *T. immigrans*, making it likely that it may not be native to Southeastern France, at least in the northernmost urban areas.

In **Chapter 4,** hybridization between *Tetramorium immigrans* and *T. caespitum* is shown to lead to fertile offspring. This introgression was revealed both by backcross detection based on 14 nuclear microsatellite loci and by mitochondrial-nuclear discordance based on comparison with mitochondrial DNA (Cytochrome Oxidase I). Results were spatially consistent, with hybrids located at latitudes where parental species are sympatric. I then investigated species recognition and discrimination between *Tetramorium immigrans*, *T. caespitum* and hybrids by comparing their cuticular hydrocarbon profiles and measuring intra- and interspecific worker aggression in both workers from zones of sympatry between species and from zones of allopatry. Differentiated cuticular hydrocarbon profiles and heightened interspecific aggression revealed well-differentiated species recognition cues. Hybrids had cuticular hydrocarbon profiles corresponding to a mixture of parental bouquets, but the chemical signal of hybrids presented higher amounts of some compounds than both parental species. Behavioral assays showed that *T. immigrans* was as aggressive towards hybrids as towards heterospecifics. Aggression between heterospecific workers was higher when protagonists came from zones of sympatry between species than from zones of allopatry. These results highlight that hybrid zones offer a unique opportunity to deeply investigate recognition mechanisms and discrimination between species. In a third study on hybrids, the analysis of microsatellite markers on 15 ant workers per colony revealed that the mating system of 28 pure colonies of *Tetramorium immigrans*, 15 pure colonies of *T. caespitum* and 27 hybrid colonies was a monogyne/polyandrous mating system, with a higher mating rate in *T. caespitum*. Hybrid queens, but no hybrid fathers, could be deduced from workers' genotypes, in accordance to Haldane's rule extended to haplodiploid organisms, which states that the haploid sex should more often be sterile or inviable. In four colonies, hybridization and multiple mating allowed the simultaneous production of both hybrid and nonhybrid offspring. Although rare, these situations hinted at asymmetrical contributions to offspring in favor of heterospecific vs. conspecific males in colonies with a *T. caespitum* queen. Such a study of polyandry within a hybrid zone is unprecedented in ants and opens the opportunity to investigate the selective processes involved in the evolution of multiple mating.

Chapter 5 briefly synthetizes the previous chapters before discussing all the results of the thesis. The patterns I describe are rather more complex that I initially supposed. Indeed, urbanization, climate and biological invasions did not act separately and individually on *Tetramorium* species distributions. I have shown that urbanization directly affects gene flow and hybridization through habitat modifications and the creation of ecotones at the edge of urban areas. Furthermore, modifications in gene flow and the interspecific hybridization newly described in this thesis have probably played a role in the invasion history of *T. immigrans*, working in conjunction with urbanization and climate.

Studies on interspecific hybridization have only occasionally been concerned with concepts such as global change or biological invasion. These concepts themselves are infrequently studied together, and their interactive effects are totally overlooked in current literature. All of my research therefore points towards new understanding of the interrelationship between biological invasions, urbanization and hybridization. Taking these interrelationships into account and exploring multiple scales are essential to better understand the processes that generate patterns of genetic exchange. The joint study of polyandry and species discrimination mechanisms within a hybrid zone is unprecedented and opens the opportunity to investigate the selective processes involved in the evolution of interspecific mating.

My thesis opens up four main questions. (i) Where did *Tetramorium immigrans* come from? Today, *Tetramorium immigrans* is known only from sporadic localities over all of Europe. A continent-wide uniform sampling mobilizing genetic tools would reveal genetic diversity patterns leading to the location of the probable source populations and should confirm that *T. immigrans* is not native to Southeastern France. Based on mitochondrial DNA barcoding, spatially explicit comparison of haplotypes could also help pinpoint source populations and the native area of *T. immigrans*. (ii) Why is *T. immigrans* a remarkable urban exploiter? *T. immigrans* may spread slowly in the Rhône-Saône valley, but further spread may likely be faster and at a broader spatial extent in the coming decades. This acceleration is expected because of “jump-dispersal” by human transport, which establishes new foci of invasion well ahead of the expanding front, rapidly advancing the invasion, and because of the combined effects of urban sprawl and climate warming promoting its establishment. Exploring the ecological and functional traits of *Tetramorium immigrans* that make it such a remarkable urban exploiter is of prime interest for the ecology of the species and the understanding of the mechanisms involved in competitive exclusion of *T. caespitum* from cities. I therefore suggest further studies take into account the role of multi-stress tolerance in *T. immigrans’* ability to be dominant in urban areas. (iii) How do *T. immigrans* and *T. caespitum* overcome the barriers to hybridization? The overlapping of reproductive phenology within the hybrid zone should be further investigated as it could be promoted by the ecological context provided by the interaction between climate and urbanization. It is also necessary to investigating if reproductive individuals are able to discriminate the identity of the partner before and during mating, as the discrimination of heterospecifics by reproductive may differ from that of workers, e.g. if fertility signals homogenize their profiles and reduce their ability to use species-specific differences. Finally, the exploration of the roles of postzygotic barriers through the comparisons between insemination bias (i.e. between heterospecific and homospecific sperm) and offspring bias observed at different stages could bring new light on the natural and sexual selection processes involved and could allow a better understanding of the viability and fertility of both hybrids and their offspring. (iv) What do the observed patterns tell us about the future of the hybrid zone? The *T. immigrans* x *caespitum* hybrid zone clinal pattern may be maintained by endogenous selection against hybrid genotypes (tension zones), by selection favoring different parental types at each end of an environmental gradient, or by selection in intermediate habitats favoring individuals of mixed ancestry (bounded hybrid superiority model). The “tension zone” model predicts that the center of the hybrid zone should correspond to an area of low population density, which could be confirmed in the *T. immigrans* x *caespitum* system by investigating the densities of pure species in the hybrid zone compared to their densities in allopatric areas. Because of the intermediate location of the hybrid zones between mostly urbanized habitats and more natural environments, hybrids could have experienced ecological conditions different from parental species in agricultural landscapes that surround the urbanized areas. It is therefore possible that specific hybrid genotypes or phenotypes have experienced relatively strong selection in this hybrid zone. Such a distribution across landscapes could be consistent with the dispersal-independent model of bounded hybrid superiority within narrow ecotones. The possibility of a specialization for agricultural habitats should induce different ecological niches in hybrids compared to parental species, either because pure species have a lower fitness in these habitats or because the fitness of hybrids is higher in these habitats.

The results and recommendations ensuing from this work are not limited to the sole *T. immigrans* x *caespitum* biological system. Although the hybrid zone presented in this study is particularly suited to the issues addressed, it would be relevant to replicate the study in other areas of hybridization between *T. immigrans* and *T. caespitum* (if such hybrid zones exist). Above all, however, these issues may be exported to other hybridization systems, to other invasive species to evaluate the robustness of the theoretical framework and to test the strength of the interactions between global changes, hybridization and invasion across taxa. Many other systems could and should be investigated in the light of these results, which strongly suggest an integrated exploration of the many global changes facing biodiversity and human populations today.

Keywords: *Aggressive behavior, Biological invasion, Chemical communication, Climate, Gene flow, Global changes, Interspecific hybridization, Introgression, Landscape genetics, Mating system, Pavement ant, Species distribution,* Tetramorium *sp., Urbanization*

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« Pendant les 12 secondes qui vont vous être nécessaires pour lire ce petit encart :
-Au moins 40 humains et 700 millions de fourmis sont en train de naitre sur Terre.
-Au moins 30 humains et 500 millions de fourmis sont en train de mourir sur Terre. »
Bernard Werber, *Le livre secret des Fourmis*

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Chapter 1. General Introduction

> *« The great challenge of the twenty-first century is to raise people everywhere to a decent standard of living while preserving as much of the rest of life as possible.»*
>
> Edward O. Wilson

1.1- PREAMBLE

When I started my PhD formation in October 2015, I joined the Ecology Ecophysiology and Behavior research team within the LEHNA - Laboratory of Natural and Human-impacted Hydrosystems. As for most of the theses, my PhD did not go exactly according to the original plan. Starting from a main focus in landscape genetics with a particular emphasis on the historic and contemporary impacts of urbanization on gene flows, I ended up working on biogeography, interspecific hybridization, and later behavioral ecology, and even chemical ecology. This manuscript compiles the work I did in the last three years and reflects the evolution of my PhD subject.

In the present Chapter, I give a general overview of the topic of my PhD: "Impacts of global changes on biological invasions and interspecific hybridization within the *Tetramorium caespitum* ant species complex". This problematic requires the mobilization of many concepts and tools to apprehend the different papers the manuscript is structured around. One of the most efficient ways to circumscribe a subject is by clarifying related issues. The structure of this introduction will thus be articulated around the developpement of some concepts and definitions of the most important issues that are "global changes", "biological invasions" and "interspecific hybridization".

I will start by introducing the concepts associated to global changes relevant to this study. The second part will introduce the different forms of biological invasions and the different features of invasive species. In the third part of the introduction, I will briefly point out the importance of genetic exchanges, with a focus on interspecific gene flows and setting up of hybrid zones. The fourth and fifth parts will introduce the specificities related to the biological and spatial models I used during my researches. Finally, a sixth part will be dedicated to the presentation of the different chapters of this thesis.

1.2- BRIEF OVERVIEW OF TWO MAJOR GLOBAL CHANGES

Environmental issues are often considered an accumulation of independent phenomena with multiple causes, e.g. biodiversity erosion, soil degradation, or chemical pollution of water. However, these concerns are deeply intertwined and aggravated by the expansion of human activities. Biodiversity faces growing pressures from human actions, including habitat conversion and degradation, habitat fragmentation, overharvesting, pollution, and climate changes (Tittensor *et al.* 2014). These large-scale environmental changes are now clearly visible at a planetary scale, hence often termed "Global changes". Global changes are thus a set of changes recorded over the environment directly or indirectly related to human activities that have global implications for human life and ecosystems. In this section, I briefly review two intensely studied global changes: climate changes and urbanization.

1.2.1- Climate changes

Climate changes are changes in the trend of a climate variable, such as temperature or rainfall, or in the frequency of climatic events, such as floods and droughts over time. Climatic changes for the 21st-century, most notably global warming, are comparable in magnitude to the largest global changes in the past 65 million years (Diffenbaugh *et al.* 2013; Kemp *et al.* 2015). Since 1880 there has been an average global warming of 0.85°C (IPCC 2014). As a result of climates changes, global assessments show that species' extinction risk is increasing on average while population sizes are declining (Pimm *et al.* 2014). Climate changes therefore may pose a serious threat to species' persistence (Parmesan 2006; Bellard *et al.* 2012). Climate projections foresee global warming, sea level rise and an increase in the frequency and intensity of extreme events (Solomon *et al.* 2007).

Such climate changes will have considerable impacts on biodiversity, from organism to biome levels (Parmesan 2006). Climate changes therefore affect fitness of individuals, populations, species and ecosystems. Changes in temperatures can affect survival, reproduction and geographical distribution of organisms (Bale *et al.* 2002).

For example, increasing temperatures induced changes in phenology, leading to an earlier apparition of butterflies, earlier return of migratory birds or precocity of plants' flowering season (Inouye *et al.* 2000; Fitter & Fitter 2002; Stefanescu *et al.* 2003).

However, the main impact of climate changes is probably the shifts in species distribution these changes generate (Fig. 1). Climate is a major determinant of the natural distribution of species, with evidence both from both the fossil records (Escarguel *et al.* 2011) and recently observed trends (Parmesan & Yohe 2003).

Figure 1. Species ranges shift due to climate changes (Stephanie McMillan, 2011)

Numerous empirical studies have already illustrated poleward shifts in distributions of species of a diverse range of taxa in response to climate modifications (e.g. Hughes 2000; Walther *et al.* 2002; Parmesan & Yohe 2003; Hickling *et al.* 2006). For example, in six studies on bird distributions reviewed by Pearce-Higgins *et al.* (2014), the leading edges of distributions shifted poleward by on average 0.76 km per year. In France, the populations of invasive pine processionary *Thaumetopoea pityocampa* spread over 87 km northward between 1970 and 2004 (Battisti *et al.* 2005). Both aquatic and terrestrial organisms are thus shifting their distributions to stay in suitable environmental conditions (Chen *et al.* 2011; Lenoir & Svenning 2015), probably more rapidly than they did in the past (Lawing *et al.* 2011).

1.2.2- Urbanization

Urbanization, i.e. the process by which urban ecosystems are created (McIntyre *et al.* 2001), is a global change of high magnitude and speed which deeply impacts biodiversity (Grimm *et al.* 2008). With around 80% of the European population living in an urban area, urbanization shows a recent but explosive growth in most European countries (see Fig. 2 for an example) first affecting the main cities, but gradually urbanization processes affect smaller settlements and even remote rural villages (Antrop 2004). Today, more than 50% of the global human population lives in urban settings. The expansion of cities is therefore a significant cause of the conversion of land to highly human-modified urban landscapes.

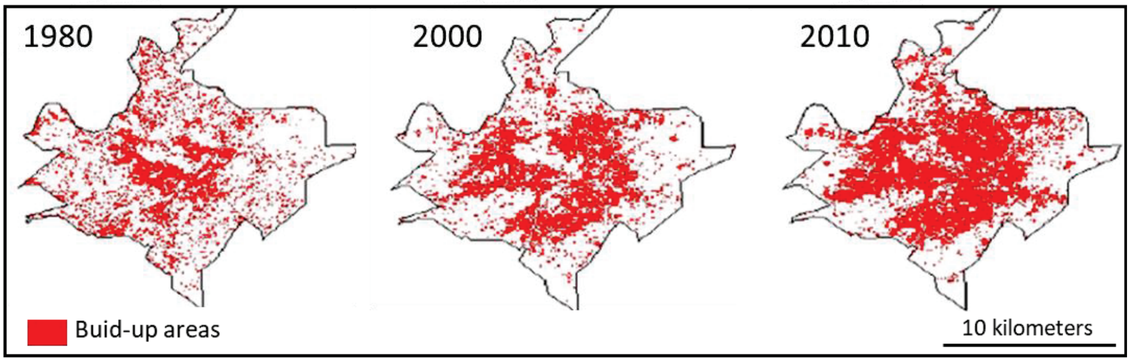

Figure 2. Example of rapid urban sprawl in Faisalabad, Pakistan, between 1980 and 2010 (extracted and shortened from Bhalli *et al.* 2012).

Urban areas are novel ecosystems (Hobbs *et al.* 2006) characterized by a high density of built areas, impervious surfaces with strong heat-retaining abilities, elevated levels of some resources (Rebele 1994) as well as by changes on a broader scale, such as the creation of vast networks of transport infrastructure or the intensification of agricultural activities on their periphery (Niemelä 1999). Urban areas therefore appear as a mosaic of land uses, including residential, commercial, industrial and infrastructural, interspersed with green spaces (Breuste *et al.* 2008). Urbanization thereby converts natural and rural landscapes into profoundly modified habitats combining artificial surfaces, industrial pollution, anthropogenic disturbance, and strongly altered energy and nutrient cycles (Seress *et al.* 2014). it therefore affects all components of the environment, from soils and hydrology to vegetation and microclimates as well as the animals living in the cities (New 2015). For instance, cities are often warmer than nearby rural habitats, with changes in the rate of warming from nighttime to maximum daytime temperatures and in the rate of warming across space being often higher in urban areas (Adams & Smith 2014).

From an ecological perspective, urbanization often has a negative impact on biodiversity. Cities are often located in naturally species-rich regions (Cincotta *et al.* 2000; Luck 2007) with serious conservation challenges (McKinney 2002). Urbanization can result in a cascade of secondary effects on habitat quality, including increased fragmentation of the remaining habitat, increased exposure to habitat edge effects and the introduction of non-native species (Marshall & Shortle 2005).

Most studies on urbanization have therefore focused on the response of organisms to pollution, disturbance, nutrient fluxes and landscape fragmentation, including a broad diversity of organisms such as vertebrates, insects, plants, fungi and micro-organisms (McDonnell & Hahs 2008). In addition, invasions of exotic species cause by human-mediated biotic exchanges, and extinctions of indigenous native species due to habitat alteration and destruction may lead to a homogenized biota across the world's cities. Although more rarely, urbanization can also have positive impacts on biodiversity. The various human influences in cities may create and maintain a variety of habitats that do not occur elsewhere (New 2015), supporting high species diversity, including threatened species (Niemelä 1999). Kark *et al.* (2007) identified traits in urban birds that enable species to dominate highly urbanized environments by becoming urban exploiters (i.e., species such as pigeons or rats that colonize highly urbanized environments using resources and shelters provided by humans; McKinney 2006), and suggested that ecological success in urbanized environments may depend on a combination of traits including diet, degree of sociality, sedentariness and preference in nesting sites.

Box 1: Ants in cities

Among the fauna present in highly urbanized environments, ants (Formicidae) are good indicators of the environmental impact of urbanization (Philpott *et al.* 2010; Heterick *et al.* 2013). Several species of ants are common in cities (e.g., *Tetramorium immigrans*; Fig. 3) and are therefore included in studies of urban ecology (e.g., Pacheco & Vasconcelos 2007; Sanford *et al.* 2009; Menke *et al.* 2011; Buczkowski & Richmond 2012; reviewed in Santos 2016). Within ants, the magnitude and direction of urban impacts depend on the life history and sensitivity to disturbance of species, but also on species interactions and dispersal ability (Garden *et al.* 2006).

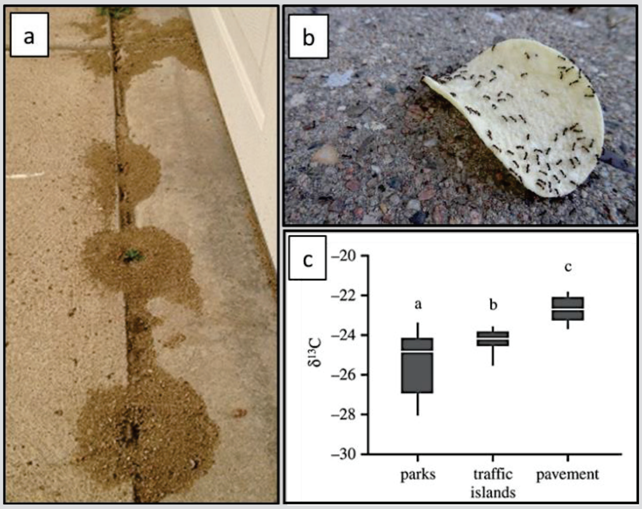

Figure 3. Two illustrations of pavement ants *Tetramorium immigrans* (a) nesting in urban ecosystems and (b) eating human food resources. (c) Changes in *Tetramorium immigrans* diet across urban habitats. Box plots show stable isotope values of carbon (median, 25–75%, and range) for *T. immigrans* workers collected from parks, traffic islands and pavements. Extracted from Penick *et al.* (2015).

Accordingly, almost all studies about impacts of urbanization on ant communities show differences in species composition in urban habitats compared to nearby natural areas (Philpott *et al.* 2010), suggesting that urbanization significantly alters ant species distributions. In addition, ants are easily transferred outside their native habitat through global scale human trade (McGlynn 1999a) and most studies published so far have shown dominance of species introduced by humans in cities (Heterick *et al.* 2013; Vonshak & Gordon 2015).

1.3- BIOLOGICAL INVASIONS

1.3.1- The major issue of non-indigenous species

Biological invasions are invasions by non-indigenous species introduced and maintained voluntarily or not outside their native range. Studies of biological invasions have grown exponentially for the last twenty years. Strictly speaking, invasions are neither novel nor exclusively human-driven phenomena. However, the geographical scope, the rate of recurrence, and the number of species involved have increased sharply subsequently to the development of transport and trade (di Castri 1989). The role of humans as dispersers of nonindigenous species could therefore have surpassed natural selection and dispersal, overcoming the natural stochastic forces and biotic resistance that would repel or remove introduced propagules (Ricciardi 2007).

Biological invasions are a leading cause of conservation concern. Ecosystems and human society have been drastically altered by the proliferation of invasive species, especially in the current age of globalization (Perrings *et al.* 2005). In terms of its rate and geographical extent, its potential for synergistic disruption and the scope of its evolutionary consequences, this current mass invasion event is without precedent and could be considered as a unique form of global change (Ricciardi 2007). Non-indigenous species adversely affect native species through competition, predation, disease, hybridization, herbivory, parasitism, and alteration of disturbance regimes (reviewed in Gurevitch & Padilla 2004). The predation by brown tree snakes *Boiga irregularis* introduced in the island of Guam, on Saipan and in Indonesia have led to the decrease of population sizes of 12 bird species and 11 lizard species, and led to the extinction of 25 birds species (Wiles *et al.* 2003). The competition between the introduced gray squirrel *Sciurus carolinensis* and the native Red squirrel *Sciurus vulgaris* in U.K. led to decrease drastically the distribution area of the red squirrel (Gurnell *et al.* 2004). Invasive ants (Box 2 hereafter) have been shown to impact many components of ecosystem functioning, ecosystem services and human societies, such as modifications of trophic web dynamics, alterations of nutrient cycling or decrease in pollination (reviewed in Bertelsmeier *et al.* 2015). Negative impacts from invasive non-indigenous species consequently include altering ecosystem functioning (e.g. Fig. 4, Wardle & Peltzer 2017), declining population sizes of other species and causing local extinctions (Moles *et al.* 2012; Pyšek *et al.* 2017).

Positive effects are sometimes associated with the presence of non-indigenous species (Ewel & Putz 2004), such as the use of non-native predators to control herbivores (Bertness & Coverdale 2013) and presence of non-native plants extending total flower duration for pollinators (Salisbury *et al.* 2015). However, studies of biological invasions highlight many more negative than positive effects. The impacts of biological invasions are difficult to assess, especially because ecological consequences are modified over time (Strayer *et al.* 2017) and because their characterization strongly depends on human perception.

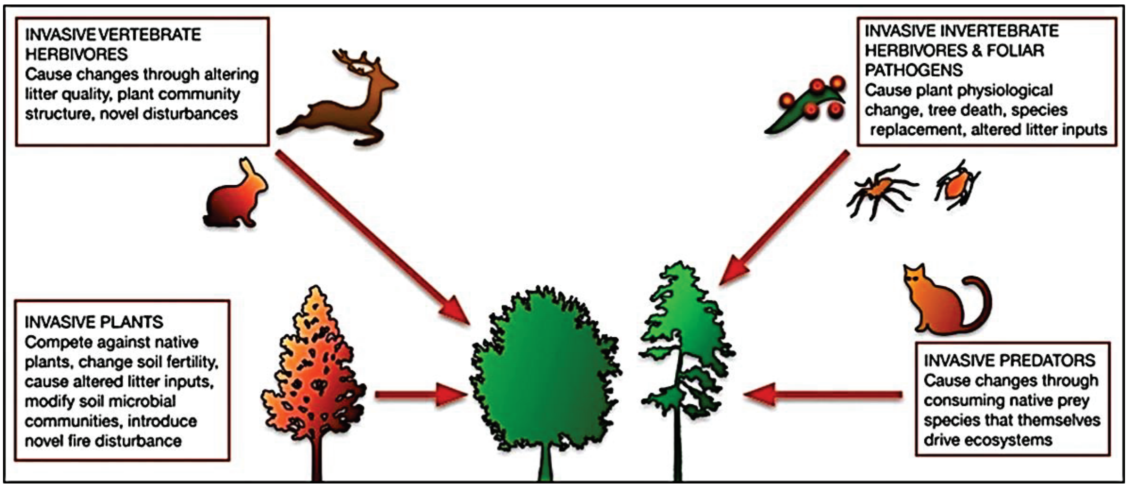

Figure 4. Upper part of the Figure 1 in Wardle & Peltzer (2017) summarizing disruption of ecosystem functioning by aboveground invasive organisms in forest ecosystems, representing all major trophic groupings, and through a wide variety of mechanisms.

Box 2: invasive ants

Social insects are among the most frequent and impactfull invasive animals. These insects live in colonies with numerous individuals, increasing their abilities to exploit resources and compete with local species and improving defenses capacities against predators (Moller 1996). Each colony produces many winged reproductives, rising dispersal abilities. These societies also favor flexibility in reproduction modes and dispersal as well as in their social structure, promoting biological invasions (Chapman & Bourke 2001 ; Holway *et al.* 2002 ; Abbott 2005). As an example, polygyny (i.e. colonies with several queens; Hölldobler & Wilson 1990) allows a faster dispersal, as the presence of many queens accelerate the growth of the colony (Vargo & Fletcher 1989) and increase the probability to introduce a group of individuals including reproductive females able to initiate new colonies (Hee *et al.* 2000).

Among invasive social insects, ants represent the most widespread and destructive taxa (Tsuitsui *et al.* 2000; Reuther 2009). According to the Invasive Species Specialist Group (ISSG, IUCN) and Lowe *et al.* (2000), 5 of the 100 most damaging invasive species in the world are ants: the Argentine ant (*Linepithema humile*; Fig. 5a), the red fire ant (*Solenopsis invicta*; Fig. 5b), the bigheaded ant (*Pheidole megacephala*; Fig. 5c), the little fire ant (*Wasmannia auropunctata*), and the yellow crazy ant (*Anoplolepis gracilipes*). Ant invasions cause significant economic costs, alter the environment and impact native biodiversity (Holway *et al.* 2002). Through competitive dominance, predation and nest raiding, native ant diversity can be significantly reduced (Human & Gordon 1997).

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Box 2: invasive ants (following)

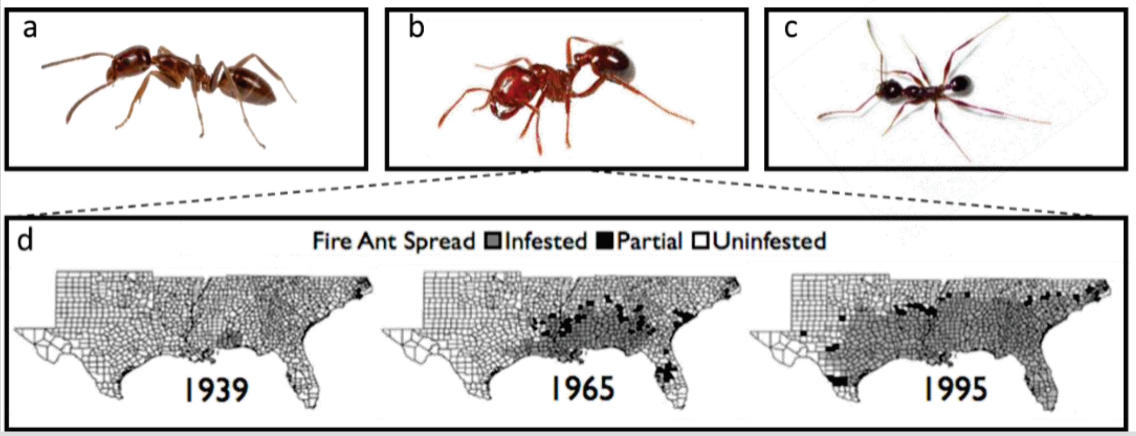

Figure 5. (a-c) Three of the main invasive species of ants (a: Argentine ant; b: Fire ant; c: Bigheaded ant). (d) Spread of the fire ants between 1939 and 1995; extracted from Callcott & Collins (1996).

Highly invasive ants are often unicolonial, like Argentine ants (*Linepithema humile*) forming supercolonies in which workers and queens mix freely among physically separate nests, reducing costs associated with territoriality, and leading to high worker densities (Tsuitsui *et al.* 2000). The red fire ant *Solenopsis invicta* is also a significant pest that was inadvertently introduced into the southern United States almost a century ago and more recently into North-America and other regions of the world (Fig. 5d). It is either monogynous or polygynous in North-America, with sometime more than 200 queens per colony (Ross & Keller 1995). Ascunce *et al.* (2011) showed that at least 9 separate introductions of *S. invicta* have occurred into newly invaded areas (e.g. Taïwan or Australia), whose main source was the main southern U.S. population.

There are many other species of invasive ants, and probably more will be observed in the coming decades in response to increasing global changes. For example, in France, 8 of the 14 invasive ant species investigated by Bertelsmeier and Courchamp (2014) were predicted to increase their potential range in response to climate change.

1.3.2- Invasion stages and evolutionary processes

Invasion pathway can be described as a series of distinct stages, and the transition between any two stages is hindered by specific barriers and can be linked to evolutionary changes (Blackburn *et al.* 2011; Bertelsmeier *et al.* 2015; Zenni *et al.* 2017; Fig. 6).

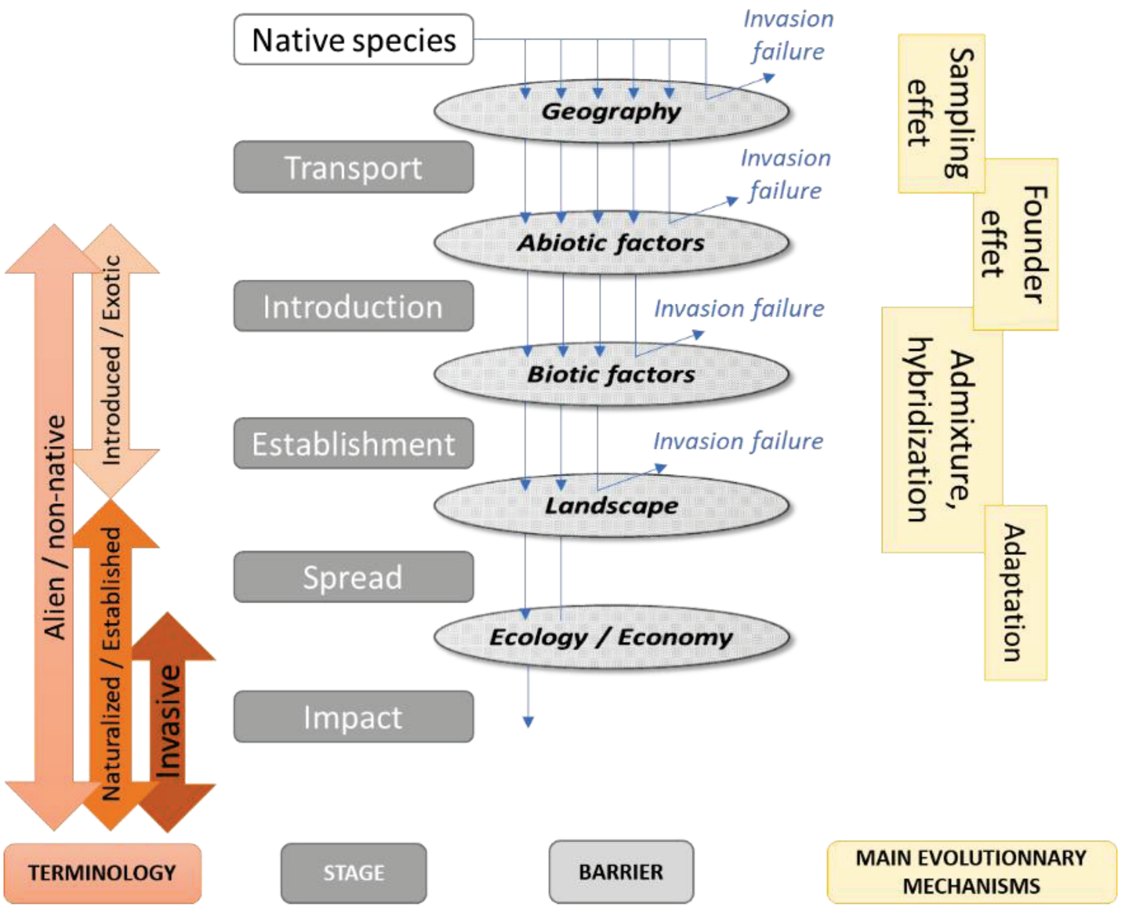

Figure 6. The simplified unified framework for biological invasions (Blackburn *et al.* 2011) expanded to incorporate the main evolutionary mechanisms associated with invasions (adapted from Zenni *et al.* 2017 and Bertelsmeier *et al.* 2015). The proposed framework recognizes that species are referred to by different terms depending on where they have reached in the invasion process, that the invasion process can be divided into a series of stages, and that in each stage barriers need to be overcome for a species or population to pass on to the next stage (Blackburn *et al.* 2011). The blue arrows describe the movement of species along the invasion framework with respect to the barriers.

First, individuals or propagules must be anthropogenically transported from one location to another, overcoming natural geographical barriers (*transport* stage in Fig. 6). In the event these individuals or propagules introduced outside their native range survive transport, they must then survive in a novel, exotic location, under the environmental conditions of the recipient area (*introduction* stage in Fig. 6). This stage can induce a founder effect, i.e., a decrease in genetic diversity within the introduction area (Allendorf & Lundquist 2003), because the genetic pool of the transported individuals only represent a small sample of the whole genetic variability inside the native area.

After colonization, populations have to be able to reproduce and to achieve a positive growth rate in order to become truly established in the new environment without new exogenous propagules or individuals incoming (*establishment* stage in Fig. 6). At this stage, selection of exapted genotypes inside the genetic pool and establishment of new genetic lineages can occur as a result of crossing between genotypes previously isolated in the native area. This stage can be followed by a lag phase with a very low population density (Williamson 1996), except for some species capable of fast adaptation or with a high plasticity (Fordyce 2006).

The last stage leading to invasion is population growth (*spread* stage in Fig. 6). Populations have to overcome interspecific interactions and other barrier linked to environmental conditions and be able to spread across the landscape. This spread can generate new founder effects, induce selections of exapted genotypes, genetic recombinations due to inbreeding and adaptation, and hybridization or introgression with local species (Ellstrand & Schierenbeck 2000, Suehs *et al.* 2004, Tiébré *et al.* 2007). Finally, the generation of impacts (e.g., altering ecosystem functioning or declining population sizes of other species) can constitute the last stage of invasion (Bertelsmeier *et al.* 2015; *Impact* stage in Fig. 6; see previous section for details).

1.3.3- How species become invasive ?

All introduced species do not become invasive (Fig. 6). Previous studies showed that only 10% of exotic populations successfully proceeded to the following invasion stage (e.g. for flatworms and marine organisms in Boag & Yeates 2001; Williamson 1996), although this success rate could be higher for exotic ant species (Suarez *et al.* 2005). Ecological, demographical and genetic factors simultaneously determine the success of the invasion. Some factors in the success of the invasion are thus not linked to the traits of the transported organisms (Box 3); for example environments with few predators or competitors favor the spread of species in a novel environment ("Enemy Release Hypothesis", Keane & Crowley 2002, but see Colautti *et al.* 2004), and high propagule pressures (i.e. the number of individuals introduced and the number of introduction attempts) increase chances of establishment (Simberloff 2009).

Significant differences in success between species also suggest that in addition to these environmental factors, multiple traits of the introduced species may influence species' invasion success. These traits can be expressed in the native population before transportation in the novel environment or can be developed after the introduction in response to evolutionary processes such as genetic drift resulting from bottlenecks (Tsutsui *et al.* 2000) or new selection pressures (Giraud *et al.* 2002). Blackburn *et al.*'s (2009) results suggested a likely influence of some species-level traits on exotic bird establishment success, e.g., success seemed greater for species with large body mass. In social insects and especially ants, many species-level traits have been found that help species to become invaders, such as nesting and feeding habits (Holway *et al.* 2002), small sizes (McGlynn 1999b), unicoloniality and reproduction by budding (Tsutsui & Suarez 2003) (reviewed in Reuther 2009; see box 2 for details).

Box 3: Urbanization promotes biological invasions

Urban environments are often seen as unique or degraded habitats that present hardships for some species. Many native species are sensitive to anthropogenic disturbances. For these species, urbanization leads to the destruction of favorable habitats (Didham *et al.* 2007). However, these ecosystems can also provide opportunities for other species. Marzluff (2001) evidenced an upward trend in the proportion of non-native species (i.e., species that did not occur before importation by humans) toward the urban core. Numerous studies have further shown that the construction and expansion of cities promote the loss of native species and their replacement by non-native species (McKinney 2006). Recently, Cadotte *et al.* (2017) showed that non-native species obtain higher abundances and greater diversity in more urbanized habitats, especially invertebrates.

Three factors may explain the increase in non-native species richness: (i) an increased rate of importation of non-native individuals, e.g., due to accidental or intentional transport (Mack *et al.* 2000; McKinney 2006); (ii) the presence of habitats favorable to the establishment of non-native species, according to the "niche opportunity" concept (Shea & Chesson 2002) where favorable combination of resources, natural enemies and physical environmental conditions, including their fluctuations in time and space, improve the invader's habitability (McKinney 2006); and (iii) the fact that non-indigenous species include species that can tolerate the unique conditions or capitalize on the opportunities found in urban environments (Cadotte *et al.* 2017).

McKinney (2006) also documented numerous examples of urban impacts on biological communities considering all three factors providing niche opportunities for non-native species: (i) by increasing food resources provided by humans (e.g., increasing densities of house mice *Mus musculus* and feral house cats *Felis catus*); (ii) by reducing or eliminating natural enemies (e.g., increasing densities of raccoons after the elimination of large carnivores in the USA); or (iii) by human alteration of the environment which can generate physical conditions detrimental to native species but favoring non-native species. Moreover, these urban beneficiaries may be those that normally cannot overcome competitive interactions in intact native communities but find the opportunity to flourish in urban habitats (Cadotte *et al.* 2017).

1.4- GENETIC EXCHANGES BETWEEN SPECIES

1.4.1- From gene flow to interspecific hybridization

Gene flow involves the movement of genes into or out of a population, due to the movement of either the whole individual organisms or genome (eggs and sperm, e.g., through pollen dispersal by a plant) from one population to another (Fig. 7). After entering a new population, immigrant genomes may become incorporated by sexual reproduction, and then be gradually broken up by recombination (Mallet 2001), adding new alleles or modifying alleles frequency in the population. Gene flow can therefore be a strong agent of evolution.

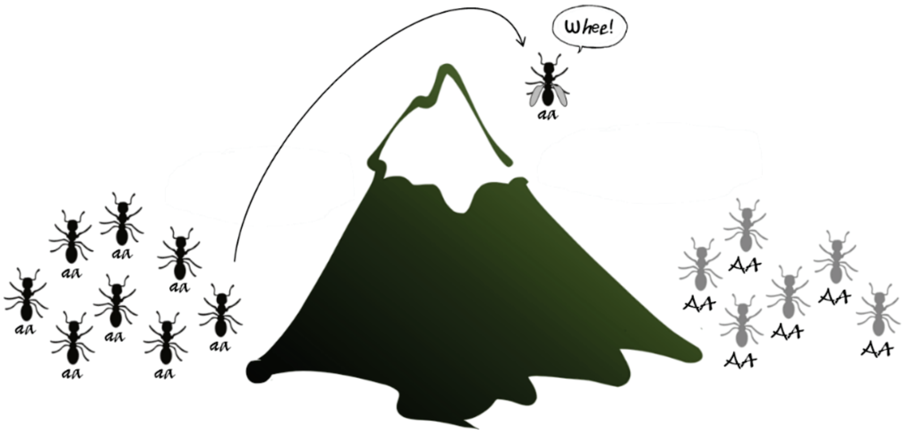

Figure 7. Illustration of limited gene flow between two population of ants isolated by a geographical barrier.

Gene flow can take place between two populations of the same species through migration (Fig. 7) and is mediated by reproduction and vertical gene transfer from parent to offspring. Alternatively, genetic exchanges can take place between two distinct species through horizontal gene transfer (Keeling & Palmer 2008), or through vertical gene transfer via interspecific hybridization and introgression (Anderson 1953). During the last decades, the use of genetic techniques and the study of hybrid zones showed that hybridization and introgression are common phenomena in animal evolution (Dowling & Secor 1997). Hybridization, i.e., reproductive interactions between individuals whose lineages show some degree of evolutionary divergence, and introgression, i.e. the durable integration of genes acquired through hybridization, have been demonstrated to be relatively common (Mallet 2007; Mallet *et al.* 2016; Larsen *et al.* 2010; Brennan *et al.* 2015), and shape species through substantial interspecific gene flow (Arnold & Kunte 2017).

1.4.2- What initiate interspecific hybridization?

Interspecific hybridization is a common natural phenomenon; it is estimated that as many as 25% of plant species and 10% of animal species hybridize naturally (Mallet 2007). Hybridization is nevertheless thought to be rare in nature between sympatric species, as implicitly suggested by many definitions of species (Mallet *et al.* 2008). Most of the time, hybridization therefore happens between previously allopatric species which have moved and met. Natural hybridization during these secondary contacts are, for a large part, related to movements driven by the last glacial periods, which gathered many species in refuges in Spain, Italy, and the Balkans (e.g., Ferrero *et al.* 2011; see also the section about the Rhône-Saône valley hereafter).

During the past decades, the role of hybridization in the extinction of species has been particularly investigated given that the impact of humans on their environment (fragmentation, species introductions, climate change) has created new opportunities for hybridization and, in some cases, led to the increase in frequency of the phenomenon where it already existed (Todesco *et al.* 2016). For the British Isles, a recent overview has already demonstrated a rise in the number of hybrids during the last few decades (Stace *et al.* 2015). Becker *et al.* (2013) showed that, consequently to climate-induced range shifts, sympatry between previously isolated species increased, potentially resulting in introgressive hybridization. Indeed, as some populations and species have spread under favourable climatic conditions, new contact zones arose between related lineages, leading to interspecific competition but also an increased likelihood of hybridization between taxa (Garroway *et al.* 2010).

Climate-induced expansions of introgression have been predicted for many terrestrial and aquatic species, especially species that are sensitive to temperature and streamflow conditions (Walther *et al.* 2002), including insects (Sánchez-Guillén *et al.* 2016). Climate change could therefore decrease worldwide biodiversity through invasive hybridization (Hoffmann & Sgro 2011). Nonetheless climate is not the sole cause of hybridization. Anthropogenic hybridization can result directly from human action, e.g. when hybridization is induced by the release of exotic individuals (e.g., Casas *et al.* 2016 for the partridge *Alectoris spp.*). As species are transported around the world with increasing intensity, barriers to gene flow between formerly geographically separated species are reduced and new hybrids between introduced and indigenous species are likely to emerge more frequently (Thomas 2013). Furthermore, biological introductions concomitant with urbanization (Cristescu 2015) could promote the establishment of contact zones between species capable of genetic exchanges because of the lack of pre-zygotic, geographical or ecological reproductive barriers, as shown by Crispo *et al.*'s (2011) illustration of the consequences of anthropogenic changes on numerous taxa.

Anthropogenic hybridization can also occur indirectly, as a result of the changes caused by human activities on their environment. For instance, human activities may impact physico-chemical properties of the environment. In Lake Victoria, the increase in water turbidity promoted hybridization between cichlid fish (*Haplochromis spp*) by making it impossible to recognize conspecifics because mate choice was based on coloration (Seehausen 1997). Urbanized areas have a greater heterogeneity than "natural" environments, creating ecotones acting as new areas of contact between species with widely differing ecological preferences (Brennan *et al.* 2015). Human activities are thus paradoxically likely to both reduce and enhance genetic exchanges, on the one hand by erecting new barriers, and on the other hand by bringing together previously distant species. Urbanization and human activities might therefore act as major qualitative and quantitative promoter of hybridization processes.

1.4.3- Long-term evolutionary consequences of hybridization and introgression

When reproductive barriers between species are insufficient, and hybridization occurs, then the outcome of hybridization (Fig. 8) can span from complete species isolation to complete admixture. The position along this continuum is determined by a combination of intrinsic genetic factors that are determined by the extent of genetic incompatibilities and extrinsic factors related to the hybrid phenotype (Sánchez-Guillén *et al.* 2016).

Several authors identified two types of models explaining how stable hybrid zones are maintained (Nielsen *et al.* 2003; Buggs 2007; Fig. 8a). When hybrids show a higher fitness than parental taxa, a hybrid zone remains stable provided that the hybrid's fitness is bounded to an ‘ecotonal’ zone corresponding to an intermediate environment to that occupied by the parental taxa (bounded hybrid superiority model; Moore 1977). When hybrids are of lower fitness than parents, a stable cline is primarily maintained by selection against intermediate genotypes. In this case, the low fitness of hybrids causes spatial mixing of the parental taxa to be minimized, leading to narrow, straight hybrid zones, usually termed ‘tension zones’ (dispersal/selection models; Barton & Hewitt 1985). In some situations, it may lead alleles and associated phenotypes to cross species boundaries and provide individuals of the recipient species with an adaptive advantage (adaptative introgression in Fig. 8; Hedrick 2013; see Whitney *et al.* 2015 or Pardo-Diaz *et al.* 2012 for examples).

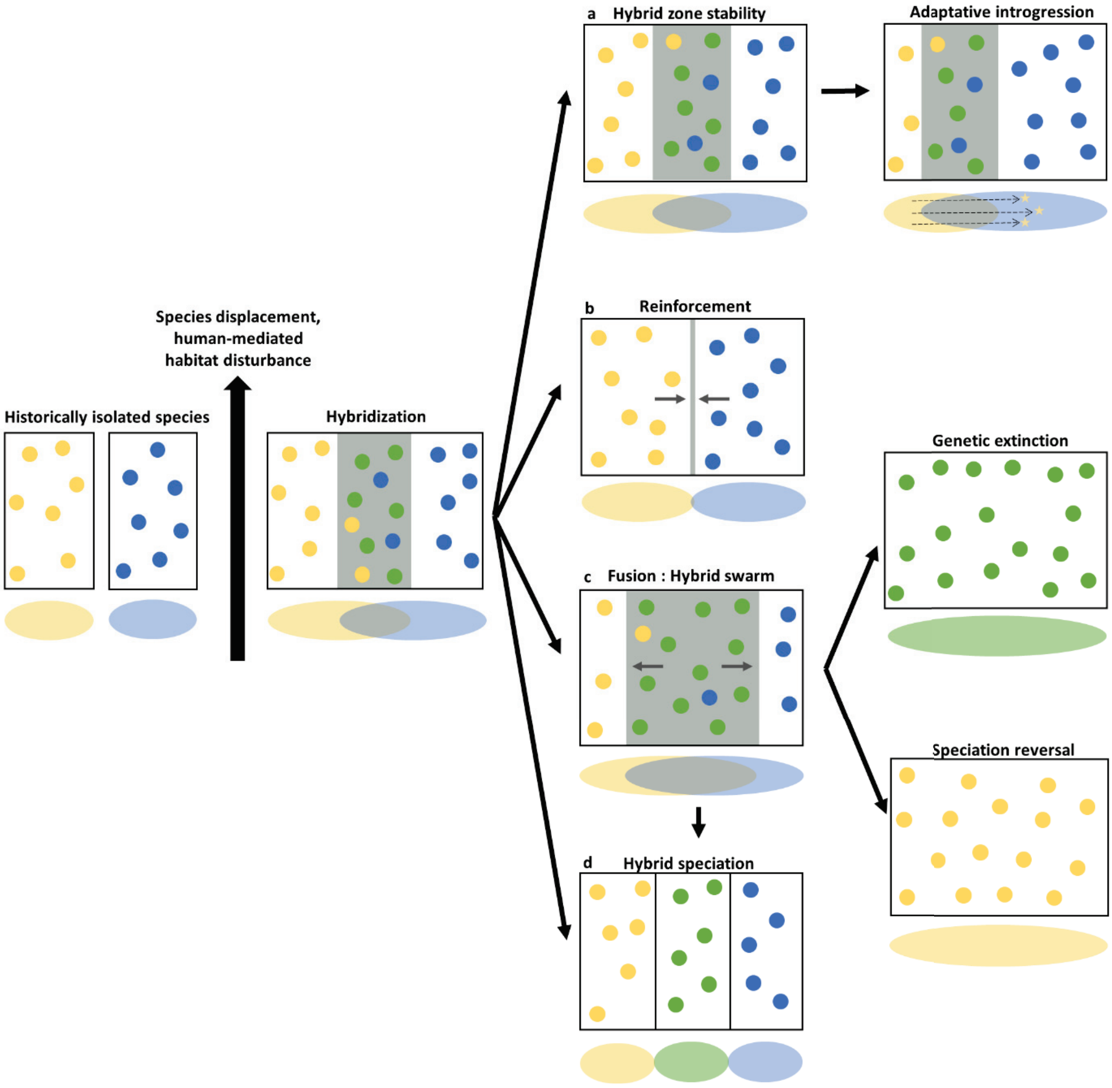

Figure 8. Different outcomes of hybridization. Boxes represent spatial outcomes, with small circles corresponding to individuals belonging to parental species (blue and yellow) or hybrids (green), and grey area characterizing the hybrid zone. Large ovales represent genetic populations, i.e. pool of individuals with the same genotype (adapted from Grabenstein & Taylor 2018). Blue and yellow ovales are parental populations, green areas of overlapping ovales represent populations with hybrid individuals of mixed ancestry and green ovales represent new species resulting of hybridization processes. Yellow stars represent alleles from the yellow genetic pool. Small dotted black arrows denote introgressed alleles. The thick vertical arrow indicates the process that initiated hybridization. Although the outcomes are depicted as a unidirectional flow chart, these outcomes are not necessarily permanent, and populations can fluctuate between outcomes over longer evolutionary timescales.

Hybrid zones can also disappear progressively through reinforcement (Fig. 8b). In this situation, hybrids initially formed have a lower fitness than either parent alone, leading over time to complete species isolation (Barton & Hewitt 1985; see Redenbach & Taylor 2003 for example). However, hybridization can also play a creative role in evolution through several processes, such as hybrid swarming or hybrid speciation. Hybrids can be just have the same fitness than either parent alone, and over time differences in parental species should weaken and hybrid populations enlarge (Fig. 8c). As a result, hybridization sometimes leads to the displacement or extinction of one or both parental taxa (Ellstrand & Elam 1993) and their replacement by a complete admixture called "hybrid swarms" ("Genetic extinction" in Fig. 8; Gilman & Behm 2011; Mallet 2007; see Ording *et al.* 2010 for an example). Hybridization can also lead to the fusion of previously divergent taxa which collapse into a single ("Speciation reversal" in Fig. 8; Seehausen *et al.* 2008).

Finally, hybrid speciation may result in a stable, fertile and reproductively isolated hybrid lineage (Arnold 2006; Mallet 2007; Abbott *et al.* 2013; Thomas 2015; Fig. 8d). This speciation process is considered rare in animals but has been reported in several occasions (e.g., Larsen *et al.* 2010; vonHoldt *et al.* 2011; Barrera-Guzmán *et al.* 2017). For instance, vonHoldt *et al.* (2011) showed that in North Carolina, endemic species of wolves may actually be the descendants of hybrids resulting from the reproduction of coyotes (*Canis latrans*) and gray wolves (*Canis lupus*).

1.4.4- Microsatellites markers and mitochondrial DNA to study hybridization

Over the last decades, several key advances in molecular genetics have greatly increased the impact of population genetics on biology, including the development of polymerase chain reaction (PCR) amplifying specified stretches of DNA to useable concentrations, and the advent of hypervariable microsatellite loci and routine DNA sequencing in biology laboratories (Sunnucks 2000). The development of genetic markers within mitochondrial DNA (mtDNA) and nuclear microsatellites have provided the potential to measure levels of gene flow and population structure (Petit & Excoffier 2009; Hermansen *et al.* 2011; André *et al.* 2011; Toews & Brelsford 2012; Lessios & Baums 2017).

Nuclear microsatellites makers

Microsatellites are DNA sequences present in high frequency in the nuclear genome of eukaryotes (Tóth *et al.* 2000). They correspond to repeats of 2 to 6 nucleotides DNA patterns (Fig. 9). The alleles of a microsatellite, which result from a high mutation rate (10^{-6} to 10^{-2} / locus & generation; Schlötterer 2000) due to slippage and errors during replication, correspond to different numbers of repeats (classically 5 to 40 repeats; Estoup & Angers 1998; Selkoe & Toonen 2006). The lengths of fragments are therefore highly polymorphic.

Microsatellites have become increasingly widespread along the last decades, notably because of their high polymorphism, but also because they are expected to be neutral with a Mendelian inheritance. Microsatellites are relatively easy to obtain, as the flanking regions surrounding the microsatellite (in black in Fig. 9) can be widely conserved across species, thus allowing the use of the same markers to study several species (Barbará *et al.* 2007). These markers are extremely abundant in most organisms (Estoup & Angers 1998; Tóth *et al.* 2000). They have been widely used to answer many different issues, e.g., to estimate genetic diversity (Garner *et al.* 2003) or population size (Rowe & Beebee 2004), to detect bottlenecks (Beebee & Rowe 2001), to estimate relatedness between individuals or populations (Janečka *et al.* 2007), or to detect hybrids (Oliveira *et al.* 2008; Godinho *et al.* 2011). However, mutation schemes of microsatellites are complex (Ellegren 2004), and the same allele may result from several evolutionary trajectories due to homoplasic processes (Selkoe & Toonen 2006). In addition, as microsatellite markers are species-specific, several microsatellite loci are needed to perform representative analyses and because these analyses are difficult to automate (Schlötterer 2004; Guichoux *et al.* 2011), the use of a large number of markers is time-consuming.

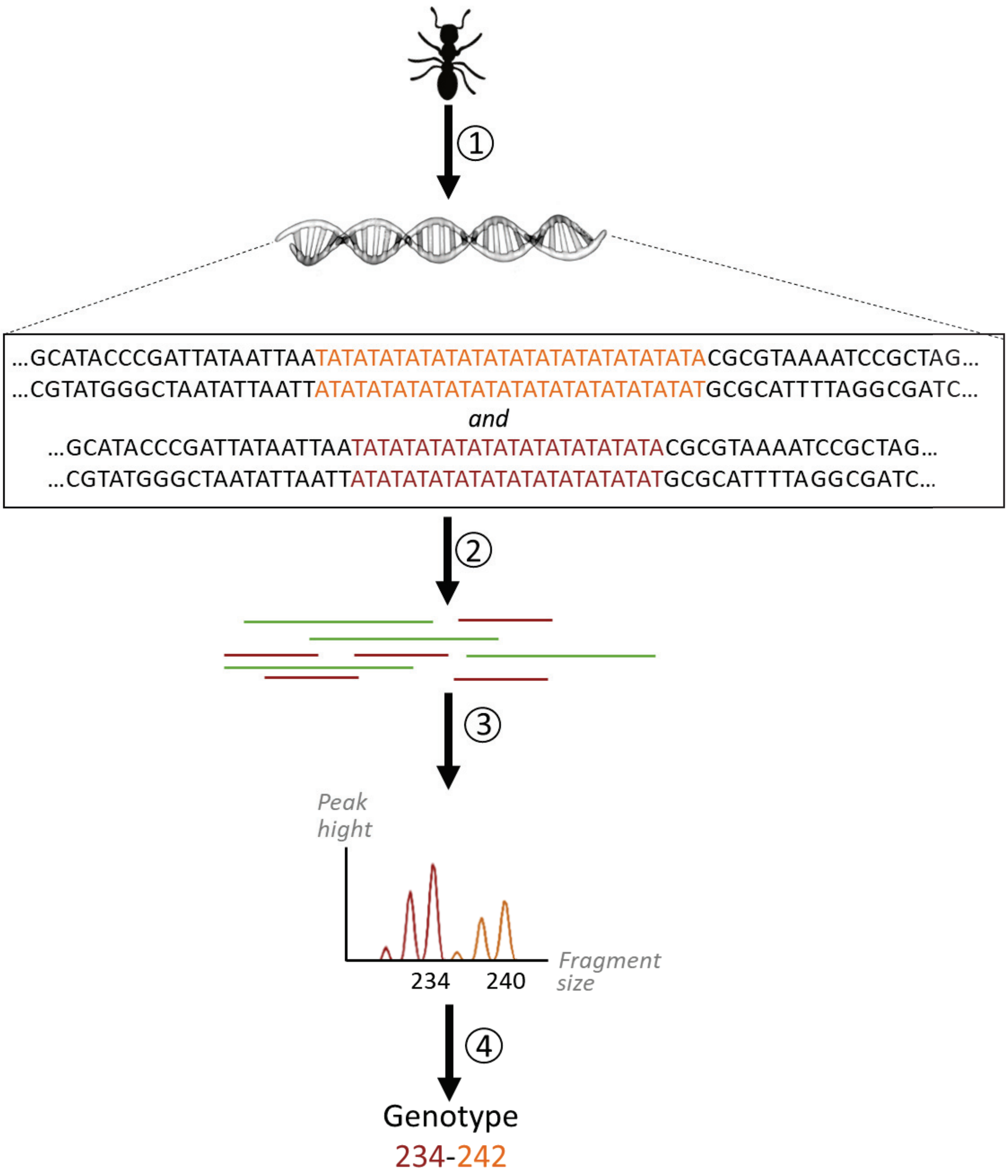

Figure 9. Representation of the different steps of genotyping of a microsatellite locus of a diploid individual. In this example, the microsatellite is a dinucleotide, i.e. the repeated motif counts two base pairs (TA). I illustrate here the case of a heterozygote individual, i.e., an individual with two distinct alleles for a locus. (1) DNA extraction; (2) PCR amplification; (3) Computer-based analyses; (4) Genotype identification.

Mitochondrial markers

In many studies, nuclear markers are combined with mitochondrial DNA, and these last decades, numerous studies about population histories were based on mitochondrial markers.

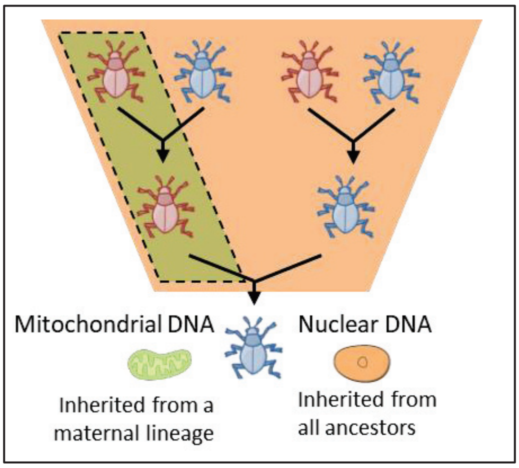

Figure 10. Nuclear DNA is inherited from all ancestors, whereas mitochondrial DNA is inherited maternally.

Mitochondrial DNA is circular, without introns in coding sequences and is not inherited according to Mendelian rules but transmitted uniparentally without recombination, typically inherited maternally (Fig. 10; Dawid & Blackler 1972). Mitochondrial DNA is expected to evolve faster than the nuclear genome (mutation rate 5 to 10 times higher; Hewitt 2001), enhancing the growth of genetic variability within and between populations over evolutionary times, and thus quickly became diagnostic of taxa (Sunnuck 2000).

All these characteristics make mitochondrial DNA highly popular to study population variability (Wahbe *et al.* 2005) or biogeography (Stöck *et al.* 2006), but also in hybridization studies, in complement to nuclear markers, as it provides important data about the direction of crosses in hybrid individuals (Avise & Saunders 1984). Recurrent backcrossing of hybrid females with males from their paternal lineage will also ultimately lead to offsprings with introgressed mitochondria, i.e., mtDNA from the maternal lineage and nuclear DNA from the paternal lineage (Darras & Aron 2015). According to the review of Toews & Brelsford (2012), when foreign mtDNA haplotypes are found deep within the distribution range of a second taxon, those mtDNA haplotypes are more likely to be at a high frequency and are commonly driven by sex-biased asymmetries or adaptive introgression. Combining microsatellite markers and mtDNA could therefore help improving discrimination between situations with hybridization and situations with introgression.

1.5- SPECIFICITIES OF ANT MODELS: THE CASE OF THE *TETRAMORIUM* GENUS

1.5.1- Why study ants?

Social insects mainly belong to two taxonomical groups, Hymenoptera and Isoptera, and are characterized by a higher level of sociality called eusociality, defined by generations overlap, cooperating broad care and specialization of individuals in reproduction through reproductive *vs.* sterile castes (Wilson 1971). Ants are an ideal model system for ecology and evolution (Lach *et al.* 2010). More than 15,000 species and subspecies of ants have been described on all continents and many other probably remain to be discovered (Guénard *et al.* 2017). Overall, their biomass is expected to account for 15% of total terrestrial biomass in some ecosystems (Schultz 2000). Because of these high abundance and species diversity, ants are particularly suited for monitoring studies. Ants are dominant in many ecosystems and are consequently keystone species due to their influence on many ecosystem functions (e.g., seed dispersal or soil chemistry; Folgarait 1998). They have colonized almost all terrestrial habitats, including tropical forests, desserts, savannah, urban areas or agricultural landscapes (Hölldobler & Wilson 1990). Due to their broad variety of their lifestyles, ants are increasingly being recognized as useful tools for monitoring ecosystem health (Underwood & Fisher 2006). For example, some species are nomads, others nest in trees, soil, or leaflitter, others construct large anthills; their diet ranges from predators to seed harvesters, including scavengers, exploiters of sugary exudates, omnivores and even fungus growers (Lach *et al.* 2010).

As ants are easily transported by humans because of their small sizes and nesting habits, over 200 species have established populations outside their native range (Lach *et al.* 2010) but it has been estimated that more than 600 ant species have already been introduced outside of their native range (Miravete 2014; Box 2). A small subset has become invasive, i.e. their establishment has been followed by a subsequent proliferation and expansion, leading to negative impacts on native biodiversity and/or human health (Holway *et al.* 2002; Lach *et al.* 2010; see section about biological invasions and especially Box 2).

As ectothermic insects, larval development and adult activities of ants are highly sensitive to climatic conditions, making their distributions strongly affected by climate (Sanders *et al.* 2007; Jenkins *et al.* 2011). Ants are therefore useful models for understanding responses to temperature variation and climate changes (Harkness & Wehner 1977; Andrew *et al.* 2013). Ants are also a suitable model to study urbanization (Box 1), with several ant species common in cities (reviewed in Santos 2016) and several others sensitive to urbanization (Philpott *et al.* 2010). Furthermore, ants are a suitable model to study genetic exchange between species, as Formicidae seem particularly predisposed to hybridization (reviewed in Feldhaar *et al.* 2008; e.g., Kulmuni *et al.* 2010; Purcell *et al.* 2016; Ueda *et al.* 2015). These hybridization events could be partly promoted by the haplodiploid reproductive system of ants (and other Hymenoptera) where haploid males produced by unfertilized eggs only inherit maternal genetic material, mitigating outbreeding depression compared to other organisms, as male fitness is preserved (Feldhaar *et al.* 2008; Kulmuni *et al.* 2010; more details hereafter). Being able to breed with a male of another species also increases the probability of successfully meeting one or more partner(s) and could thus even prove reproductively advantageous (Rosenthal 2013).

1.5.2- Key elements of ant biology

According to Lach *et al.* (2010), the ecological success of ants can be attributed to the benefits of division of labor and morphological specialization among adults. Functional differences between queens and workers are amplified by morphological differences: winged queens start new colonies and produce offspring, whereas non-winged sterile workers raise the brood, build the nest and forage for food. Passera and Aaron (2005) suggested that chemical communication also plays a key role in the evolution of social organization and is an essential driver of the ecological success of ants. In this section, I briefly review these aspects and highlight the main biological characteristics of ants.

Colony life histories, haplodiploidy and the minefield of mating systems

Hölldobler and Wilson (1990) and Lach *et al.* (2010) reviewed the incredible diversity in life histories and mating patterns in ants. All ants live in perennial colonies that exhibit three phases: foundation (initiation of new colonies), growth (production of workers), and reproduction (production of sexuals). Because a broad variety of life cycles exists in ants, I have chosen to only detail the most classical situation (Fig. 11).

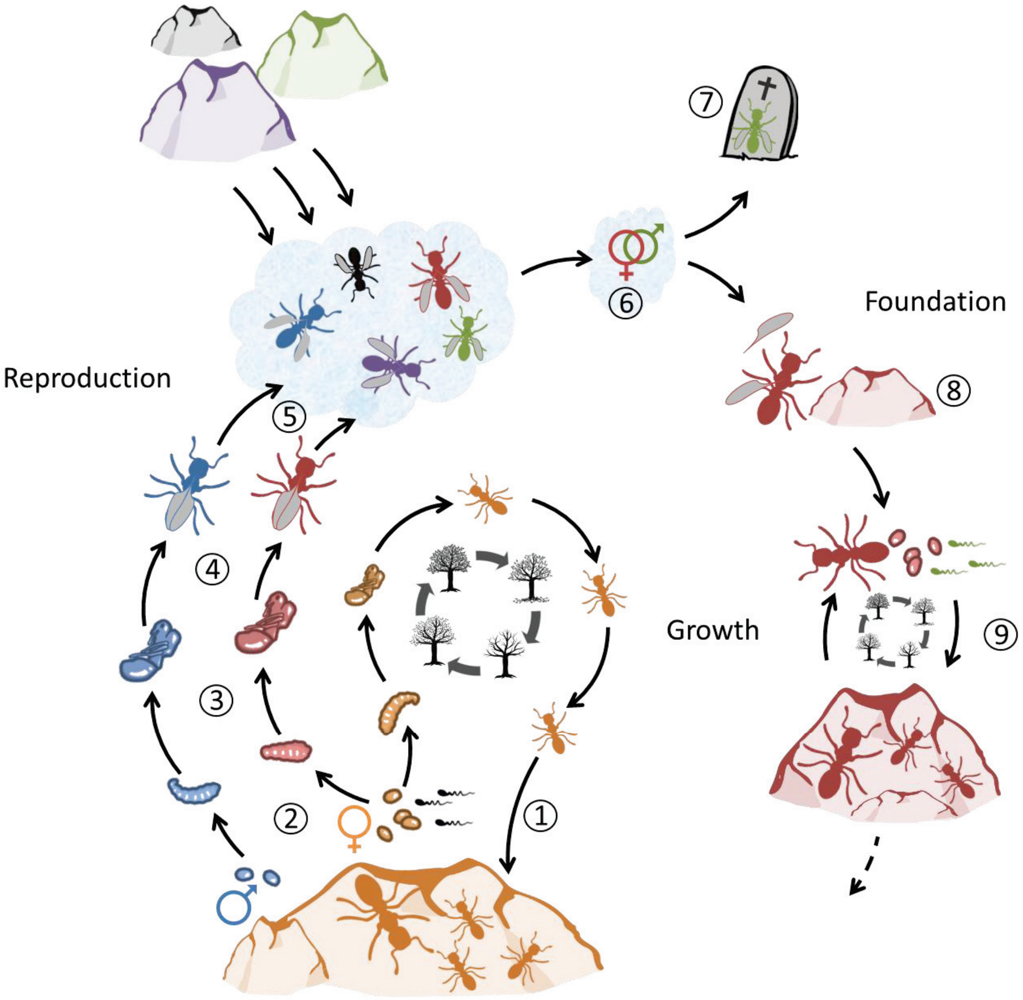

Figure 11. Stages of the life cycle of a monogynous (one queen is the mother of all offspring of the colony) monandrous (only one male mated per queen) colony. 1- Generations of workers raise the brood and take care of the queen. 2- Queen fertilizes eggs to produce females (sexual reproduction) or produces males with unfertilized eggs (arrhenotokous parthenogenesis). 3- The brood grows. 4- Adults emerge. 5- Gynes and males leave the colony for the nuptial flight. 6- Gynes and males copulate. 7- Males die. 8- Female wings are dropped, and the new queen found a nest. 9- The colony grows with many generations of workers during several years before producing winged individuals able to reproduce.

In this system, young winged queens and males are reared once a year (stages 2-4 in Fig. 11). Following a change in weather conditions (e.g. temperature, humidity), gynes and males exit the nests synchronously for the nuptial flight (stage 5 in Fig. 11) and copulate (stage 6 in Fig. 11). Males die (stage 7 in Fig. 11) and only their genes “survive”, sometimes for decades, as sperm stored in a queen's spermatheca. Queens work alone for several weeks up to a few months, feeding and caring for the brood until these become adult workers (independent colony foundation stage; stage 8 in Fig. 11). Colonies of most species must grow to a large size before producing individuals being able to reproduce, which may take several years (stage 9 in Fig. 11). Because of ants’ life cycle, the queen of a colony only copulates during her brief mating period and never mates again.

In ants, sexual determination is haplodiploid (Fig. 12). Females are produced through sexual reproduction (stage 2 in Fig. I. 11) after egg fertilization with sperm stored in the spermatheca (Fig. 12). Unfertilized eggs develop into males through arrhenotokous parthenogenesis (stage 2 in Fig. 11). These males are haploid and only contain genetic material (both nuclear and mitochondrial DNA) of maternal inheritance. Consequently, in a monogynous monandrous system (i.e., one queen mating with one male and being the mother of all the offspring of the colony), all workers inherit their father’s complete nuclear DNA (corresponding to 50% of their own nuclear genome) and 50% of their mother’s nuclear DNA (corresponding to 50% of their own nuclear genome), but their whole mitochondrial DNA is maternally inherited (Fig. 12).

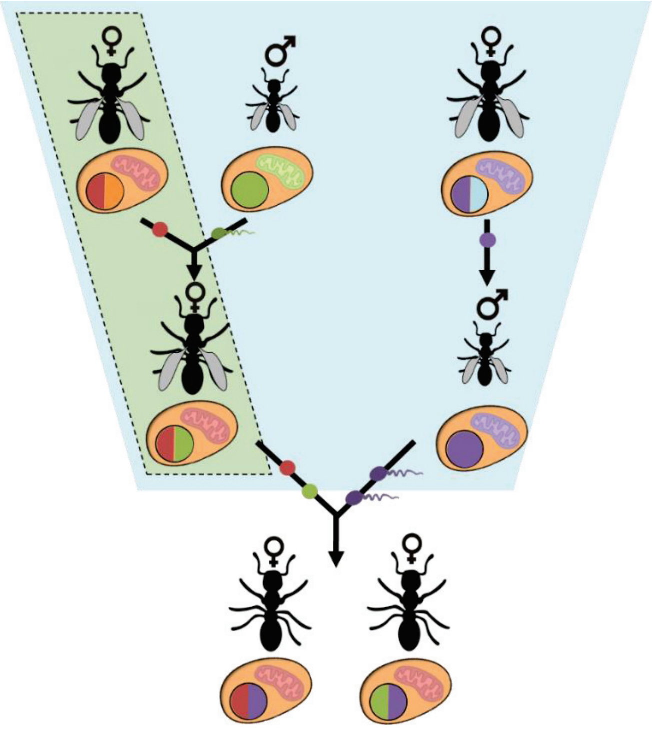

Figure 12. Adaptation of Fig. 10 for the haplodiploid system of ants in a monogynous monandrous mating system. Males and females have wings whereas worker are wingless individuals. Mitochondria color indicate mtDNA haplotypes and nuclear color indicate nuclear DNA allele(s) for a locus. The green area corresponds to mitochondrial inheritance whereas the blue area corresponds to nuclear inheritance.

Although ant colonies are classically headed by a single queen mated to a single male (Figs. 11 and 12), their actual kin structure can be more complex. Colonies of many ant species have multiple queens (polygyny; Hölldobler & Wilson 1977). In addition, in some species, queens mate with multiple males (polyandry; Hardy *et al.* 2008). Polygyny and polyandry both have costs (Hughes *et al.* 2008). Polygyny requires a queen to share her colony's reproductive output with other queens. Polyandry involves energy expenditure and increased risks of predation, parasitism and damage by male partners. However, Hughes *et al.* (2008) suggested that fitness benefits resulting from increased intracolonial genetic diversity have played a significant role in the evolution of polyandry and possibly polygyny in ants (Fraser *et al.* 2000). Because of the important effect of these multiple mating on the relatedness among offspring and the genetic diversity of colonies, the study of the variation in mating frequency in ants is a crucial issue, which has received much attention in the last decades (e.g. Strassmann 2001; Baer & Boomsma 2004; Kellner *et al.* 2007).

Communication and chemical signal

Understanding communication processes within ants has advanced significantly during the last decades (Liebig 2010; Ozaki & Hefetz 2014; Sturgis & Gordon 2012). Most of the interactions among ants are based on the exchange of olfactory information involving compounds constitutive of the cuticle of individuals or actively produced by various exocrine glands present mainly on the legs, thorax and head (Blomquist & Bagnères 2010). Chemical cues are detected by the peripheral nervous system (i.e. the antenna) and relayed towards the higher nerve centers. In social Hymenoptera, a particular class of chemical compounds is involved in the regulation of social interactions: cuticular hydrocarbons (CHCs; Blomquist & Bagnères 2010). Primarily acting as a barrier against desiccation and pathogens, CHCs serve in ants at least as signature mixtures and pheromones at various levels (individual, within colony, between colonies; d'Ettorre *et al.* 2017). CHCs are stored in the post-pharyngeal gland and exchanged between members of the colony by trophallaxis, allogrooming, and physical contact (Lenoir *et al.* 2001; Chapuisat *et al.* 2005). The resulting chemical mix typically includes alkanes, alkenes and methyl branched alkanes (Martin & Drijfhout 2009) which contribute to the creation of a colony-specific odor template (Frizzi *et al.* 2015).

Within species, different colonies usually have the same blend of CHCs but differ in their relative concentrations (van Wilgenburg *et al.* 2006; Foitzik *et al.* 2007; Brandt *et al.* 2009). Workers inspect the hydrocarbon profiles of other workers with their antennae and are thought to discriminate according to the degree to which it overlaps their own template (Suarez *et al.* 2002; van Zweden *et al.* 2009). Colony mates are usually ignored, whereas other hetero- and conspecifics typically elicit an aggressive response (Fadamiro *et al.* 2009; Menzel *et al.* 2009). Such mechanisms thus allow recognizing nestmate and limiting aggressiveness between nestmates, which is crucial in regulating colony cohesion and interactions with other colonies.

The CHCs chemical mix can be modified by the environment, including diet, pollution, construction materials of the nest, microorganisms associated with the colonies, or physical contacts among individuals (Sorvari & Eeva 2010; Chen & Nonacs 2000; Liang & Silverman 2000). Environmental factors therefore play a key-role in inter-individual recognition and can alter behavior, specifically generating increases or decreases in aggression towards interacting individuals (Frizzi *et al.* 2015).

1.5.3- *Tetramorium* ant species in France

Tetramorium is a ubiquitous genus of small generalist ants including several important invasive pests such as *T. bicarinatum* (Nylander, 1846) (Garcia & Fisher 2012), *T. immigrans* Santschi, 1927 (Wagner *et al.* 2017) and *T. tsushimae* Emery, 1925 (Steiner *et al.* 2010). Twelve *Tetramorium* species have been recorded in France, including *T. semilaeve* André, 1883, *T. meridionale* Emery, 1870, two species of the *T. chefketi* complex (*T. forte* Forel, 1904, *T. moravicum* Kratochvil, 1941), and five species of the *T. caespitum* complex: *T. impurum* (Foerster, 1850), *T. alpestre* Steiner, Schlick-Steiner & Seifert, 2010, *T. immigrans*, *T. indocile* Santschi, 1927, and *T. caespitum* (Linnaeus, 1758) (Schlick-Steiner *et al.* 2005, 2006; Guesten *et al.* 2006; Steiner *et al.* 2010; Csösz *et al.* 2014; Borowiec *et al.* 2015; Wagner *et al.* 2017). Morphological identification can be used to discriminate among *Tetramorium* species (e.g. Guesten *et al.* 2006; Borowiec *et al.* 2015; Wagner *et al.* 2017), but difficulties arise within the *T. caespitum* complex where species appear very similar in color and form (Schlick-Steiner *et al.* 2006; Wagner *et al.* 2017). As a consequence, it is necessary to use genetic methods to securely identify *Tetramorium* species in France.

In comparison to, and probably because of the systematic work on these *Tetramorium* ants (e.g. Schlick-Steiner *et al.* 2006; Wagner *et al.* 2017), comparatively little has been done so far to study the biology, life history and ecology of these species, especially in recent times. As the present PhD work is strongly focused on the cryptic species *T. immigrans* and *T. caespitum*, I briefly review below the relevant previous studies on these taxa. To my knowledge, nothing is known about the fine-scale distributions of these species in France, even if they are known to differ in thermal specificity. Wagner *et al.* (2017) indicate that the thermal niche of *T. caespitum* corresponds to an average standard air temperature of 16.1°C *vs.* 19.9°C for *T. immigrans*. Brian *et al.* 1967 used mark-recapture techniques to estimate colony size of *T. caespitum* in England, and the 22 colonies studied averaged populations of 14 448 ± 1440 with territories found to average at 43 ± 4 m^2. Gippet *et al.* (2017) investigated urbanization impacts on *T. immigrans* and *T. caespitum* presence, concluding that *T. immigrans* (formerly called *T.* sp. E; Wagner *et al.* 2017) occurred in fragmented and warmer areas, contrary to *T. caespitum* (formerly called *T.* sp. U2; Wagner *et al.* 2017), but this study not detect any urbanization effect on these taxa, focused on a single urban area and did not investigate how land-cover and scale explained their spatial distribution. *Tetramorium immigrans* and *T. caespitum* are monogynous, but to my knowledge, mating systems have never been investigated in these species. Hybridization is suspected between *T. immigrans* and *T. caespitum*, based on mitochondrial-nuclear discordance found in two individuals with a *T. immigrans* mtDNA and which clustered with *T. caespitum* for Amplified Fragment-Length Polymorphism (Wagner *et al.* 2017). However, this hybridization has not been further investigated to date. Recognition cues through chemical signals or aggression behavior have also been little studied in these species. *Schlick-Steiner et al. (2006)* showed that the chemical signal of *T. caespitum* differs from *T. immigrans* but did not identify which chemical compounds were implicated in these differences. Sano *et al.* (2018) combined behavioural tests and cuticular hydrocarbons assessments to investigate the use of these compounds in recognition of conspecifics *vs.* heterospecifics in a *Tetramorium* species from the USA and called *T. caespitum* in their paper. However, according to its geographical location, it is likely *T. immigrans*, as *T. caespitum* only occurs in Europe according to the literature, and because *T. immigrans* and *T. tsushimae* are the only pavement ants described so far from North America and *T. tsushimae* is morphologically distinguishable (Schlick-Steiner *et al.* 2006, Steiner *et al.* 2006, Wagner *et al.* 2017, Steiner *et al.* 2008). According to Sano *et al.* (2018), *T. immigrans* (?) responded with same levels of aggression to conspecific and heterospecific non-nestmates. They therefore suggested that *T. immigrans* (?) workers simply excluded all non-nestmate ants regardless of their species membership.

Tetramorium immigrans is particularly worth studying because it is an invasive species in North America, where it was introduced in cities in the 19th century or earlier (Steiner *et al.* 2006, 2008). It has also been found in South-America, notably in Valparaiso (Chile) from where the current lectotype originates (Wagner *et al.* 2017). Wheeler (1927) was the first to discuss the occurrence of pavement ants in North America and its possible routes of introduction into the Continent from Europe. Wheeler speculated that they came over during the colonial era (1748), but the reason why this spread in the United States was so slow until 1924 is still not understood.

To conclude, it must be noted that many issues have been neglected in these species, especially regarding their ecology. In the current context of ecological research, it is somewhat surprising that so little is known on such widespread and ecologically important species. The recent taxonomical revision of these species calls into question most of the previous findings, making crucial to bridge the gaps in the understanding of the ecology of these taxa.

1.6- THE RHÔNE - SAÔNE VALLEY: AN OPEN-AIR LABORATORY TO ASSESS GLOBAL CHANGES

The valley of the middle and lower Rhône River and of the Saône River occupy a central place in the “Grand Sud-Est”, France, formed by the administrative regions of Auvergne-Rhône-Alpes, Provence-Alpes-Cote d'Azur and the eastern part of Occitanie. This valley spans a steep North-South climatic and environmental gradient (mean annual temperature difference of 5°C over 460 km) across the boundary between the Mediterranean and Continental biogeographical regions in South-eastern France, Western Europe (Metzger *et al.* 2005, 2008). The Mediterranean/Continental biogeographical boundary is especially pronounced in this valley, with a steep latitudinal temperature gradient concurrent with marked differences in vegetation and habitats. Rueda *et al.* (2010) showed that this biogeographical boundary results from marked differences in tree, bird and butterfly communities and, to a lesser extent, in mammal and amphibian communities. It is also a classical boundary between the Mediterranean and Circumboreal second order phytoregions as defined by Takhtajan (1986).

Araújo *et al.* (2006) have shown that a great proportion of amphibian and reptile species unable to disperse are projected to reduce their distribution range in response to climate changes mainly in South-western Europe, because of the loss of suitable climate space. The Rhodanian valley is especially sensitive to these losses according to projections for 2050 (Fig. 13.). Thuiller *et al.* (2005) founded the same pattern in plant species. South-eastern France could therefore be especially sensitive to climate changes in the near future.

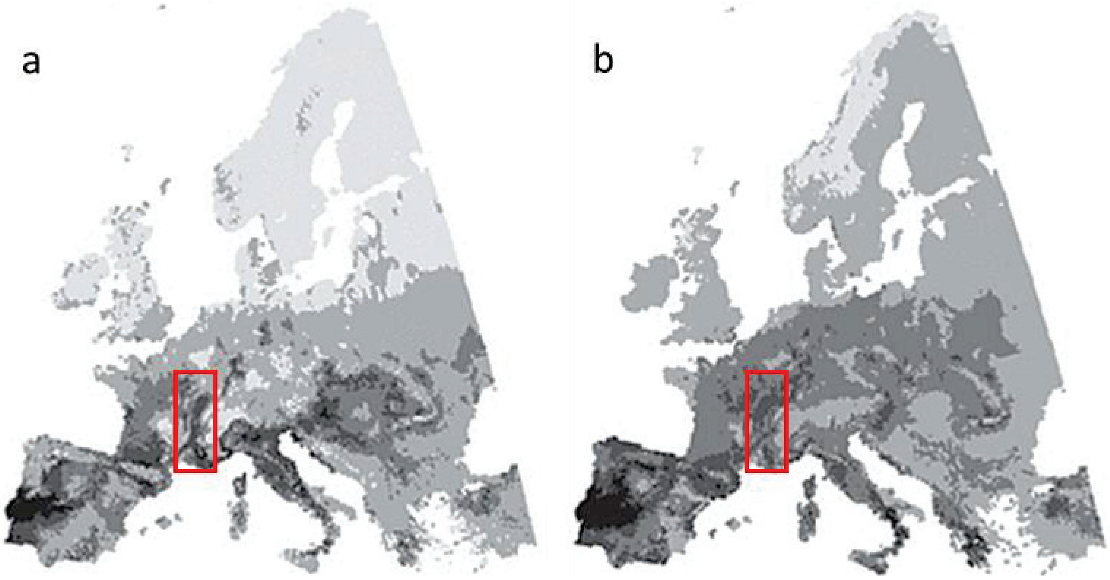

Figure 13. Projected amphibian (a) and reptiles (b) species losses by 2050 across Europe (projections with feed-forward artificial neural networks technique implemented in the climate envelope modelling implementation 'BIOMOD'; six-class scale where increasing intensities of grey represent increasing losses). Adapted from Araújo *et al.* (2006). The Rhône-Saône valley area is indicated in red.

Due to its intermediate location between glacial refugees of the Iberian, Italian and Balkan peninsulas (Hewitt 2001; Fig. 14), this valley also corresponds to a major postglacial recolonization route. Indeed, the range of many temperate species appear to have been restricted to the southern peninsulas during one or more glacial periods, allowing genetic diversity to be preserved in refuge areas. In the central and northern regions of Europe, temperate species frequently exhibit lower levels of mtDNA genetic diversity than found in southern refugia, due to rapid post-glacial recolonization and repeated founder events during interglacial periods (Hewitt 2004). The postglacial re-colonisation of Central and Northern Europe by Mediterranean species mostly followed four model patterns (Habel et al 2005): (i) the hedgehog (postglacial expansion from all three southern European differentiation centres), (ii) the bear (expansion of the western and the eastern lineage, but trapping of the Adriatic-Mediterranean lineage by the Alps), (iii) the butterfly (expansion of the Adriatic- and the Pontic-Mediterranean lineages, but trapping of the Atlantic-Mediterranean lineage by the Pyrenees), and (iv) the grasshopper (major expansion to Central Europe only from the Balkans and trapping of the Atlantic- and Adriatic-Mediterranean lineages by the Pyrenees and Alps, respectively).

These patterns have been reported in many animal and plant species, e.g., the "hedgehog" paradigm in the silver fir *Abies alba* (Konnert & Bergmann 1995), the "bear" in the shrew *Crocidura suaveolens* and the water vole *Arvicola terrestris* (Taberlet *et al.* 1998), the "grasshopper" in the black alder *Alnus glutinosa* (King & Ferris 1998); the "butterfly" is currently only described in three butterfly species (Schmitt 2007; e.g., the marbled whites *M. galathea/lachesis* complex; Habel et al 2005). In most of these patterns, recolonization routes cross western Europe through Southeastern France (Petit *et al.* 2002; Fritz *et al.* 2005), precisely in the valley of the middle and lower Rhône River and of the Saône River. As a consequence, a network of contact zones is spread over this valley as the Alps mountain often act as a dispersal barrier, inducing secondary contact between populations and species, and leading to hybridization areas in this valley (Schmitt 2007; H2 and H3 in Fig. 14).

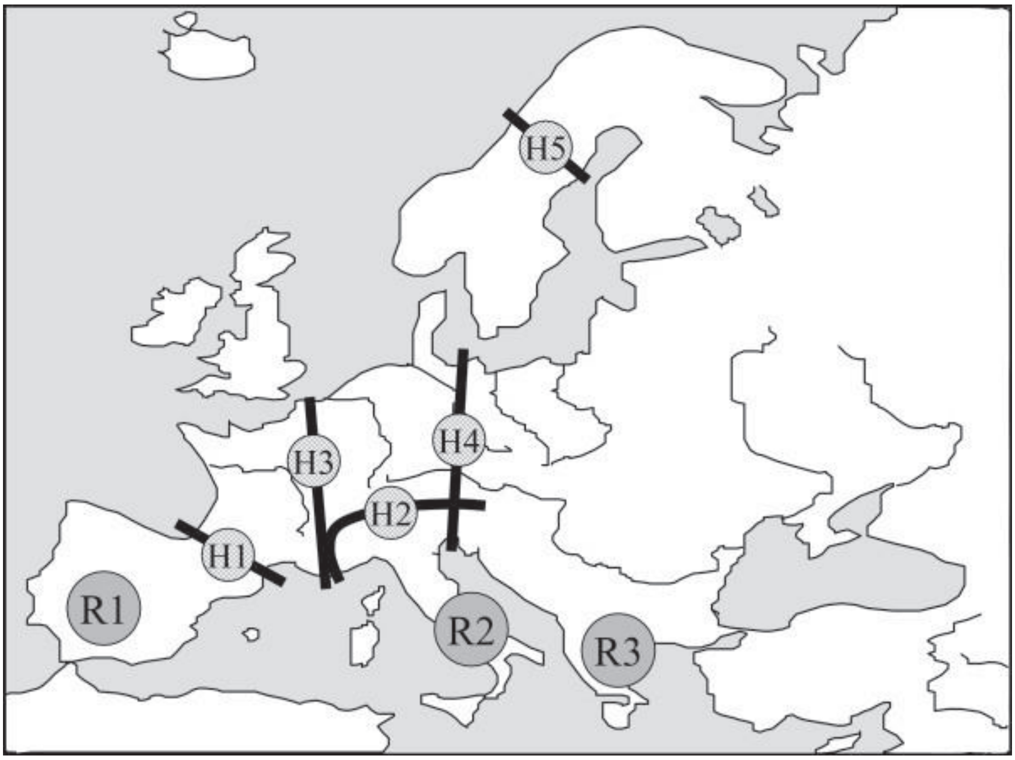

Figure 14. The three main Mediterranean refugies and differentiation centers of Southern Europe during the last Ice Age (R1-R3) and the geographical location of the five main contact and hybridization areas where different biota came into secondary contact during the post-glacial range expansion processes (H1- H5). From Schmitt (2007); based on Taberlet *et al.* (1998) and Hewitt (1999).

The Rhône-Saône valley is also a relevant area to study urbanization, as the "Grand Sud-Est" regions have a dynamic demography, with for example 35% of its inhabitants born outside this area in 1999 (Dou *et al.* 2001). The valley has been urbanized since Roman times and urbanization processes are still important today, giving birth to large and constantly expanding urban areas. Today, the constraints on urban planning associated with the river have been much reduced by various developments, giving the towns new options for urban planning (Delahaye 2004).

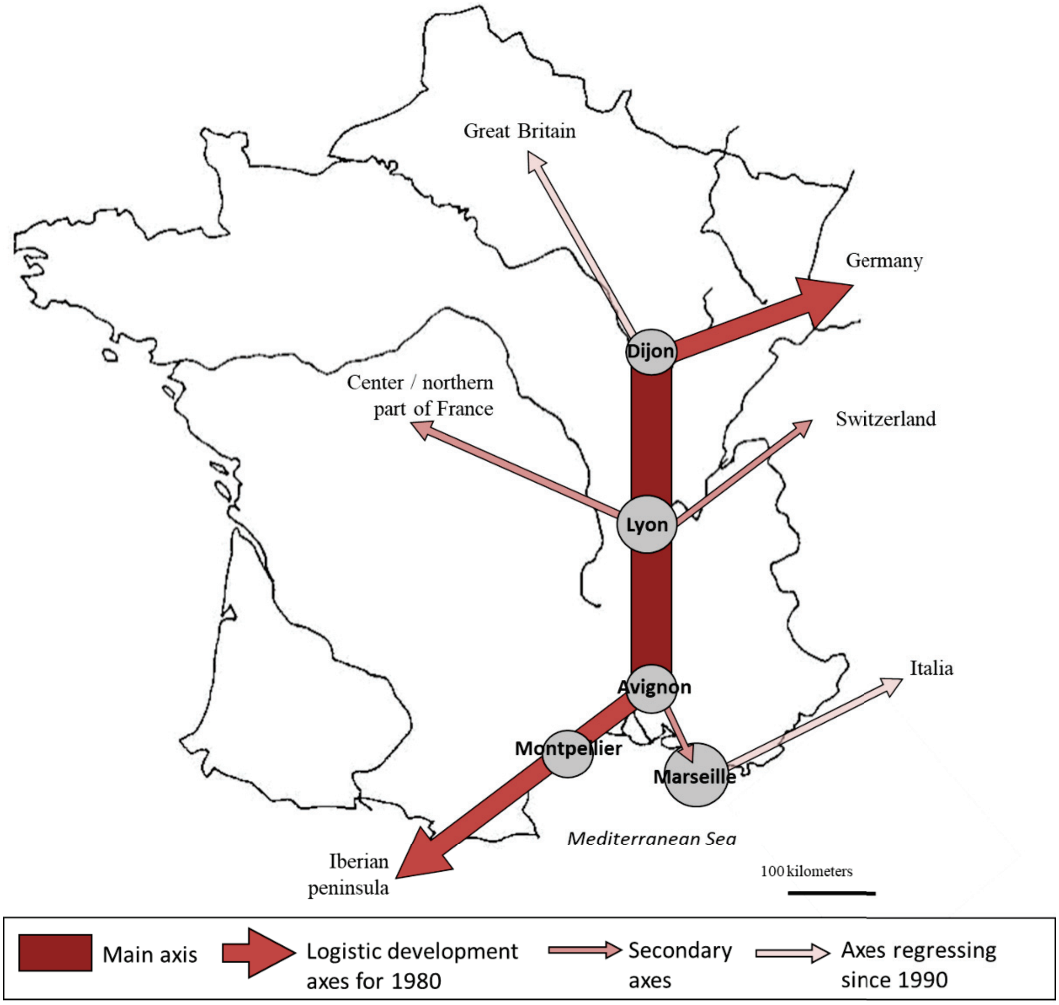

Figure 15. Transport structures and spatio-logistic dynamics in South-eastern France. Simplified from Piquant (2003; Fig. 2).

Last, the valley is located between Central European countries, Italy and the Iberian Peninsula, and is experiencing a growth in its transport and logistic activities. Over the last twenty years, trade between European countries has boomed, intensifying the flow of goods and people between Germany and the Iberian Peninsula through the Grand Sud-Est, France (Fig. 15). The Lyon area is becoming more and more central in South-eastern France and is now considered a European logistics center (Piquant 2003). Such dynamic development of transport and trade increases the probability of introducing non-native species in this area (di Castri 1989). Piquant (2003) also suggested that this dynamism still has huge room for growth and profit, and flows should further intensify in the decades to come. The Rhône-Saône valley must therefore be considered as an area of major environmental stakes, particularly with regard to the increased risk of biological invasions.

In conclusion, many issues related to global changes, biological invasions, species distribution and genetic exchange between species are of concern in this area, making it an open-air laboratory to assess ecological issues in a changing world.

1.7- THESIS OVERVIEW

1.7.1- Aims of the thesis

Interspecific hybridization is becoming more and more common around the world, favored by climate changes inducing shifts in species distribution as well as human-induced translocations of organisms and habitat modification, and therefore often associated with urbanization. Besides, hybridization may sometimes threaten the conservation of local biodiversity, for example by altering the genetic integrity of native species through introgression (Taylor *et al.* 2015). Therefore, the acceleration of climate changes and urbanization makes it utgent to study hybridization in order to understand the consequences of the erosion of reproductive barriers between distinct evolutionary lineages (Vallejo-Marín & Hiscock, 2016).

As these global changes act at multiple spatial scales on the environment, the investigation of a sufficiently large biogeographical area is of prime importance: (i) to identify relevant patterns of distribution, adaptation, and intra- and interspecific genetic exchanges, but also (ii) to address issues related to the impacts of hybridization and to the direction of gene transfer between taxa. From this perspective, the Rhône-Saône valley provides a major opportunity to further understand the relationships between biological invasions, climate, urbanization, and hybridization. Indeed, studying urbanization while integrating other global changes such as global warming should prove essential to unravel the processes involved. In addition, the *Tetramorium* ant genus, and more specifically the two species I have described above, *T. immigrans* and *T. caespitum*, seem particularly suitable for conducting such an integrative study.

In this context, my thesis has the main objective to provide an integrated perspective on the responses of biodiversity to global changes through the assessment of the relationships between climate, urbanization, biological invasions and hybridization patterns between ant species of the *Tetramorium caespitum* complex (Fig. 16).

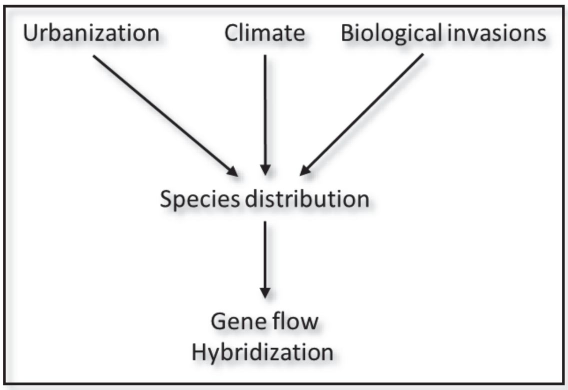

Figure 16. Schematic overview of the main objective of the thesis.

More specifically, the objectives of the thesis are:

<u>*– Regarding the identification of species and distribution patterns –*</u>

- To map the range limits of cryptic *Tetramorium* species while avoiding errors attributable to both hybridization and rarity
- To identify climatic factors limiting the distribution of *Tetramorium* species across a major biogeographical boundary
- To assess at what scales urbanization impacts *Tetramorium* species distributions

– Regarding global changes and biological invasions –

- To investigate how the interaction between climate and urbanization impacts *Tetramorium immigrans* occupancy
- To examine the role of human activity in the spread of *T. immigrans* through the study of its genetic structuration
- To gather evidence on the native or invasive status of *T. immigrans* in Western Europe

– Regarding interspecific hybridization –

- To investigate hybridization patterns between *T. immigrans* and *T. caespitum* and to detect potential introgression between them
- To determine if chemical profiles and behavioral assays reveal that species recognition cues are both present and perceived in the hybrid complex of *T. immigrans* x *T. caespitum*
- To unravel the mating system of *T. immigrans* and *T. caespitum* and to assess how it could play a role in a hybridization context through potential pre- or post-copulatory sexual selection

– And ultimately –

- To provide a synthesis of my results and to show how they bring clarity into the complex interactions between biological invasions, climate, urbanization and hybridization
- To offer short and longterm perspectives for further researches

1.7.2- Thesis outline

This thesis includes five chapter.

- Present ***Chapter 1*** is a general introduction and provides a general overview of the thesis' subjects. This introduction is followed by three research axes (Chapters 2 to 4) combining six papers, each of them using a variety of multi-disciplinary approaches, and by a general discussion of the results and perspectives of the thesis (Chapter 5).
- In ***Chapter 2***, I study *Tetramorium* ant species along a large-scale climatic gradient and 19 urban gradients in the Rhône-Saône valley (France). I investigate environmental factors that limit the distribution of these species from the local scale to the biogeographical scale. I combine genetic tools based on nuclear and mitochondrial DNA to identify *Tetramorium* ant species and give a first description of the climatic niche of four *Tetramorium* species (**Paper 1**). I further investigate how urbanization structures the distribution of these species at four nested spatial scales (**Paper 2**).
- In ***Chapter 3***, I focus on *Tetramorium immigrans*, mobilizing multidisciplinary approaches to investigate the geographical distribution, genetic diversity and structure of this species in the Rhône-Saône valley. I test the hypothesis that urban warming, through Urban Heat Island effects, may lead to biological responses similar to climate warming in rural areas and I investigate the status of *T. immigrans* in the study area (**Paper 3**).
- In ***Chapter 4***, I investigate the processes involved in establishing the hybridization zone between *Tetramorium caespitum* and *T. immigrans*. I combine approaches based on simulations with Bayesian and Maximum-likelihood genetic clustering to highlight the existence of backcrosses between hybrids and parental species, showing fertility of hybrids (**Paper 4**). I also address the issue of inter-individual recognition mechanisms, mobilizing both behavioral biology and chemical ecology approaches (**Paper 5**). Finally, I use paternity analysis techniques to test the existence of pre- or post-mating sexual selection (**Paper 6**).
- Finally, in ***Chapter 5***, I discuss results of the thesis and how the relationships between global changes, hybridization and biological invasion within the studied species are structured. I then discuss the implications of these conclusions and I offer perspectives for future researches.

1.7.3- Articles accepted, submitted or in prep.

Paper 1

Cordonnier, M., Bellec, A., Dumet, A., Escarguel, G., Kaufmann, B. Range limits in sympatric cryptic species: a case study in *Tetramorium* pavement ants (Hymenoptera: Formicidae) across a biogeographical boundary. *Insect conservation and diversity* DOI: 10.1111/icad.12316

Paper 2

Cordonnier, M., Gibert, C., Bellec, A., Kaufmann, B., Escarguel, G. Spatial scaling of the impacts of urbanization on species distribution within the genus *Tetramorium*. Submitted to *Landscape Ecology*

Paper 3

Cordonnier, M., Bellec, A., Escarguel, G., Kaufmann, B. Urbanization-climate interactions promote the expansion of species ranges: a case study in the invasive pavement ant *Tetramorium immigrans*. Submitted to *Journal of Animal Ecology*

Paper 4

Cordonnier, M., Gayet, T., Escarguel, G., Kaufmann, B. From hybridization to introgression between two closely related sympatric ant species. Submitted to *Journal of Zoological Systematics and Evolutionary Research*

Paper 5

Cordonnier, M., Mondy, N., Simon, L., Escarguel, G., Kaufmann, B. Discriminating conspecifics from heterospecifics in a hybrid zone: from behavioral cues to chemical signals. In prep for *Behavioral Ecology*

Paper 6

Cordonnier, M., Escarguel, G., Dumet, A., Kaufmann, B. Multiple mating and sexual selection in an interspecific hybridization context. In prep for *Evolution*

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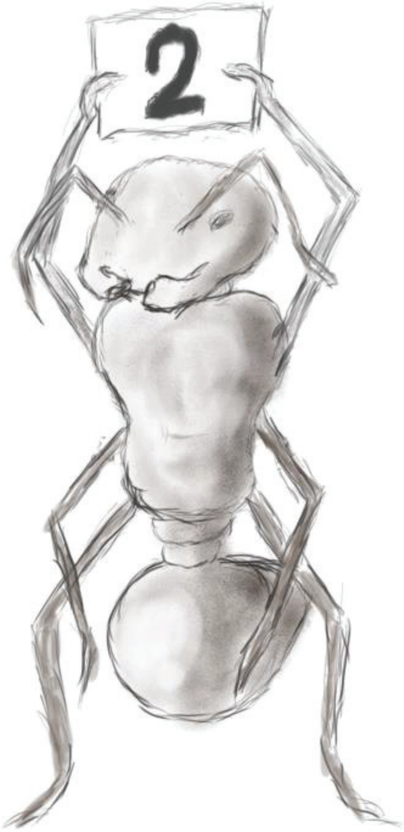

Chapter 2. Species identification and distribution patterns

« I had the great idea of using markers to gently color the ants, so I could tell them apart, but I learned that this is exactly like somebody trying to gently color on you with a thirty-story building.»

Jim Bentoncitation

PAPER 1. Range limits in sympatric cryptic species: a case study in Tetramorium pavement ants (Hymenoptera: Formicidae) across a biogeographical boundary

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Abstract

1- In the context of climate changes, factors that determine the distribution patterns of European species of the ubiquitous ant genus *Tetramorium* were investigated.

2- The study took place along a steep North-South climatic gradient across the boundary between the European Continental and Mediterranean biogeographical regions spanning 460 km along the Rhône valley, France.

3- Ants from 1690 *Tetramorium* colonies were collected at 19 sampling zones. Species have been identified using an integrative approach based on a two-step process combining nuclear DNA (14 microsatellite markers), morphological examination and mitochondrial DNA cytochrome oxidase I sequencing. The impact of climate on species distribution patterns was tested using bioclimatic variables.

4- Species discrimination was successful, despite a complex situation with cryptic species, interspecific hybridization and uneven sampling across species. Our results showed a strong effect of latitude, temperature and rainfall on the distribution of three of four species. *T. semilaeve* was found only south of the boundary, in warmer and dryer sites; *T. caespitum* extended north and 70 km south of the boundary and favoured colder sites with strong seasonal variation. *T. immigrans* was absent from the northernmost sampling zones and favoured warmer and wetter sites. *T. moravicum* was mostly found close to the boundary, but without significant climatic preferences.

5- The fundamental role of climate as a factor limiting the ranges of these species at a major biogeographical boundary is confirmed. Monitoring range limits of these strongly climate-dependant species may offer exciting insights on the impact of climate changes on species distributions.

Keywords: Bayesian clustering, Climate, Range limits, Species distribution, Species identification, Temperature gradient

INTRODUCTION

Because of global climate change, major shifts in species distributions and range limits are expected in the coming decades (Parmesan & Yohe, 2003; Chen *et al.*, 2011; Pecl *et al.*, 2017). Climate is a major determinant of the natural distribution of species, with evidence both from the fossil record (Escarguel *et al.*, 2011) and from recently observed trends (Parmesan & Yohe, 2003). It has long been known that for terrestrial organisms, temperature and precipitation are among the most important abiotic factors that may set range limits (Phillips 1860; Wiens 2011). Ectotherms such as insects are especially likely to be vulnerable to climate factors because their basic physiological functions such as locomotion, growth, and reproduction are strongly influenced by environmental temperature (Deutsch *et al.*, 2008; Kingsolver *et al*., 2013). Their range limits are therefore determined by the capacity of species to match their thermal tolerance to the temperature of their habitat (Sunday *et al.*, 2012).

Accurate data on species ranges are needed to track range shifting over time (Yalcin *et al.*, 2017) or to design conservation areas (Rondinini *et al.*, 2006). Such data are rarely available for inconspicuous taxa such as most insect species, with negative consequences for conservation planning and serious biases in meta-analyses (Maclean & Beissinger, 2017; Pironon *et al.*, 2017). Following the centre-periphery hypothesis (CPH; Pironon *et al.*, 2017), species' occupancy decreases close to range limits, leading to lower detection probabilities away from the distribution centre. This generates a major challenge in delineating range limits due to their intrinsically mobile and progressive nature (Brown *et al.*, 1996, Sexton *et al.*, 2009). To overcome this challenge, at least partially, sampling should extend deep into the expected ranges on both sides of the biogeographical boundary, as well as through a diversity of environments spanning the habitat niche of the species of interest. One further challenge for most inconspicuous species relates to taxonomy (which may also be the case for more conspicuous taxa; e.g., Cordes *et al.*, 2017). Traditionally, species have been identified using morphological traits, but such characters may undergo convergent evolution under similar selective pressure, leading to underestimating the actual number of species and failing to identify cryptic species (Yang & Rannala, 2010; Morard *et al.*, 2016). Genetic methods may therefore help support species identification (McKendrick *et al.*, 2017).

Mitochondrial cytochrome oxidase I (COI) partial sequencing is widely used in taxonomy for species identification (e.g., Schlick-Steiner *et al.*, 2006; Dinsdale *et al.*, 2010; Wilson-Wilde *et al.*, 2010). However, identification results based only on mitochondrial DNA barcodes might sometimes be biased (e.g., due to mitochondrial introgression; Whitworth *et al.*, 2007), and particularly in case of hybridization where the use of mitochondrial DNA cannot detect hybrids between taxa (McKendrick *et al.*, 2017). Wilson-Wilde *et al.*, (2010) also suggested that using COI for species identification is insufficient when dealing with closely related species. Approaches based on population assignment can palliate these problems using multilocus genotype data to identify groups of genetically isolated individuals which constitute potential species (Yang & Rannala, 2010). Overall, the use of a combination of methods seems the best way to avoid misidentification in complex situations, i.e., involving cryptic species or interspecific genetic exchanges (Rellstab *et al.*, 2011; Dantas-Torres *et al.*, 2013; Schmidt-Roach *et al.*, 2014; Wagner *et al.*, 2017).

The present study spans a steep North-South climatic and environmental gradient (mean annual temperature difference of 5°C over a distance of 460 km) across the boundary between the Mediterranean and Continental biogeographical regions in South-eastern France, Western Europe (Condé *et al.*, 2002; Metzeger *et al.,* 2005, 2008) along the middle and lower Rhône River valley and further north along the Saône River, its main tributary. The Mediterranean/Continental biogeographical boundary is especially pronounced in this valley, with a steep latitudinal temperature gradient concurrent with marked differences in vegetation and habitats. Rueda *et al.* (2010) showed that this biogeographical boundary results from marked differences in tree, bird and butterfly communities, to a lesser extent in mammal and amphibians communities, and does not correspond to a transition zone for reptilian species. It is also a classical boundary between the Mediterranean and Circumboreal second order phytoregions, as defined by Takhtajan (1986). Across this major biogeographical transition, the present study focuses on range limits of *Tetramorium* ant species, a ubiquitous genus of small generalist ants including several important invasive pests such as *T. bicarinatum* (Nylander, 1846) (Hita-Garcia & Fisher, 2011), *T. immigrans* Santschi, 1927 (Wagner *et al.*, 2017) and *T. tsushimae* Emery, 1925 (Steiner *et al.*, 2010).

T. immigrans has an invasive status in North America, where it was introduced in the 19th century or earlier (Steiner *et al.,* 2008a), and is considered native in Europe where its status has never been investigated. So far, species of *Tetramorium* have been recorded in France, including *T. semilaeve* André, 1883, *T. meridionale* Emery, 1870, two species of the *Tetramorium chefketi* complex (*T. forte* Forel, 1904, *T. moravicum* Kratochvíl, 1941)*,* and five species of the *Tetramorium caespitum* complex: *T. impurum* (Foerster, 1850), *T. alpestre* Steiner, Schlick-Steiner *et* Seifert, 2010, *T. immigrans*, *T. indocile* Santschi, 1927, and *T. caespitum* (Linnaeus, 1758) (Schlick-Steiner *et al.*, 2005; Schlick-Steiner *et al.*, 2006; Güsten *et al.,* 2006; Steiner *et al.*, 2010; Blatrix *et al.*, 2013; Borowiec *et al.*, 2015; Csösz *et al.*, 2014; Wagner *et al.*, 2017). Morphological identification can be used to discriminate *Tetramorium* species (e.g., Güsten *et al.,* 2006; Borowiec *et al.*, 2015; Wagner *et al.*, 2017), but difficulties arise within the *Tetramorium caespitum* complex where species appear very similar in colour and form (Schlick-Steiner *et al.*, 2006; Wagner *et al.*, 2017). Furthermore, hybridization is already known between two species of this complex, namely *T. alpestre* and *T. indocile* (Steiner *et al.*, 2010) and is strongly suspected between *T. immigrans* and *T. caespitum* (Wagner *et al.*, 2017), which significantly complicates species identification. As a consequence, it is necessary to use a combination of methods to identify *Tetramorium* species in France. Finally, to our knowledge, nothing was known about the fine-scale distributions of these species in the study area, even if some of them differ in ecological specificity – e.g., Wagner *et al.* (2017) indicate that the thermal niche of *T. caespitum* corresponds to an average standard air temperature of 16.1°C, 19.9°C for *T. immigrans* or 8.6°C for *T. alpestre.* At a finer scale, such niche differences may result in a strong spatial structure of these species along the study north-south climatic gradient.

On this ground, the two major questions addressed by the present study are: (i) How feasible is mapping range limits for these inconspicuous taxa including cryptic species, while avoiding errors attributable to both hybridization and rarity? (ii) How do *Tetramorium* species' range limits respond to climatic factors across a major biogeographical boundary? To address these questions, a stratified sampling scheme was used to collect individuals from 1690 *Tetramorium* ant colonies. We applied a two-step approach to identify species: (i) Bayesian clustering based on nuclear DNA (14 microsatellite markers), and (ii) species assignment of subsamples of these clusters using morphology and mitochondrial DNA (Cytochrome Oxidase I). We subsequently analyzed the range limits of the detected species using climatic data, expecting a strong climate-driven spatial structure of the different species with broad changes in species occurrences across the Mediterranean/Continental biogeographical boundary.

METHODS

Biological model, study area and sampling scheme

Tetramorium taxon concepts (sensu Packer *et al.*, 2018) used here are based on Güsten *et al.* (2006) for *T. moravicum* and *T. forte*, and on Wagner *et al.* (2017) for the *T. caespitum* complex (including *T. immigrans* and *T. caespitum*) and on Borowiec *et al.* (2015) for *T. semilaeve*. Sampling was carried out along a 460 km climatic gradient located in France, east of the Saône and Rhône Rivers, extending from the city of Langres in the North (47° 51′ 12″ N, 5° 20′ 02″ E) to the city of Tarascon in the South (43° 48′ 21″ N, 4° 39′ 37″ E). The gradient extends over the limit between two major European biogeographical regions (Mediterranean and Continental, Condé *et al.*, 2002), which is located ca. 200 km North of Tarascon and 260 km South of Langres (Fig. 1). The Mediterranean region is characterized by cool winters, warm dry summers, and wet autumns. The Continental region has cold winters, warm dry summers and rainfall spread over most of the year.

We collected at least 30 worker ants per colony sample from altitudes between 0 and 470 m a.s.l. in 19 sampling zones which were at least 20 km apart and comprised cities with their adjoining peri-urban and rural areas (Fig. 1). In order to capture habitat heterogeneity, we collected 30 colony samples distributed homogeneously throughout contiguous urbanized areas (as defined by the classes 1.1.1, 1.1.2 and 1.2.1 in Corine Land Cover 2012) and 60 colony samples distributed homogeneously along a 15 km long and 3 km wide transect to the east of the urbanized areas. The 1:100,000 scale of this land cover data, which lumps small agricultural and semi-natural patches with artificialized surfaces, is not designed to allow analyses of the impact of urban gradients on species distribution. Corine Land Cover data was used here to ensure that a representative diversity of habitats were sampled in the study. Samples were collected in diverse environments including urban pavements, roadsides, public parks, orchards, farmlands, fields, vineyards, meadows, riverbanks and forest paths. Sampling was conducted along a predefined path in 2015 and 2016 from April to September on non-rainy days with temperatures ranging from 16 to 28°C, with a minimum distance of

200 m between two colonies. Sampling was performed by teams of two persons conducting a direct search for *Tetramorium* nests followed by hand collection with an entomological aspirator. Collected ants were stored in 96% ethanol. Individuals from 1710 colonies were collected for this study and deposited as voucher material in the collection UCBLZ, CERESE, Université de Lyon, Université Claude Bernard Lyon1.

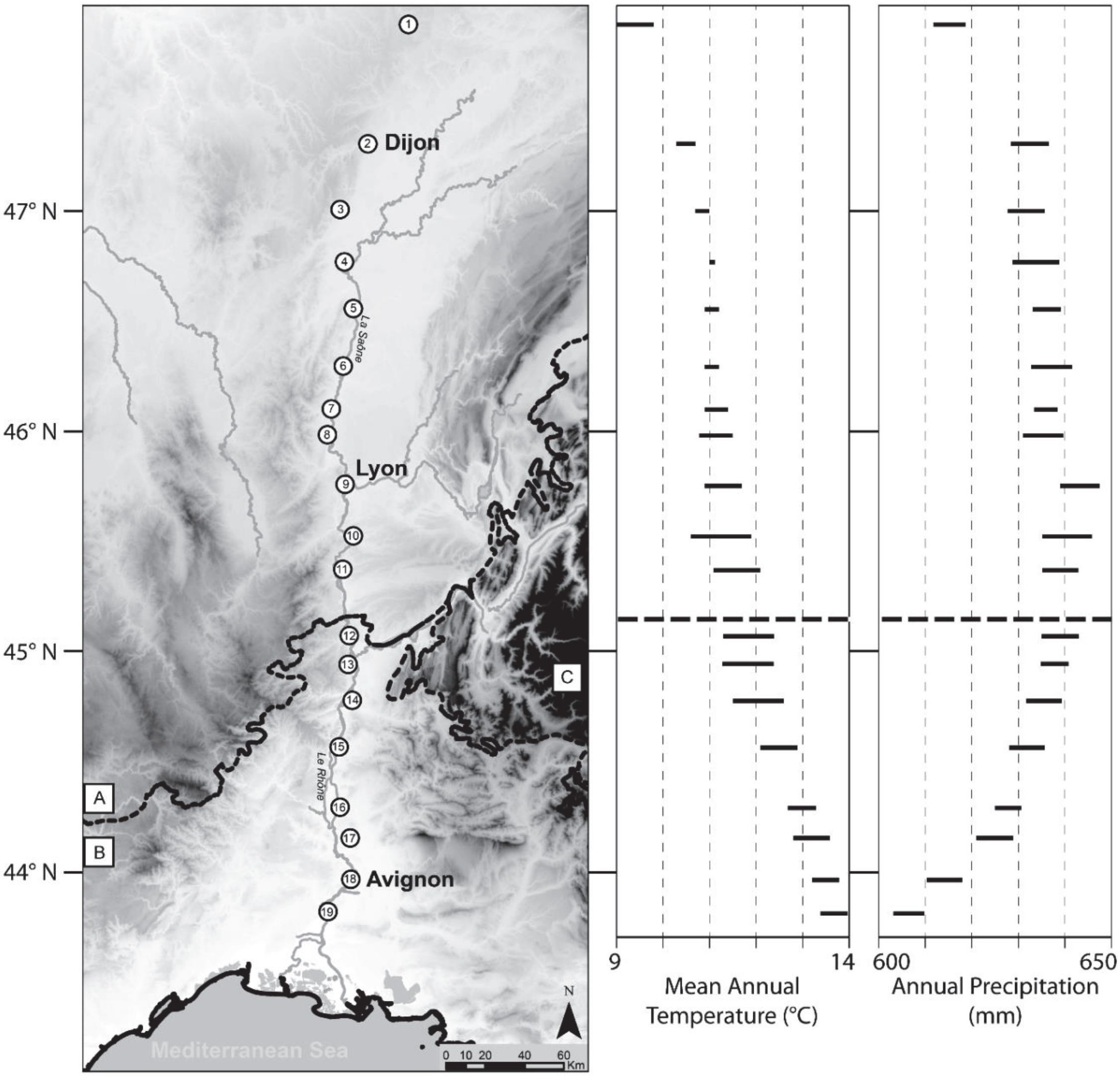

Figure 1. Map of the sampling zones (90 samples from each zone). 1: Langres, 2: Dijon, 3: Beaune, 4: Châlon-sur-Saône, 5: Tournus, 6: Mâcon, 7: Belleville, 8: Villefranche-sur-Saône, 9: Lyon, 10: Vienne, 11: Péage-de-Roussillon, 12: Tournon sur Rhône, 13: Valence, 14: Livron, 15: Montélimar, 16: Bollène, 17: Orange, 18: Avignon, 19: Tarascon. Main rivers are indicated in dark grey, altitude is indicated by grayscale (black = high altitude). The boundaries of the three major climatic zones are indicated by a dotted line. A: Continental zone, B: Mediterranean zone and C: Alpine zone. Mean annual temperature (°C) and mean precipitation (mm) as well as the associated variances are indicated on the right for each sampling zone.

Microsatellite genotyping

One randomly selected individual per colony was used for genetic analyses. DNA was extracted from whole individuals, which were crushed and then mixed with 150 µL of Chelex® 100 and 10 µL of proteinase K (15 mg.mL^{-1}) at room temperature; the solution was incubated at 55°C overnight (Casquet *et al.*, 2012). For genotyping, 17 microsatellites developed for *T. immigrans* or *T. tsushimae* by Steiner *et al.* (2008b) were organized in three multiplex PCR mixes. All three mixes had a total volume of 10 µL with 1X MasterMix (kit type-it microsatellite PCR Qiagen n°206246) and 2 µL DNA. Mix1 contained 0.08 µM of tspE53a primers, and 0.2 µM of tspE52b, tspE52d, tspE52k, ttsU55a and ttsU56d primers. Mix 2 contained 0.15 µM of tspE51oR3 primers, 0.2 µM of tspE51a, tspE51b, tspE51d, ttsU58i and ttsU59j primers, and 0.3 µM of tspE51i primers. Mix 3 contained 0.08 µM of ttsU54e, 0.15 µM of tspE52a, and 0.2 µM of tspE53b and ttsU57l. Amplifications consisted in 5 min at 95°C, then 32 cycles (30s at 95°C, 90s at 60°C, 30s at 72°C), and 30 min at 60°C. All PCR products were analyzed with an ABI 3730xl sequencer (service provided by GENOSCREEN). Electropherograms were read and interpreted with Genemarker 1.95 (SoftGenetics). Three markers were discarded because they presented too many disparities in allele sizes and risks of misinterpretation at the reading stage. In addition, samples where alleles were not clearly legible for at least 12 markers were removed from the analysis, resulting in 1690 genotypes for 14 markers. For each microsatellite marker we computed null allele frequency, Fis (GENEPOP v. 4.2; Rousset, 2008), deviation from Hardy-Weinberg equilibrium, observed and expected heterozygosity and number of alleles and effectives alleles (GENALEX v.6; Peakall & Smouse, 2006) (Table S1).

Identification of clusters

To determine the number of genetically homogeneous groups using microsatellite data, the Bayesian clustering algorithm implemented in the software STRUCTURE v. 2.3.1 (Pritchard *et al.*, 2000) was used, based on the admixture model with correlated allele frequencies and the LOCPRIOR model with prior location of samples (Hubisz *et al.*, 2009), and with a number of a priori unknown clusters (K) varying from K = 1 to K = 20, running 10 iterations for each K-value. Each run consisted of 500,000 replicates of the MCMC after a burn-in of 500,000 replicates.

To investigate the 10 independent runs, clustering results were analyzed using CLUMPAK (Kopelman *et al.*, 2015) based on a Markov clustering algorithm which identifies sets of highly similar runs grouped together in modes and separating these distinct groups of runs to generate a consensus solution for each distinct mode. For any given K, the different runs were either consensual with a single mode or resulting in both a majority mode consisting of most of the iterations and one or more minority modes consisting of the remaining iterations. Next, we used CLUMPAK to identify an optimal ordering of inferred clusters across different values of K, and then to define the optimal K-value using the method of Evanno *et al.*, (2005). Finally, we calculated the mean of the logarithm of the data probability and associated per K variance using Structure Harvester (Earl & vonHoldt, 2012). We then retained the most conservative value of K compatible with these different criteria – concordance between runs, Evanno *et al.*'s (2005) method, and mean lnP(K). Based on the consensus solution of the majority mode, we obtained K distinct *Q-values* for each individual corresponding to their membership coefficient for each cluster.

Identification of species by Sanger sequencing of mtDNA and morphological examination

For each of the K clusters identified by genotyping, 10 individuals with a *Q-value* higher than 0.95 were Sanger sequenced for a stretch of mitochondrial gene cytochrome oxidase I (mtDNA). COI was amplified by PCR using specific primers developed from longer stretches of COI from the literature (Schlick-Steiner *et al.*, 2006; using two sequences: Tetra_F: TAGCATCTAATRTCTTTCAYAGAGG, Tetra_R: AGTATCAGGATAATCTGAGTAYCGAC) in a 30 µL total volume of 170 µM dNTPs, 0.1 µg.µL^{-1} BSA (Biolabs, B9001S), 0.16 µM of primers, 1.5 mM MgCl2, 2 µL DNA, 1.2 U Taq Polymerase (Eurobio, GAETAQ00), and 1X PCR Buffer (Eurobio, GAETAQ00). Amplifications consisted in 5 min at 94°C, then 40 cycles (30 s at 94°C, 30 s at 48°C, 30 s at 72°C), and 5 min at 72°C. After purification, products were sequenced (service provided by BIOFIDAL, ABI 3730xl sequencer) and compared to reference sequences published by Schlick-Steiner *et al.* (2005), Steiner *et al.* (2005), Schlick-Steiner *et al.* (2006) (accession numbers available in Fig. S3), and obtained from GenBank using Blast-n to identify the sequenced species.

For each cluster identified by genotyping, 10 colonies containing individuals with a *Q*-value higher than 0.95 were identified to the species level by morphological examination following Seifert (2007) and Güsten *et al.* (2006) to confirm genetic identification. Only individuals belonging to species outside the *T. caespitum* complex (i.e., *T. forte*, *T. moravicum*, and *T. semilaeve*) were identified at the species level; individuals belonging to *T. immigrans* and *T. caespitum* were identified as members of the *T. caespitum* species complex.

Individual species assignment

Clusters corresponding to sub-populations of the same species were grouped together. An individual was assigned to a species if its *Q*-value was greater than 0.95. Individuals for which no status could be determined (i.e., without any *Q*-value > 0.95) were considered unidentified individuals, interspecific hybrids or otherwise unidentified species and therefore removed for subsequent analyses.

Analysis of geographical and climatic ranges

All analyses were conducted using R v. 3.3 software (R Development Core Team, 2004). To understand how climate affects the regional-scale distribution of species, we first investigated interspecific differences in latitudinal distributions. As latitudinal locations of samples are not normally distributed we used nonparametric Kruskal-Wallis tests coupled with Mann-Whitney-Wilcoxon tests and Kolmogorov-Smirnov tests for contrasts (including a simple Bonferroni correction).

We next investigated the effect of temperature and precipitations on the presence of species. For each colony sample, climatic data of the sample site were extracted with ArcGIS 10.1, using the WorldClim 1.4 dataset at 30’ cell size (~1 km^2; www.worldclim.org; Hijmans *et al.*, 2005). To establish a set of uncorrelated climatic variables for the analysed geographic area, we ran an exploratory data analysis and a collinearity analysis, eliminating one of the variables in every pair with a Pearson correlation value > 0.7 (Dormann *et al.*, 2013; Fig. S1).

The final data set included mean annual temperature (bio01; mean = 11.78 ± 1.17°C, range: 9-14°C), seasonality of temperature (bio 04; mean = 6318.71, range: 6033-6476), annual precipitation (bio12; mean = 787.21 ± 51.71 mm, range: 670-898 mm) and precipitation of the coldest quarter (bio19; mean = 179.56 ± 17.29 mm, range: 146-242 mm).

We then used partial models to describe the impacts of climatic factors on the presence of each species using a two-step process:

- First, bioclimatic covariates were log-transformed and scaled to reduce the influence of extreme values and improve model convergence as suggested by Diez and Pulliam (2007). We used logistic linear mixed models treating species presence (presence = 1; absence = 0) as a repeated measurement of species occurrence. Sample sites (n = 19) were introduced in the model as a random effect. The bioclimatic variables (bio1, bio4, bio12 and bio19) and the interaction between mean annual temperature and annual precipitation (bio1:bio12) were introduced as explanatory terms in the fixed part of the model. This model was then used in a model selection procedure using the *dredge* function (R v.3.3 package MuMin; Bartoń 2013), performing automated model selection with subsets of the supplied initial model generated with all possible combinations. The sample-size corrected version of the Akaike information criterion (AICc) was used to rank the models and to obtain model weights;

- Finally, the simplest model having a $\Delta_{\mathrm{AICc}} < 2$ was retained and used for parameter estimation. The significance of each explanatory term was tested using a Wald test (Luke, 2017) and looking at confidence intervals on the estimates. Homoscedasticity, independence and normality of residues were checked for each model (package *statmod*; Giner and Smyth, 2016). The absence of spatial autocorrelation in the residuals of the final models has been assessed based on Moran's I values and associated *p values* for each model using the *ncf* package (Bjornstad, 2018).

RESULTS

The Bayesian clustering analysis revealed consistent modes for models with K = 1, 2, 6, 7, 13 and 18 clusters (major mode recovered in at least 9/10 runs). Although methods based on ΔK suggested retaining K = 2 clusters (ΔK = 112.698), K = 6 clusters also appeared suitable (ΔK = 28.912, next max ΔK obtained for K = 3: ΔK = 1.930). Based on mean lnP(K), K = 6 was markedly better than any other K value with a lower associated deviation (mean lnP(K) = -121,453.380, Stdev LnP(K) = 64.132). All these metrics can be consulted in Table S2 for all K values tested. A 6-cluster model appeared substantially better regarding the different metrics as well as consistency between iterations.

Analysis of the 57 COI sequences (length 472 to 756 bp; GenBank accession numbers MH398246 to MH398302) as well as morphological examination suggest that the K = 6 clusters correspond to 5 distinct *Tetramorium* species, identified as *T. forte* (n = 8 sampled specimens), *T. moravicum* (n = 60) and *T. semilaeve* (n = 95), and genetically identified as *T. caespitum* (n = 698) or *T. immigrans* (n = 544, separated into two clusters whose nature and characteristics will necessitate further analyses) (Fig. 2, Table S3, Fig. S2). Individuals unassigned to a cluster after Bayesian clustering (n = 285; simultaneously associated to several groups in Fig. 2a, including 240 individuals whose *Q-values* were mixed between *T. immigrans* and *T. caespitum*) were considered hybrids or belonging to unidentified species. Therefore, these individuals were removed for subsequent analyses, as the precise identity of these specimens is beyond the scope of this paper and will be the subject of further study (Cordonnier *et al.*, ongoing work).

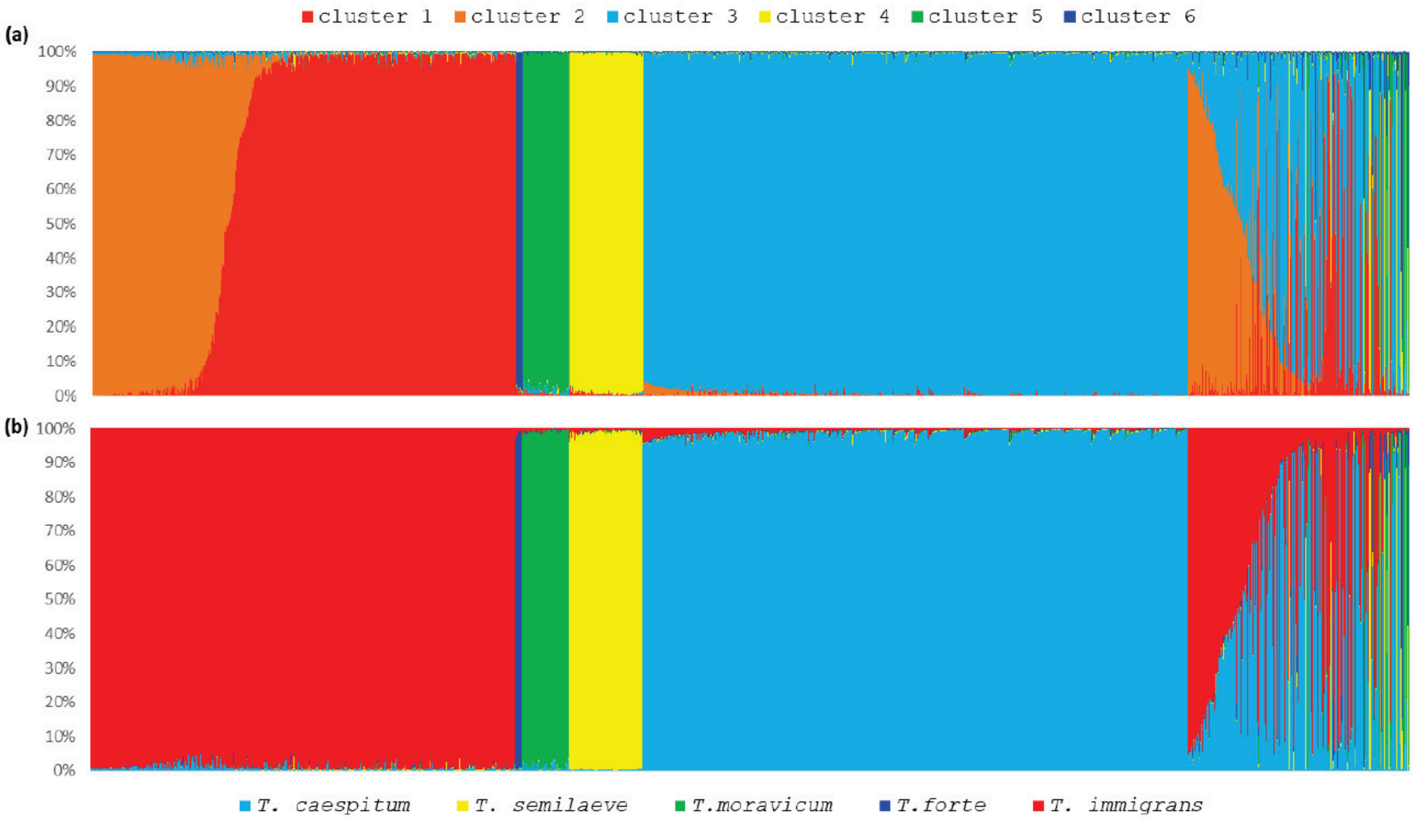

Figure 2. Structure bar plots. **(**a) Each individual is represented by a vertical line, which is partitioned into k coloured segments that represent each individual's estimated membership fractions in k clusters (*Q*-values) from the consensus solution of the majority mode for the K = 6 bayesian clustering assignment. **(**b) The five colored segments represent each individual's estimated membership fractions in five species identified by Sanger sequencing and morphological examination of 10 randomly selected individuals per cluster. The boxed area includes 285 individuals (16.9%) for whom identification has not been conclusive of a definite cluster and who have been omitted from subsequent analyzes.

In the study area, the ranges of the identified *Tetramorium* species showed a strong effect of latitude (Fig. 3). *T. caespitum* was found in the northernmost parts of the Continental area (sampling zones 1 and 2 in Fig. 1 and Fig. 3), and was absent from southernmost parts of the Mediterranean area (sampling zones 16 to 19 in Fig. 1 and Fig. 3). *T. semilaeve* occurred in the Mediterranean area only (sampling zones 15 to 19 in Fig. 1 and Fig. 3) and was completely absent from the Continental area. *T. forte* was restricted to the southernmost part of the Mediterranean area (sampling zones 17 to 19 in Fig. 1 and Fig. 3). *T. immigrans* appeared widely distributed, missing only from the 3 northernmost sampling zones (1 to 3 in Fig. 1 and Fig. 3).

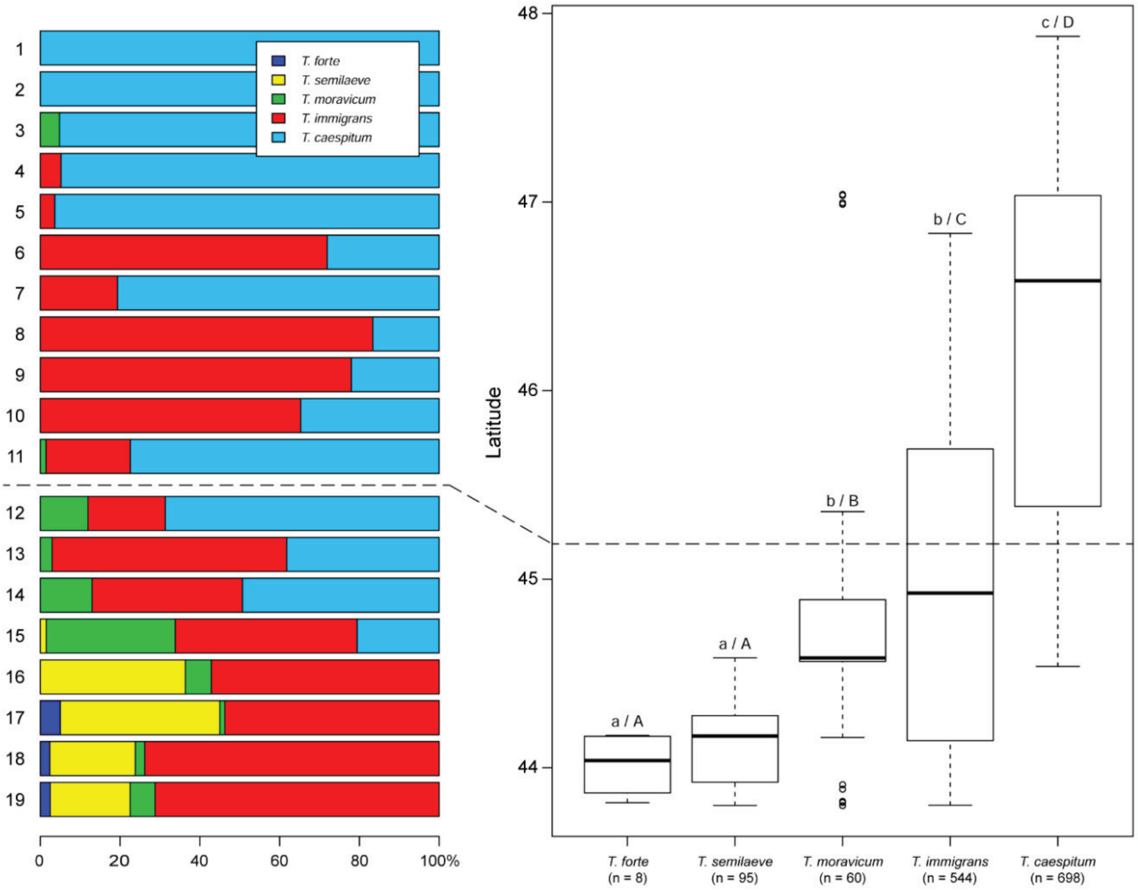

Figure 3: Proportion of each species in each sampling zone (left). Latitudinal distributions of the five species (right). White dots are outlier individuals; thick black horizontal line: median value; box ends: upper and lower quartiles; whiskers: max and min values. Horizontal dashed lines represent the limit between continental and Mediterranean areas (A-B limit in Fig. 1). Letters a, b, c indicate the results of the nonparametric Mann-Whitney-Wilcoxon tests; Letters A, B, C, D indicate the results of the nonparametric Kolmogorov-Smirnov tests (different letters when the difference of impervious area between the taxa is significant at 0.05 Bonferroni-corrected level, all tests in Tab S5).

T. moravicum was more largely distributed than *T. immigrans* (sampling zones 3 to 19 in Fig. 1 and Fig. 3) but most occurrences were found in sampling zone 15 (Fig. 1 and Fig. 3), close to the boundary between the Mediterranean and Continental areas.

Table 1: Logistic linear mixed models resulting from the model selection procedure (see text for details). For each tested species and parameter, estimate corresponds to the estimated parameter value, z and P correspond to the associated statistical test, and C.I corresponds to the 95% confidence interval around this parameter (bio1: mean annual temperature, bio4: seasonality of temperature, bio12: annual precipitation, bio19: precipitation of the coldest quarter). The parameter bio1: bio12 corresponds to the interaction between the variables bio1 and bio12. All the variables used in the models were previously log-transformed and scaled.

Parameter		*T. immigrans*	*T. caespitum*	*T. semilaeve*	*T. moravicum*
bio1	estimate	3.516	-3.508	3.773	---
	z	7.995	-7.547	0.6803	---
	P	$1.29*10^{-15}$	$4.44*10^{-14}$	$2.93*10^{-08}$	---
	CI	2.684; 4.4379	-4.506; -2.611	2.654; 5.434	---
bio4	estimate	---	1.385	-11.184	-0.712
	z	---	3.066	-4.736	-1.401
	P	---	0.0022	$2.18*10^{-06}$	0.161
	CI	---	0.507; 2.317	16.263; -6.971	-1.900; 0.208
bio12	estimate	1.662	---	-1.588	---
	z	3.612	---	-2.608	---
	P	0.0003	---	0.0091	---
	CI	0.723; 2.608	---	-2.824; -0.425	---
bio19	estimate	-2.866	1.122	7.149	---
	z	-7.165	3.975	2.467	---
	P	$7.77*10^{-13}$	$7.04*10^{-05}$	0.0136	---
	CI	-3.690; -2.076	0.550; 1.687	1.632; 13.073	---
bio1:bio12	estimate	---	---	6.576	---
	z	---	---	6.561	---
	P	---	---	$5.34*10^{-11}$	---
	CI	---	---	4.757; 8.705	---

Regarding relationships between species presence and quantitative bioclimatic variables within the study area, the selection model process resulted in different optimal logistic linear mixed models for each species (Table 1, Table S4). These models showed that compared to other species, *T. semilaeve* was present in sites with higher mean annual temperature (bio1; $z = 0.6803$, $P = 2.93\times10^{-8}$) and lower levels of changes in temperatures over the course of the year (bio4: $z = -4.736$, $P = 2.18\times10^{-6}$). It was present in dryer sites (bio12: $z = -2.608$, $P = 0.0091$) but with high level of precipitations during the coldest quarter (bio19: $z = 2.467$, $P = 0.0136$), and occurred in warmer sites where annual rainfall is higher (bio1:bio12; z = 6.561; P = 5.34×10^{-11}). Compared to other species, *T. immigrans* was present in sites where mean annual temperature is high (bio1; z = 7.995, P = 1.29×10^{-15}). Sites with *T. immigrans* received significantly more annual rainfall (bio12; z = 3.612, P = 0.0003) but lower precipitations during the coldest quarter (bio19; z = -7.165, P = 7.77×10^{-13}) than other species. *T. caespitum* was present in sites where mean annual temperature is lower (bio1; z = -7.547, P = 4.44×10^{-14}), with high levels of changes in temperatures over the course of the year (bio4: z = 3.066, P = 0.0022) and high level of precipitations during the coldest quarter (bio19: z = 3.975, P = 7.04×10^{-5}). The distribution of *T. moravicum* was not affected significantly by any climatic variable in the study area. *T. forte* was not analyzed here as only 8 samples were collected. The results for all species and climatic variables are summarized in Tab. 2.1.

DISCUSSION

This study investigated range limits over a climatic gradient for a group of inconspicuous species including cryptic taxa, avoiding errors attributable to both hybridization and rarity. We identified five *Tetramorium* species in the Saône and Rhône valleys: *T. forte*, *T. moravicum*, *T. semilaeve*, *T. immigrans*, and *T. caespitum*. The spatial distributions of the last three were strongly correlated to climatic conditions. Most species had a distribution whose limits corresponded with the biogeographical boundary, except for *T. immigrans* whose distribution covered both the Continental and Mediterranean parts of the study area. *T. caespitum* was mostly found North of the boundary. *T. semilaeve*, *T. forte* were found South of the biogeographic boundary. *T. moravicum* was found mostly close to the boundary, with few locations further North.

The present study is an example of an effective and relevant approach to discriminating species within a group with interspecific hybridization and cryptic species (Schlick-Steiner *et al.*, 2006; Wagner *et al.*, 2017). Each species detected was represented by at least 60 individuals (except *T. forte* with only 8 individuals). Three further species have been identified close to the study area but have not been detected here: *T. alpestre* is a high-altitude species (above 900 m a.s.l.) in the sampled latitudes (Steiner *at al*. 2010; Wagner *et al.*, 2017); *T. indocile* is described as generally rare in Southern and Western Europe (Csösz *et al.*, 2014) but has been found in the Swiss Alps as well as in western France (Wagner *et al.*, 2017); *T. impurum* was found from Greece to Spain, and as far north as Belgium, with widely differing annual average temperatures (Wagner *et al.*, 2017). These species are most likely absent from the study area, or at least very rare and therefore undetected.

Regarding the Bayesian clustering approach used in the two-step procedure of species identification, the method used to select the number of clusters was efficient at discriminating species where taxonomy is clear and avoids some of the problems inherent to selecting the value of K that best fits the data. A simple hierarchical structure is often assumed, most of the time with individuals clustered within populations and populations clustered within geographical regions, but the different levels are not necessarily hierarchically structured: a species may be structured into geographical regions, but also into ecotypes across regions (Meirmans 2015). Therefore, Meirmans (2015) suggested discussing all clustering results that warrant a biological interpretation because clustering analysis is an exploratory analysis with interpretations at multiple levels. In this study, considering all K values between 1 and 20 helped avoid missing biologically relevant structures. This is all the more appropriate here as the clusters are of unequal sizes, a situation where it is more difficult to accurately estimate K across an existing population structure (Puechmaille 2016). Finally, a problem commonly raised in the literature is the overrepresentation of the $K = 2$ situation when using ΔK approaches (Gilbert 2016; Janes *et al.*, 2017). These points all emphasize the need for validation of clustering results through multiple decision criteria, using comparisons of outcomes obtained from different methods to improve confidence in the results, and to use a conservative approach based on biological assumptions. The multi-criterion approach proposed here is both efficient and able to solve the problems associated with $K = 2$ and cluster size heterogeneity; it proved particularly appropriate in a taxonomically complex situation including hidden biodiversity.

As expected, the *Tetramorium* species distributions in the study area correlate to latitude, except for *T. moravicum*. We therefore investigated which climatic factors were associated with these distributions. Species from the *T. caespitum* complex have already been the subject of a study of thermal niches, but only standard air temperature (i.e., altitude-corrected mean air temperature of the nearest three meteorological stations from May, 1st to August, 31st) was considered (Wagner et al., 2017), except for *T. immigrans* whose climatic niche had been previously studied using climatic variables, but without a detailed analysis of individual variables (Steiner *et al.*, 2008). The present results suggest that *T. immigrans* favors warm environments, as also suggested by Wagner *et al.*, (2017), and requires high levels of precipitation, especially during its activity period as it may tolerate a dry period during the coldest months corresponding to the winter phase of inactivity. Conversely, *T. caespitum* was found in predominantly cold sites of the study area, with significant seasonal temperature variations and wet conditions in winter. *T. semilaeve* was found in warmer environments with little temperature variations, overall dry but with important winter precipitations. *T. moravicum* seemed to be a ubiquitous species, with no significant climatic driver. For *T. forte*, the small number of samples was insufficient to investigate climatic preferences.

Other factors not considered in this study may also be influential. At the habitat level, temperature and precipitation differences between urban areas and their rural surroundings (i.e., urban heat island effects) are certainly the more pertinent example. The impact of urbanization on climate is locally comparable to global climate change signal, suggesting that urbanization could strongly enhance climate change at local scales (Argüeso *et al.*, 2014). Specifically, ectotherms from warmer, urban environments should not withstand colder temperatures and should tolerate heat better than ectotherms from cooler, rural environments (McLean *et al.*, 2005). Indeed, in the set of *Tetramorium* species studied here, *T. immigrans* has been found to favor urban parts whereas *T. caespitum* has mainly been found in rural areas (Gippet *et al.*, 2017); here we show that these species prefer warmer and cooler environments along the latitudinal gradient, respectively. Most of the species common in urbanized areas have more southern distributions and greater tolerance to dry conditions and warmer temperatures than species found in more natural sites (Menke *et al.*, 2011), which corroborates our results regarding the latitudinal distributions and climatic preferences of *T. immigrans* and *T. caespitum*. It would be worth knowing if minimal or maximal temperatures limit the distributions of these species, as tolerance to maximal temperatures is often highlighted but sometimes minimal temperatures are actually more relevant (Warren and Chick, 2013). Finally, it could also be important to consider the tolerance to dry conditions of these species, as *T. immigrans* seems to favor wetter conditions than other species.

At the colony level, the location and construction of nests play a key role in regulating temperature and humidity (Hölldobler & Wilson, 1990; Blüthgen & Feldhaar, 2010). For example, nests can provide a thermal refuge in hot environments, allowing workers to retreat into a cool nest in the hottest part of the day (Ward 2007). In the detected species, *T. caespitum* is the only species known to build mounds higher than 10 cm, which give it access to increased spring insulation raising nest temperatures (Wagner *et al.*, 2017). Soil hygrometry and granulometry, as well as plant cover, could also strongly affect species distributions, even at a regional scale (Seifert, 2017). In addition, local biotic interactions (e.g. competition, predation, host-parasite interaction or mutualism) may affect species distributions and realized species assemblages across spatial scales (Wisz *et al.*, 2013), including at macroecological scales (Araújo & Luoto, 2007). For example, competition between species at the same trophic level can affect both range limits and geographic diversity patterns (Wisz *et al.*, 2013). Competitive interactions are common between ant species and can alleviate spatial habitat partitioning (Parr & Gibb, 2010). Therefore, while Lessard *et al.*, (2012) have shown that climate and biogeographical history are more important than competition in shaping ant communities, the role of competition in structuring ant assemblages should not be neglected (Cerda *et al.*, 2013) and should be taken explicitly into account in future studies.

CONCLUSION

This study used an efficient method to discriminate species in a complex admixture situation including interspecific hybridization and cryptic biodiversity, while dealing with problems such as hierarchical cluster structuring due to uneven sampling across species. The identified species presented distinct range limits suggesting a strong effect of temperature and rainfall in distribution patterns, mostly following the Mediterranean-Continental biogeographical boundary. These results highlight the fundamental role of climate as a factor limiting the species ranges at a well-known biogeographical limit. On that ground, monitoring the distribution ranges of these species should offer exciting insights into the impact of ongoing climate changes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article

Data S1. Genotypes table

Data S2. Tables S1 to S5 and Figures S1 and S2

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PAPER 2. Spatial scaling of the impacts of urbanization on species distribution within the genus *Tetramorium*

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Abstract

Context

Urbanization is a global change which deeply impacts landscapes. Long studied through transects along urbanization gradients, ecological response to urbanization can now be investigated precisely using direct GIS-based measures. Ecological responses are strongly scale-dependent as both large- and fine-scale environments drive the species distribution.

Objectives

To bridge the gaps regarding the effect of scaling on the distribution of biodiversity in the context of urbanization, the present study questions how urbanization structures the distribution of *Tetramorium* ants at different spatial scales.

Methods

Based on model averaging procedures, we investigated ~1400 individuals belonging to four *Tetramorium* species at four distinct spatial scales, from urban microhabitat (1 meter around the nest) to urban landscape (500 meter around the nest) in 19 urban gradients in South-eastern France.

Results

The probabilities of occurrences of *Tetramorium caespitum and T. immigrans* simultaneously depended on urbanization at the landscape and local scales, with *T. caespitum* avoiding urban microhabitats and impervious landscapes whereas *T. immigrans* favored them. These scaling impacts of urbanization were species-dependent as *T. moravicum* was associated with nonurban landscapes only, and *T. semilaeve* showed no association whatever the tested variables.

Conclusions

These results highlight the importance of considering several spatial scales simultaneously to study the impact of urbanization on species distribution. The highly contrasted responses to urbanization of *T. immigrans* and *T. caespitum* may indicate niche partitioning processes driven by urbanization. Monitoring the distribution range dynamics of these two species should provide insightful information into the impact of urbanized landscapes on species distribution.

Keywords: Landscape, Microhabitat, Spatial scaling, Species distribution, *Tetramorium*, Urbanization

INTRODUCTION

Urbanization is a global change of high magnitude and speed, which deeply impacts biodiversity (Grimm et al. 2008). Urban areas are characterized by a high density of built areas, as well as by broader scale changes such as the creation of vast networks of transport infrastructure or the intensification of agricultural activities on their periphery. Urbanization thereby converts natural and rural landscapes into profoundly modified habitats combining artificial surfaces, industrial pollution, anthropogenic disturbance, and strongly altered energy and nutrient cycles (Seress et al. 2014). Kark et al. (2007) identified traits in urban birds that enable species to dominate highly urbanized environments by becoming urban exploiters (i.e., species that colonize highly urbanized environments using human-provided resources and shelters; McKinney 2006), and suggested that ecological success in urbanized environments may depend on a combination of traits including diet, degree of sociality, sedentariness and preference in nesting sites. Marzluff et al. (2001) found that with increasing urbanization, bird species richness and evenness decreased whereas density increased. They also evidenced an upward trend in the proportion of non-native species (i.e., species that did not occur before importation by humans) toward the urban core. Numerous studies further show that the construction and expansion of cities promote the loss of native species and their replacement by non-native species (McKinney 2002, 2006), and favor generalist non-native and native species (Müller et al. 2013).

The gradient approach proposed by McDonnell & Picket (1990) provides a useful tool for untangling complex urban dynamics (McDonnell & Hahs 2008). However, transitions within urban gradients diverge abruptly between completely different habitats, such as woodlands and croplands, and therefore do not necessarily indicate gradual shifts in habitat quality (Warren et al. 2018). A few years earlier, urbanization gradient studies had already been criticized for being too simplistic due to the prevalence of the transect approach to represent the urbanization gradient (Alberti et al. 2001; McKinney 2006; Alberti 2008). The increased utilizes of geographic information systems has favored the use of direct measures of urbanization to characterize local landscape contexts. Today, thanks to these improvements, gradients can be defined with various levels of precision including broad measures of urbanization such as the proportion of built or impermeable surfaces, therefore providing a more precise measure of the characteristics of the urban area that are likely to have a direct influence on ecological responses (McDonnell & Hahs 2008). Such measurements are now often found in the literature (e.g., Seress et al., 2014 for birds). Thogmartin & Knutson (2007) showed that avian species-habitat relationships respond to factors at multiple spatial scales. Such an impact of scaling is all the more relevant for smaller animals (e.g., insects or micro vertebrates), as weakly mobile species should respond to landscape patterns at finer spatial scales than would more mobile taxa (Concepción et al. 2015). Utilization of fine-scale artificial structures provides wildlife with alternative forms of shelter in urban environments (Lowry et al. 2013). Microhabitats also appear to be a determining factor for some taxa such as insects that are sensitive to micro-environmental variations (e.g. insolation, temperature or soil properties) (Mehrabi et al. 2014). Hartley et al. (2010) showed for example that fine-scale variations in temperature restrict brood development in the invasive Argentine ant (*Linepithema humile*) at the edge of its distribution, limiting populations spread. However, although species richness has been extensively studied throughout different spatial scales, much less is known about how species distributions vary across spatial scales, especially in arthropods (Hortal et al. 2010). As far as we know, such questions have never been addressed to measure the impact of urbanization on insect distribution.

Studies on urbanization have included a broad diversity of organisms such as vertebrates, insects, plants, fungi, and micro-organisms (McDonnell & Hahs 2008). Among the fauna present in highly urbanized environments, ants (Formicidae) are good indicators of the environmental impact of urbanization (Philpott et al. 2010; Heterick et al. 2013; Gippet et al. 2017). In the case of ants, the magnitude and direction of urban impacts depend on species life history and sensitivity to ecological disturbance, but also on species interactions and dispersal ability (Garden et al. 2006). Accordingly, almost all studies on the impacts of urbanization on ant communities have shown differences in species composition in urban habitats compared to nearby natural areas (Philpott et al. 2010), suggesting that urbanization significantly alters ant species distributions. In Lessard & Buddle’s (2005), urban ant assemblages were characterized by several competitively dominant species, including one introduced species. Menke et al. (2011) showed that urban areas may facilitate the movement of some species, leading urban adapted ants to be particularly successful at tracking future climate change. Within the ant genus *Tetramorium*, only the study of Gippet et al. (2017) has investigated urbanization impacts on species' presence. However, this study considered only two cryptic *Tetramorium* species, concluding that *T. immigrans* (formerly called *T*. sp. E; Wagner et al. 2017) occurred in fragmented and warmer areas, contrary to *T. caespitum* (formerly called *T*. sp. U2; Wagner et al. 2017). It was focused on a single urban area and did not investigate how land-cover and scale explained their spatial distribution.

To bridge the methodological gaps regarding the effect of scaling on the study of biodiversity distribution in the context of urbanization, the present study focuses on *Tetramorium* species at three distinct spatial scales, from urban microhabitat to urban landscape. We question how urbanization structures the distribution of *Tetramorium* species at these different spatial scales, and which scale is the most impacting for the studied species. We address this question using a sample of about 1400 individuals (one individual per colony) sampled along 19 urban gradients and belonging to four common species in South-eastern France: *T. immigrans* Santschi, 1927, *T. semilaeve* André, 1883, *T. moravicum* Kratochvíl, 1941, and *T. caespitum* (Linnaeus, 1758) (Schlick-Steiner et al. 2005; Schlick-Steiner et al. 2006; Borowiec et al. 2015; Wagner et al. 2017; Cordonnier et al. 2018). We test here the hypothesis that urbanization affects differentially the distribution of these four species, and that the impact of urbanization depends on the scale considered.

METHODS

Biological model, study area, and sampling scheme

We collected one worker ant per colony sample in 19 sampling zones which were at least 20 km apart and comprised cities with their adjoining peri-urban and rural areas (Fig. 1). For each sampling zone, 30 colony samples were collected homogeneously throughout contiguous urbanized areas (as defined by the classes 1.1.1, 1.1.2 and 1.2.1 in Corine Land Cover 2012; CLC 2012©, Copernicus (https://land.copernicus.eu/)) and 60 colony samples were collected homogeneously along a 15 km-long × 3 km-wide transect to the east of the urbanized areas. The 1:100,000 scale of Corine land cover data lumps small agricultural and semi-natural patches with artificialized surfaces and therefore does not allow analyses of the impact of urban gradients on species distribution but ensured that a representative diversity of habitats was sampled in the study. For each sample collected, the occurrences of main components of microhabitats within a radius of 1 m around the nest (Fig. 1) were recorded and classified into nine categories: (1) full soils with vegetation (e.g. grass, herbaceous plants), (2) pavements (concrete and impervious coating), (3) unstabilized material (sand, gravels), (4) trees or roots, (5) litter (leaves, plant debris, woodchips), (6) curb (i.e. along granite or concrete slabs), (7) buildings or walls, (8) individual features (e.g. stones, rocks, lamp posts or any other material which can support a nest), (9) slopes (e.g. ditches, banks). Among these nine microhabitats, categories (2), (6) and (7) are classically considered as urban whereas categories (1), (4) and (5) are typically related to nonurban settings. Categories (3), (8) and (9) can be found in either urban or nonurban environments. Sampling was conducted in 2015 and 2016 from April to September on non-rainy days with temperatures ranging from 16 to 28°C, with a minimum distance of 200 m between two sampled colonies. Sampling was performed by teams of two persons conducting a direct search for *Tetramorium* nests followed by hand collection with an entomological aspirator. Collected ants were stored in 96% ethanol. Individuals from 1710 colonies were collected for this study and deposited as voucher material in the collection UCBLZ, CERESE, Université de Lyon, Université Claude Bernard Lyon1. Cordonnier et al. (accepted) identified the sampled species based on mitochondrial DNA barcoding and genotyping at 14 microsatellite markers. Among the identified species, the present study focuses on 1397 samples corresponding to the four most abundant *Tetramorium* species in the study area (i.e., with at least 60 sampled colonies): *T. immigrans* (n = 544), *T. caespitum* (n = 698), *T. moravicum* (n = 60), and *T. semilaeve* (n = 95).

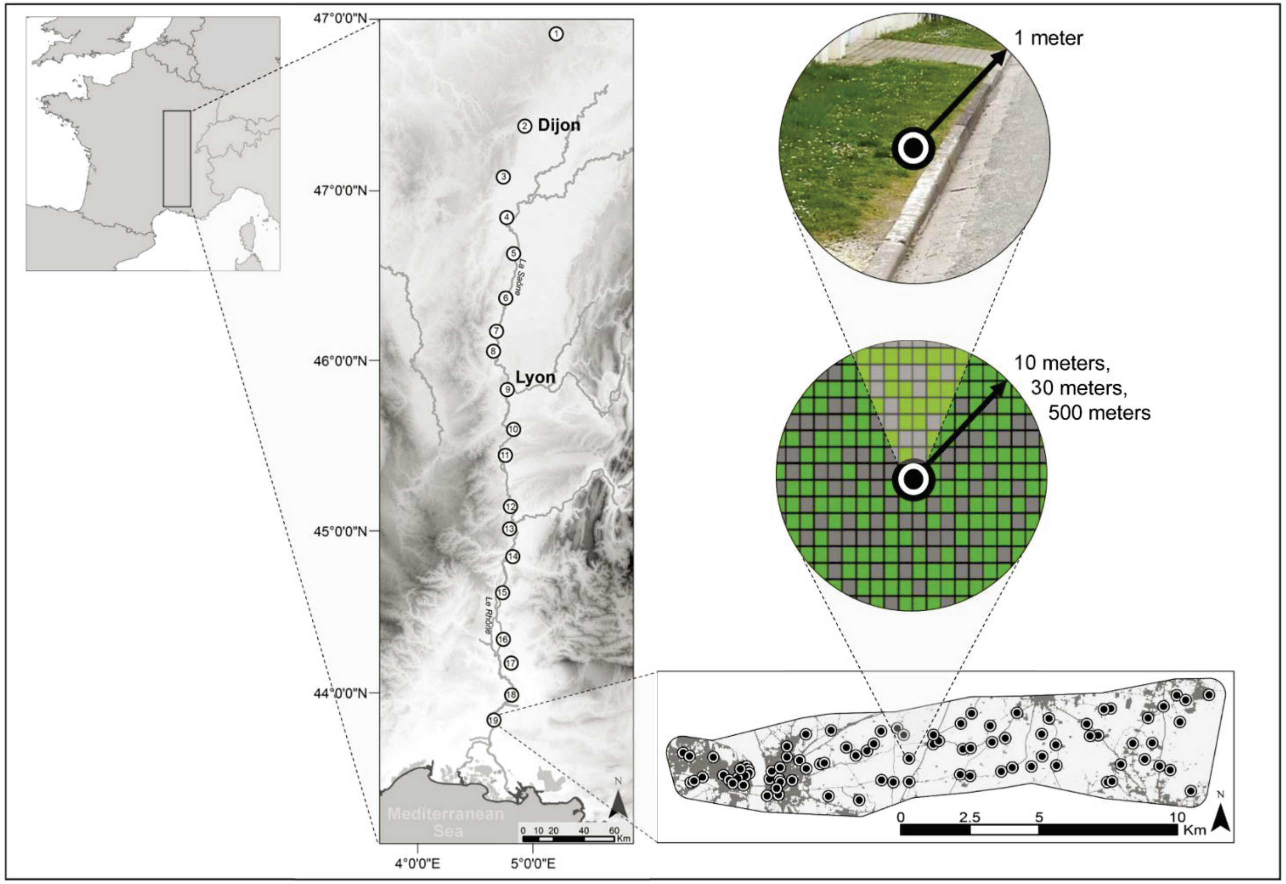

Figure 1. Experimental design of the study. Left: location of the study area and picture of one of the biological models. Center: Map of the 19 sampling zones, each of them including 90 samples. 1: Langres, 2: Dijon, 3: Beaune, 4: Chalon-sur-Saône, 5: Tournus, 6: Mâcon, 7: Belleville, 8: Villefranche-sur-Saône, 9: Lyon, 10: Vienne, 11: Péage-de-Roussillon, 12: Tournon sur Rhône, 13: Valence, 14: Livron, 15: Montélimar, 16: Bollène, 17: Orange, 18: Avignon, 19: Tarascon. Main rivers are indicated in dark grey, altitude is indicated by grayscale (black = high altitude). Bottom right: Example of sample distribution within a sampling zone. Impervious areas are indicated in dark grey and vegetalized areas in light grey. Top right: Scales of urban metrics used in the study

Spatial data

Landcover maps were obtained by pixel-based classification method using ArcGIS v.10.1 and ENVI 5.2. SPOT6 imagery (spatial resolution: 1.5 m; spectral band: R, G, B, NIR) obtained by GEOSUD EQUIPEX were used to identify impervious (e.g., building, roads and parking lots) and pervious surfaces (e.g., vegetated areas, bare soil and water) based on spectral index NDVI (Normalized Difference Vegetation Index), DVI (Differential vegetation Index), and BSI (Bare Soil Index). We used external data for the Lyon area (BDTOPO® 2013, Graphic Parcel Register (RPG2012); French National Geographic Institute IGN) to pre-classify SPOT imagery. The urban context was calculated as the proportion of impervious surfaces in three buffers around each nest: a 10 m radius buffer (area ensuring to include the total nest territory; Brian et al., 1974), a 30 m buffer representing the foraging area and potential biotic interactions, and a 500 m buffer representative of the landscape area of influence (Fig. 1).

Analyses

First, run tests (Sokal & Rohlf 1995) computed using PAST v3.12 software (Hammer et al., 2001) were used to ensure that the nine microhabitat variables and the three urban buffer variables were randomly distributed along the latitudinal gradient; i.e. that the patterns being analyzed were not attributable to other processes, e.g., linked to the climate gradient. We then selected the four microhabitat elements with at least 10% of occurrences (lmt1: vegetation, lmt2: pavement, lmt3: unstabilized material and lmt6: curb). The four species were tested for differences in the four microhabitat elements using PAST v3.12 software through an ANOSIM procedure (Clarke 1993) based on the Bray-Curtis distance with 99,999 permutations, using a simple Bonferroni correction. A SIMPER analysis (Clarke 1993) allowed for the computation of the relative contribution of each of the four microhabitat elements to the overall average dissimilarity (OAD) observed between the four species. ANOSIM analyses (Bray-Curtis distance, 99,999 permutations, simple Bonferroni correction) were finally computed for each microhabitat element to separately assess the effect of each of them in microhabitat differences between species.

To establish a set of uncorrelated variables for the proportions of impervious surfaces (variables u10, u30 and u500 for the 10 m, 30 m and 500 m buffer), we ran a collinearity analysis, eliminating the u30 variable as the Spearman correlation value was >0.7 with both u10 and u500 (Online Resource Fig. 1 and Fig. 2). Habitat selection for each species was assessed with generalized logistic linear models to investigate the effect of urbanization on the presence of each species at different spatial scales using the package lme4 (Bates et al. 2014) in R v. 3.3 software (R Development Core Team 2004). These models treated species presence in the sampling site as a repeated measurement of species occurrence (presence = 1, absence = 0). The percentages of impervious area in the 10 m and 500 m buffers around the nest (u10 and u500) and the microhabitat elements previously identified as relevant by ANOSIM+SIMPER analyses (lmt1: vegetation, lmt2: pavement; see results for details) were introduced as explanatory terms in the fixed part of the model. To select the best model, we used the Bayesian Information Criterion (BIC) that selects for the most consistent and parsimonious model (Aho et al. 2014). Model parameters were then estimated through a model averaging procedure on models with ∑ weight BIC = 0.95, using the MuMIn package (Bartoń 2016) (Burnham & Anderson 2004). The significance of each explanatory term was tested using a Wald test on the full model and looking at confidence intervals on the estimates. Homoscedasticity, independence and normality of residues were checked for each model.

RESULTS

The ANOSIM analysis based on the four selected microhabitat elements revealed a significant global difference in species microhabitats ($R = 0.031$, $p \leq 10^{-5}$) mainly driven by a significant difference between the sites with *T. immigrans* and those with *T. caespitum* ($p = 0.0006$; Fig. 2).

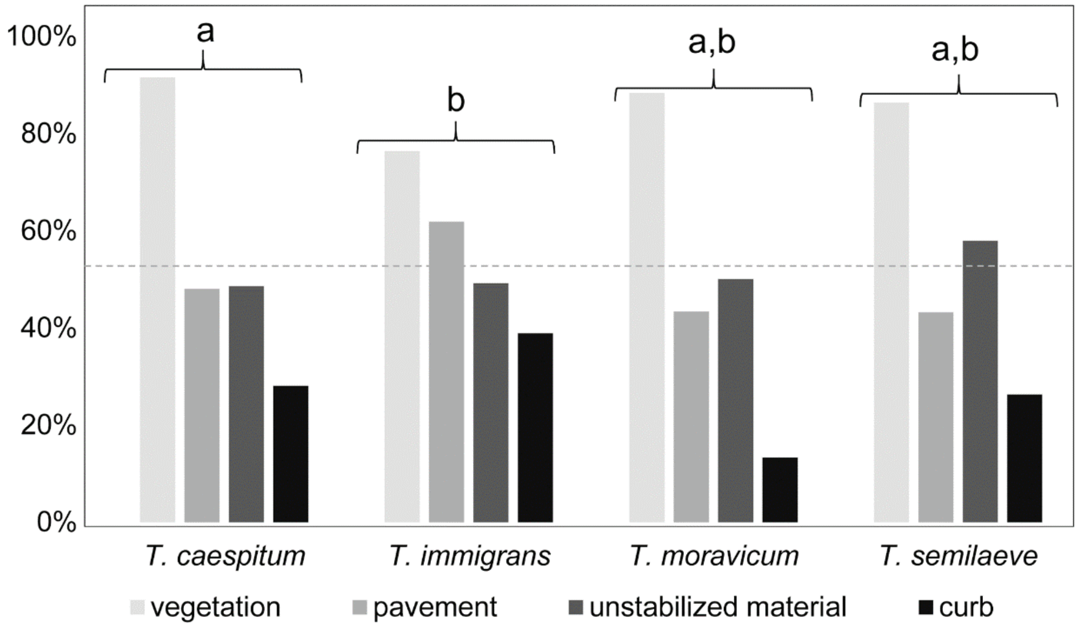

Figure 2. Proportions of sites with each microhabitat element in the four studied *Tetramorium* species. Different letters indicates significant differences in microhabitats between species according to global ASOSIM analysis after simple Bonferroni correction

According to the SIMPER analysis, the four tested elements contribute to the overall average microhabitat dissimilarity between species (OAD) in broadly similar ways, with relative contributions to OAD ranging from ~18% to ~30% (Table 1).

Table 1. SIMPER analysis average dissimilarities and percentage of contribution of each microhabitat element to the observed overall average dissimilarity between *Tetramorium* species' microhabitats

Microhabitat element	Av. dissimilarity	Contribution to OAD (%)
lmt2: Pavement	12.35	29.96
lmt3: Unstabilized material	12.29	29.81
lmt6: Curb	9.264	22.46
lmt1: Vegetation	7.322	17.76

The separate ANOSIM analyses on each microhabitat element are significant only for the presence of full soil with vegetation (lmt1: R = 0.0338, $p = 10^{-4}$) and pavement (lmt2: R = 0.0153, $p = 10^{-4}$). The post-hoc contrast analyses revealed a significant difference in relative occurrence of the vegetation element between sites with *T. immigrans* and sites with *T. caespitum* (p = 0.0006), as well as in relative occurrence of the pavement element between sites with *T. immigrans* and sites with *T. caespitum* (p = 0.0006), *T. moravicum* (p = 0.0222) and *T. semilaeve* (p = 0.0042) (Fig. 3).

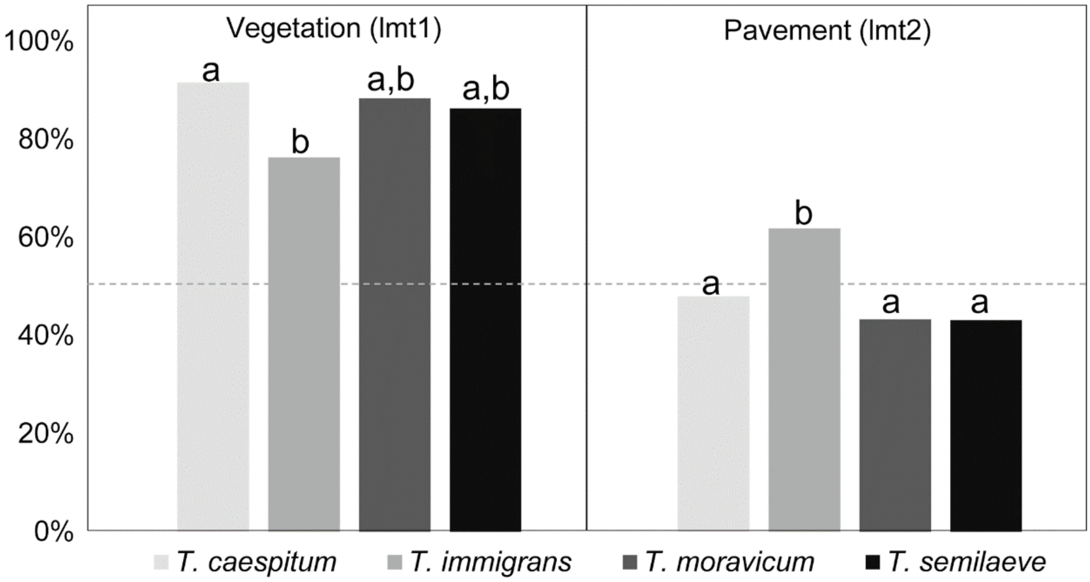

Figure 3. Proportions of sites with vegetation (lmt1; left) or pavement (lmt2; right) for the four studied *Tetramorium* species. Different letters indicates significant differences in relative occurrence of microhabitat elements according to ANOSIM analysis after simple Bonferroni correction

Model averaging over the 95% confidence set of best-ranked logistic models allowed the selection of different models for the four studied species (see Online Resource Table 1 for detailed list of models). The Wald tests computed on parameter estimates for these models revealed that the presence of *T. semilaeve* was not associated with any of the tested variables. Urbanization at landscape scale (500 m buffer) was the only variable that negatively affected the presence of *T. moravicum* (est. = -0.0484, z = 3.944, p = 8.03^{-05}). The presence of *T. caespitum* was negatively associated with urbanization at landscape scale (500 m buffer; est. = -0.0195, z = -7.149, p = 8.72^{-13}) but positively associated with sites with local-scale vegetation (1 m buffer; est. = 0.8547, z = 5.045, p = 4.54^{-07}). Finally, the presence of *T. immigrans* was significantly explained both by presence of urbanization at landscape scale (500 m buffer; est. = 0.0237, z = 8.693, $p < 2\times10^{-16}$) and absence of local-scale vegetation (1 m buffer; est. = -0.8597, z = -5.316, p = 1.06×10^{-07}). All these results are summarized in Fig. 4.

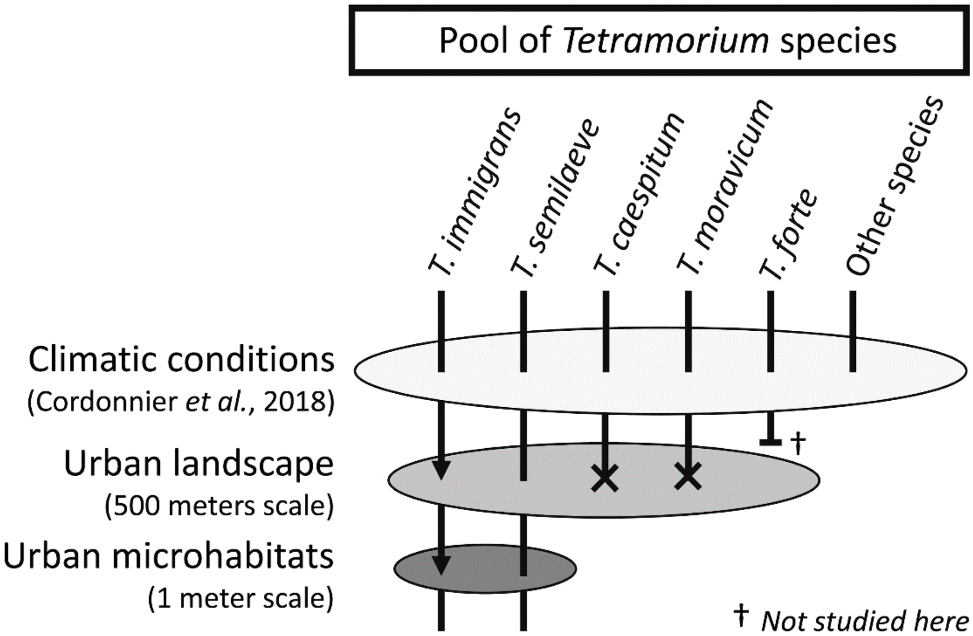

Figure 4. Schematic representations of filters endured by *Tetramorium* species in the study area. An arrow indicates a significant positive impact of variables linked to urbanization at the considered scale. A cross indicates a significant negative impact of variables linked to urbanization at the considered scale

DISCUSSION

The species studied here respond to landscape patterns at fine spatial scale as both vegetation and pavement presences have been identified as factors shaping species' occurrence. Microhabitats therefore appear to significantly influence species presence, as suggested in Mehrabi et al. (2014). Nevertheless species distributions were not shaped by fine-scale factors only, according to the results from model averaging. The occurrences of *T. caespitum and T. immigrans* simultaneously depended on urbanization at the landscape and local scales, with *T. caespitum* avoiding urban microhabitats (pavement, curb) and impervious habitats in the 500 meters surrounding the nest, whereas *T. immigrans* favored them. These scaling impacts of urbanization were species-dependent as *T. moravicum* was only associated with nonurban landscapes and *T. semilaeve* showed no association whatever the tested variables. As also suggested by Thogmartin & Knutson (2007) for birds, species-habitat relationships of the studied *Tetramorium* species respond to factors at multiple spatial scales. These results highlight the importance of considering several spatial scales simultaneously to study the impact of urbanization on species distribution patterns. McIntyre (2000) had already pointed out how little was understood about the mechanisms accounting for distribution and abundance patterns of urban arthropods. Only five of the 300 papers reviewed by McDonnell & Hahs (2008) addressed the measures used to quantify the urbanization gradient itself and only fifteen investigated cryptic organisms, highlighting the lack of investigation regarding these topics. These findings remain of immediate relevance. McGarigal et al. (2016) reviewed studies on habitat selection between 2009 and 2014 and concluded that the majority of habitat studies were still not using a multi-scale framework, and very few evaluated environmental variables across different grains. These authors nevertheless confirmed that distinct species select habitats at different scales, and therefore suggested to use multi-scale approaches, encouraging the use of generalized linear models and model selection methods based on Information Criteria to model different scales of habitat selection. Building on these studies, the present work contributes to untangling the effects of scales in studies of species distribution in response to urbanization processes.

The highly contrasted responses to urbanization observed between *Tetramorium immigrans* and *T. caespitum* may be indicative of niche partitioning processes driven by urbanization between these two species. Among the species studied, *T. immigrans* is the only one clearly associated with urban areas where it faces a modified habitat dominated by human-made structures, exhibiting thermal, pedological and hydrologic conditions that markedly depart from those of nearby natural areas (reviewed by Forman 2014). In urban habitats, *T. immigrans* exploits anthropogenic resources (here pavement) and could therefore be categorized as an urban exploiter or synanthropic species (McKinney 2006; Forman 2014). Pavement plays a very important role in the urban thermal balance as it absorbs solar and infrared radiation and dissipates part of the accumulated heat through convective and radiative processes (Santamouris et al. 2011). At the microhabitat scale, pavements could therefore offer to *T. immigrans* an alternative form of shelter in urban environments (Lowry et al. 2013), providing warmer nesting areas than microhabitats with vegetation cover only, which could give *T. immigrans* an advantage if these warmer temperatures actually offered a wider annual time-window than species inhabiting vegetated microhabitats such as *T. caespitum*. Wagner et al. (2017) showed that winged reproductive alates of *T. immigrans* were found in nests between March, 17 and September, 29, whereas reproductive alates of *T. caespitum* were present in nests between

May, 28 and August, 19, which corroborate the hypothesis of a larger phenological niche for *T. immigrans*. Wagner et al. (2017) indicated that contrary to *T. immigrans*, *T. caespitum* builds soil mounds higher than 10 cm. Although they are costly to build (e.g., Mikheyev et al. 2004), domes intercept incident solar radiation, therefore maintaining ideal thermal conditions in the nest (Kasimova et al. 2014). The anthropized habitats of *T. immigrans* may allow this species to overcome the construction of such structures by nesting in microhabitats generating sufficient heat. Grant et al. (2011) evidenced that amphibian and reptile species that persist in urban habitats tend to have broad diet requirements, high mobility and reproductive capacity, small body size and tolerance to human disturbance. *Tetramorium* ant species have similar body sizes according to their respective cephalic sizes: *T. immigrans*: 834 ± 56 [713, 943] µm; *T. caespitum*: 761 ± 50 [591, 867] µm (Wagner et al., 2017); *T. moravicum*: 878 ± 41 [807, 951] µm (Schlick-Steiner et al. 2005); *T. semilaeve*: 707 ± 36 [622, 760] µm (Borowiec et al. 2015); therefore, size alone probably cannot generate strong differences in habitat preferences between these species. Concepción et al. (2015) showed that urban expansion especially impacts highly mobile and specialized butterfly species, which were negatively affected by urban areas even at great distances. However, very little is known about the diet, dispersal abilities, reproductive fitness, and responses to human disturbance in these species. In-depth investigation of these traits should uncover the drivers of their habitats preferences.

The results of the present study could have indirect applications and interest for further studies. For instance, Menke et al. (2011) showed that urban environments may facilitate the movement of ant species adapted to warmer and drier environments and suggested that urban adapted ants may be particularly successful at tracking future climate change. *Tetramorium immigrans* should therefore be further investigated from a climate change perspective as it could be a highly relevant species to study species and organism responses facing global climate warming. Munshi-South & Kharchenko (2010) showed that the urban white-footed mouse, *Peromyscus leucopus,* exhibited little genetic structure in natural areas over even regional scales but strong genetic structuration in New York City populations, suggesting that urbanization is a strong driver of genetic differentiation compared to natural fragmentation. As far as the ant species studied here are concerned, compared genetic structures and diversities at the urban scales identified as relevant should allow a more precise quantification of the impacts of urbanization.

This study used an effective method to study the multi-scale impacts of urbanization on the distribution of four *Tetramorium* species by investigating spatial scales from urban microhabitat to urban landscape. It showed that the four species of interest presented distinct response to urbanization. As *T. immigrans* was particularly associated with urban areas, monitoring the dynamics of its distribution range should provide insightful information into the impact of urbanization on species presence and distribution. In addition, as urbanization could enhance the climate change signal at the local scale, we suggest simultaneously considering the impact of urban land use and climate in subsequent studies about drivers of species presence and distribution. Factors such as stress tolerance (e.g., resistance to extreme temperatures and/or to dry conditions) should also be taken into account, as well as the role of competition in structuring ant assemblages.

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SUPPORTING INFORMATION

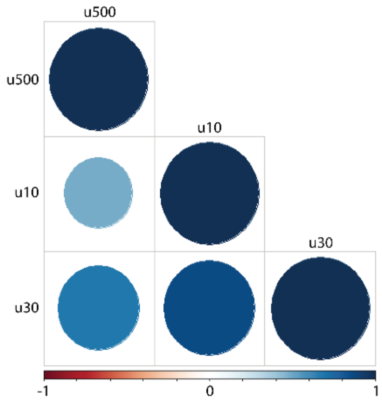

Figure S1. Correlation coefficients between percentages of impervious surfaces in 10, 30 and 500 m buffers around sampled colonies

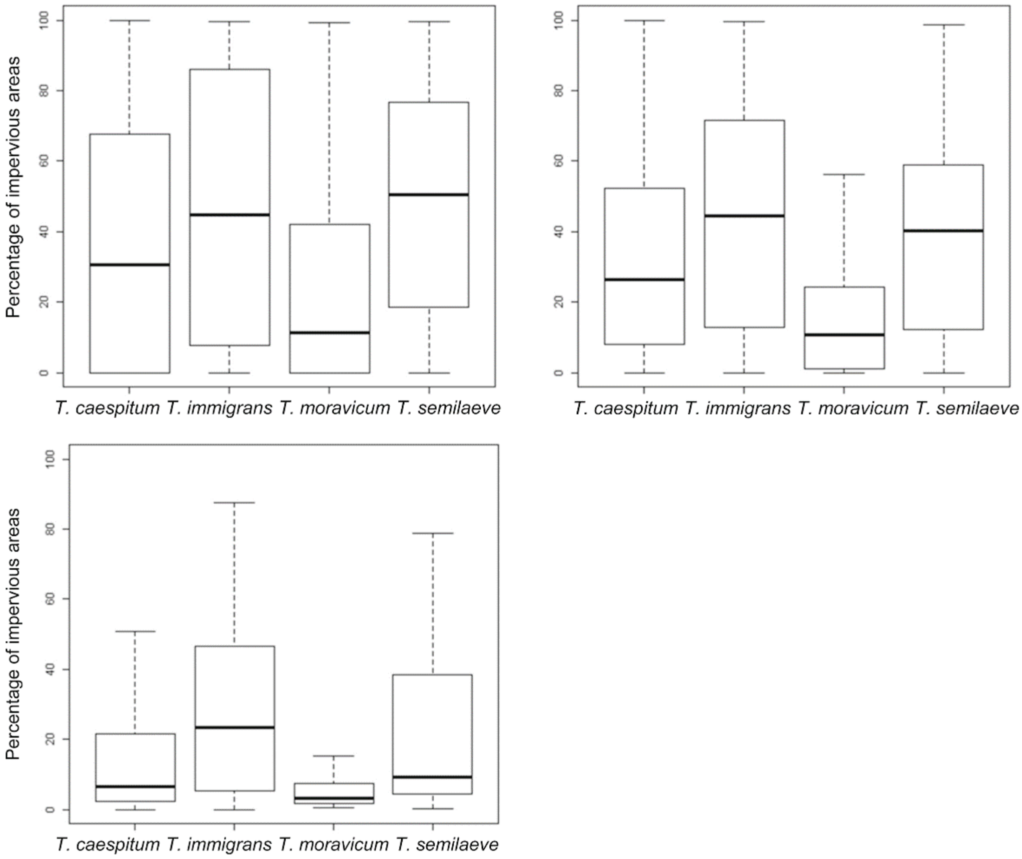

Figure S2. Percentages of impervious surfaces in 10, 30 and 500 m buffers around sampled colonies)

Table S1. Model selection tables for each species (models ranked by increasing BIC-value)

```
Tetramorium semilaeve
   (Intrc) pvmnt      u10     u500 vgttn df   logLik   BIC delta weight
1   -2.616                                1 -343.258 693.7  0.00  0.688
2   -2.427    +                           2 -341.598 697.7  3.91  0.097
3   -2.842       0.005062                 2 -341.737 697.9  4.19  0.085
4   -2.674    + 0.007263                  3 -338.750 699.2  5.44  0.045
5   -2.732                0.005454        2 -342.620 699.7  5.95  0.035
9   -2.693                             +  2 -343.216 700.9  7.14  0.019
6   -2.560    +           0.010100        3 -339.722 701.1  7.39  0.017
11  -3.021       0.005398              +  3 -341.543 704.8 11.03  0.003
10  -2.437    +                        +  3 -341.597 704.9 11.14  0.003
7   -2.857       0.004534 0.001810        3 -341.683 705.1 11.31  0.002
8   -2.702    + 0.005801 0.005937        4 -338.224 705.4 11.62  0.002
12  -2.792    + 0.007444               +  4 -338.671 706.3 12.52  0.001
13  -2.881                0.005949     +  3 -342.483 706.7 12.91  0.001
14  -2.655    +           0.010330     +  4 -339.670 708.3 14.51  0.000
15  -3.053       0.004766 0.002244     +  4 -341.462 711.8 18.10  0.000
16  -2.848    + 0.005957 0.006164     +  5 -338.107 712.4 18.62  0.000
```

```
Tetramorium immigrans
    (Intrc) pvmnt        u10    u500 vgttn df   logLik    BIC  delta weight
13 -0.23810                  0.02367     +  3 -855.935 1733.6   0.00  0.861
14 -0.33660     +            0.02196     +  4 -854.409 1737.7   4.18  0.107
15 -0.19860       -1.126e-03 0.02456     +  4 -855.744 1740.4   6.85  0.028
16 -0.28740     + -1.595e-03 0.02310     +  5 -854.032 1744.2  10.65  0.004
5  -1.01100                  0.02592        2 -870.279 1755.0  21.46  0.000
6  -1.11000     +            0.02379        3 -868.009 1757.7  24.15  0.000
7  -1.01000       -4.156e-05 0.02595        3 -870.278 1762.2  28.69  0.000
8  -1.09600     + -5.918e-04 0.02422        4 -867.955 1764.8  31.27  0.000
10  0.08302     +                        +  3 -884.368 1790.4  56.87  0.000
12 -0.08973     +  3.930e-03             +  4 -881.483 1791.9  58.32  0.000
11  0.12720        5.590e-03             +  3 -888.848 1799.4  65.82  0.000
9   0.44630                              +  2 -895.201 1804.9  71.30  0.000
4  -0.97100     +  5.312e-03                3 -899.207 1820.1  86.54  0.000
2  -0.80320     +                           2 -904.754 1824.0  90.41  0.000
3  -0.77580        7.244e-03                2 -908.678 1831.8  98.25  0.000
1  -0.46620                                 1 -920.040 1847.3 113.75  0.000
```

```
Tetramorium caespitum
     (Intrc) pvmnt        u10     u500 vgttn df   logLik    BIC delta weight
13 -0.354700                  -0.01953     +  3 -907.669 1837.0  0.00  0.943
15 -0.388300        9.741e-04 -0.02032     +  4 -907.517 1844.0  6.93  0.030
14 -0.340100     +            -0.01925     +  4 -907.630 1844.2  7.15  0.026
16 -0.372400     +  1.062e-03 -0.02004     +  5 -907.453 1851.1 14.03  0.001
5   0.425900                  -0.02183        2 -921.172 1856.8 19.78  0.000
6   0.452700     +            -0.02119        3 -920.971 1863.6 26.60  0.000
7   0.426700       -3.014e-05 -0.02181        3 -921.172 1864.0 27.01  0.000
8   0.449900     +  1.276e-04 -0.02129        4 -920.968 1870.9 33.83  0.000
11 -0.650600       -4.514e-03              +  3 -930.189 1882.1 45.04  0.000
10 -0.698900     +                         +  3 -930.733 1883.2 46.13  0.000
9  -0.906100                               +  2 -934.550 1883.6 46.53  0.000
12 -0.540900     + -3.641e-03              +  4 -928.112 1885.1 48.12  0.000
3   0.246000       -6.049e-03                 2 -948.238 1910.9 73.91  0.000
4   0.351000     + -4.924e-03                 3 -944.998 1911.7 74.66  0.000
2   0.199700     +                            2 -950.023 1914.5 77.48  0.000
1  -0.005797                                  1 -956.537 1920.3 83.28  0.000
```

```
Tetramorium moravicum
   (Intrc) pvmnt       u10     u500 vgttn df   logLik   BIC delta weight
5   -2.485                 -0.04895        2 -229.584 473.6  0.00  0.876
7   -2.359       -0.006809 -0.04113        3 -228.562 478.8  5.19  0.065
6   -2.532    +            -0.05037        3 -229.467 480.6  7.00  0.026
13  -2.303                 -0.04998     +  3 -229.480 480.7  7.02  0.026
8   -2.418    + -0.007270 -0.04263        4 -228.325 485.6 11.94  0.002
15  -2.102       -0.007133 -0.04216     +  4 -228.372 485.7 12.04  0.002
3   -2.598       -0.015500                 2 -236.327 487.1 13.49  0.001
14  -2.355    +            -0.05132     +  4 -229.372 487.7 14.04  0.001
16  -2.165    + -0.007583 -0.04364     +  5 -228.144 492.4 18.81  0.000
4   -2.571    + -0.015190                 3 -236.290 494.3 20.64  0.000
11  -2.596       -0.015500              +  3 -236.327 494.3 20.72  0.000
1   -3.109                                 1 -243.706 494.6 21.02  0.000
2   -2.933    +                            2 -242.790 500.0 26.41  0.000
9   -3.342                              +  2 -243.475 501.4 27.78  0.000
12  -2.560    + -0.015200               +  4 -236.290 501.5 27.87  0.000
10  -3.117    +                         +  3 -242.665 507.0 33.39  0.000
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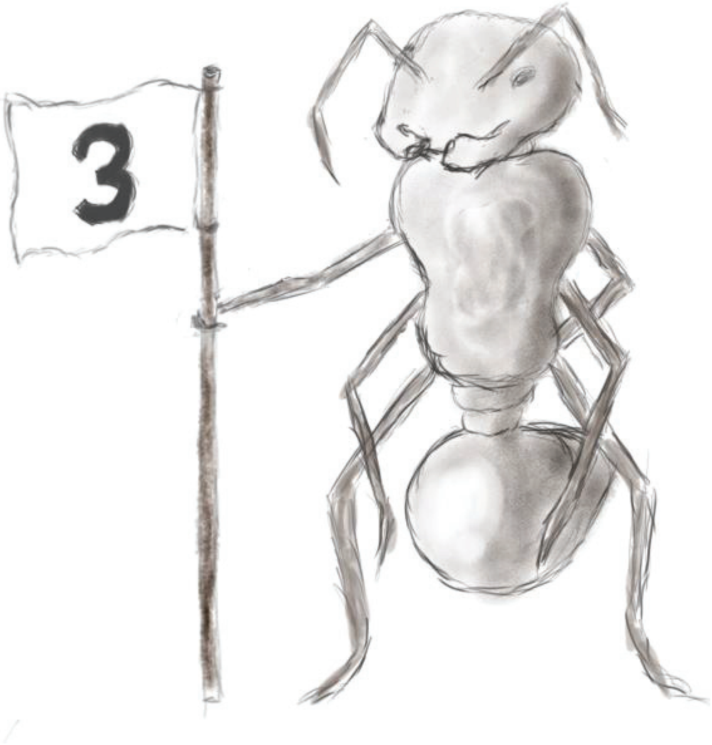

Chapter 3. Distribution, genetic structure and status of *Tetramorium immigrans*

« Ultimately we need to recognize that while
humans continue to build urban landscapes,
we share these spaces with other species »
David Suzuki, 2012

PAPER 3. Urbanization-climate interactions promote the expansion of species ranges: a case study in the invasive pavement ant *Tetramorium immigrans*

This article has been submitted in Journal of Animal Ecology

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Abstract

1- Global changes, such as climate and urbanization, are widely studied as they have global implications for people and ecosystems. Global changes are strongly entwined and aggravated by the development of human activities, which also intensifies the human-mediated dispersal of species. However, few studies have explicitly considered the combined influence of urbanization and climate change on species expansion.

2- As anthropogenic introductions are common in cities and occur in numerous ecologically impactful ant species, we investigated the combined roles of climate, urbanization and human mediated dispersal in the expansion of *Tetramorium immigrans*, an invasive pavement ant in North America, in urban areas of South-eastern France where it is supposed native.

3- A total of 544 *T. immigrans* individuals were sampled from 16 urban gradients and genotyped at 14 microsatellite markers. We combined molecular ecology methods (Bayesian and frequency-based analyses), and statistical modelling to evaluate the impact of interaction between climate and urbanization on the pavement ant distribution patterns.

4- The occurrence probabilities of *T. immigrans* depended on the interaction between climatic and urban factors. Two latitudinally distinct clusters of *T. immigrans* were hierarchically structured in two sub-clusters each, suggesting different colonization histories. Strong founder effects indicated introductions from external sources followed by colonization favoured by human activities in the northern urban areas.

5- The effect of climate-urbanization interaction on species distribution, an interaction between two global changes mostly overlooked in the literature, was clearly evidenced, suggesting that in the north of its range, *T. immigrans* thrives under harsher climate by colonizing the most urbanized areas. Many taxa may conform to such pattern, making the combined study of global changes a necessary challenge for future studies. Distribution patterns concurred with similar observations in the invasive range of *T. immigrans*, making it likely that it may not be native to South-eastern France, at least in the northernmost urban areas. Cryptic invasions or discreet range shifts are likely to occur in many taxa, especially in ants, and deserve increased attention from researchers and managers alike.

Keywords: Biological invasion, Climate changes, Pavement ant, Species expansion, *Tetramorium immigrans*, Urbanization

INTRODUCTION

Urbanization is a global change of high magnitude and speed, which deeply impacts biodiversity (Grimm et al., 2008). Urban climates differ from surrounding natural areas because of the Urban Heat Island effect (Grimm et al., 2008), affecting species distributions and biotic interactions (Parris & Hazell, 2005; Youngsteadt et al., 2015). Many plant and animal species in cities have been therefore documented outside their natural climatic range (Youngsteadt et al., 2015). Climatic conditions however are a major determinant of the natural distribution of species, with evidence both from the fossil record (Escarguel, Fara, Brayard & Legendre, 2011) and from recently observed trends (Parmesan & Yohe, 2003), and therefore remain crucial in determining species distributions, even in cities (Aronson et al., 2016). Few studies explicitly considered this combined influence of urbanization and global climate change when investigating the impacts of climate change in the context of global land use changes (Diamond et al., 2014). In urban areas, species composition is shaped by hierarchical series of filters including regional climate and biogeography, land use and human mediated biotic interchange, urban form and development history, local human facilitation as well as species interactions (Aronson et al., 2016). Urban biological communities have been filtered from the regional species pool, but also from a global pool of species transported by human activities, either intentionally or not, which find suitable habitats within urban areas (Aronson et al., 2016). Marzluff, Bowman and Donnelly (2001) evidenced an upward trend in the proportion of non-native species (i.e., species that did not occur before importation by humans) toward the urban core. Numerous studies have further shown that urbanization promotes the replacement of native species by non-native ones (McKinney, 2006). This increasing non-native species richness could be due to (i) an increased rate of introduction of non-native individuals, e.g., due to accidental or intentional transport for human use, and (ii) to the presence of favorable habitats for the establishment of non-native species, according to the “niche opportunity” concept (Shea & Chesson, 2002) where a combination of resources, fewer natural enemies and environmental conditions, including their fluctuations in time and space, improve habitability for the invader (McKinney, 2006).

Among the fauna present in highly urbanized environments, ants (*Formicidae*) are easily transported outside of their native habitat through global scale human trade (McGlynn, 1999) and most studies published so far have shown the dominance in cities of ant species introduced by humans (Heterick, Lythe & Smithyman, 2013; Vonshak & Gordon, 2015; but see Gippet et al., 2017). The urban-adapted ant assemblages comprise species with biogeographic distributions that differ from those found in natural or rural areas and these species tend to have ranges that extend into areas with both drier and warmer climates (Menke et al., 2011). The present study focusses on *Tetramorium immigrans* in France. *Tetramorium immigrans* is an invasive species in North America, where it was introduced in cities in the 19th century or earlier (Steiner et al., 2006, 2008a). In France, a previous study in the Lyon urban area showed that *T. immigrans* frequently occurs in fragmented and warmer areas habitats, contrary to its phylogenetically closest species in the same area, *T. caespitum* (Gippet et al., 2017). We used an individual-based sampling scheme (Prunier et al., 2013) to collect 544 individual samples of *T. immigrans* from 16 urban landscape gradients in the Rhône valley (France), and applied landscape genetic tools based on nuclear DNA (14 microsatellite markers) and semi-automated interpretation of SPOT 6 imagery to investigate the impact of interaction between climate change and urbanization on the expansion of *T. immigrans*. As suggested by Diamond, Dunn, Frank, Haddad and Martin (2015) for insects, we expected that at higher latitudes, the niche of *T. immigrans* should narrow towards urban core areas. Using linear mixed models, we therefore tested the hypothesis that *T. immigrans* occupancy in the study area would depend on climate and urbanization, but also on their interactions. In addition, given the invasive status of *T. immigrans* in cities of North America (Steiner et al., 2006, 2008a), its presence in highly fragmented areas in France (Gippet et al., 2017), and as anthropogenic introductions are very common in urbanized area (Marzluff et al., 2001; McKinney, 2006; Cristescu, 2015; Youngsteadt et al., 2015; Aronson et al., 2016) and very common in ants (McGlynn, 1999; Heterick et al., 2013; Vonshak & Gordon, 2015), we examined here the role of human-mediated dispersal in the introduction of this species in urban areas. We therefore studied the impact of urbanization on the genetic structuration of this species. In the case of a northwards gradual expansion, such as post-glacial recolonization, we expected a linear differentiation pattern, with clues of more pronounced founder effects in the colonization front. The opposite hypothesis, which would support a colonization favoured by human activities could result in situations characterized by latitudinally distinct sub-clusters hinting at different colonization histories, by nonlinear patterns in fixation indices or by reduction in population sizes suggestive of human mediated introduction.

METHODS

Biological model, study area and sampling scheme

Tetramorium immigrans was sampled in 16 urban landscapes along a steep climatic gradient crossing the boundary between the European Continental and Mediterranean biogeographical areas extending from the city of Chalon-sur-Saône in the North (46° 78′ 99″ N, 4° 85′ 11″ E) to the city of Tarascon in the South (43° 48′ 21″ N, 4° 39′ 37″ E). In all 16 sampling zones, individuals belonging to the genus *Tetramorium* were collected in 2015 and 2016 from April to September on non-rainy days with temperatures ranging from 16 to 28°C from 30 locations distributed homogeneously throughout contiguous urbanized areas (as defined by the classes 1.1.1, 1.1.2 and 1.2.1 in Corine Land Cover 2012) and 60 locations samples distributed homogeneously along a 15 km long and 3 km wide transect to the east of the urbanized areas, leading to a total of 1436 sampled colonies, with a minimum distance of 200 m between two colonies. Collected ants were stored in 96% ethanol. Samples were deposited as voucher material in the collection UCBLZ, CERESE, Université de Lyon, Université Claude Bernard Lyon1. Individuals were identified to the species level using both morphology and genetics (barcoding based on mitochondrial DNA and genotyping at 14 microsatellite markers), resulting in the preliminary removal of 285 colonies whose species identity could not be determined unambiguously (Cordonnier, Bellec, Dumet, Escarguel and Kaufmann, 2018). *Tetramorium immigrans* was present in 544 and absent in 607 locations from the 16 sampling zones. DNA of the 544 *T. immigrans* individuals was extracted from whole individuals which were crushed and then mixed with 150 µl of Chelex 100 and 10 µl of proteinase K (15 mg ml^{-1}) at room temperature; the solution was incubated at 55 °C overnight (Casquet, Thebaud & Gillespie, 2012). DNA was genotyped at 14 of the microsatellite loci developed by Steiner, Arthofer, Schlick-Steiner, Crozier, and Stauffer (2008b) organized in three multiplex PCR mixes. All three mixes had a total volume of 10 µl with 1X MasterMix (kit type-it microsatellite PCR Qiagen no. 206246) and 2 µl

DNA. Detailed quantities of each primers in each mix can be found in Cordonnier et al. (2018). Amplifications consisted in 5 min at 95 °C, then 32 cycles (30 s at 95 °C, 90 s at 60 °C, 30 s at 72 °C), and 30 min at 60 °C. All PCR products were analyzed with an ABI 3730xl sequencer (service provided by Genoscreen, Lilles, France). Electropherograms were read and interpreted with Genemarker 1.95 (Softgenetics, State College, PA, USA). Statistics (expected and observed heterozygosity, allelic richness, F_{is}, Hardy-Weinberg Equilibrium HWE) of microsatellite markers were computed using GENALEX (Peakall and Smouse, 2006) (Supporting information Table S1) in reconstructed populations defined by Bayesian clustering (explained below).

Spatial data

Landcover maps were obtained by pixel-based semi-classification using ArcGIS v.10.1®, ESRI, Redlands, CA, USA and ENVI 5.2®, Exelis Visual Information Solutions, Boulder, CO, USA. SPOT6 imagery (spatial resolution: 1.5m; spectral band: R, G, B, PIR) were used to identify impervious (e.g., building, roads and parking lots) and pervious surfaces (e.g., vegetated areas, bare soil and water) based on spectral index NDVI (Normalized Difference Vegetation Index), DVI (Differential vegetation Index) and BSI (Bare Soil Index). We used external data for the Lyon area (BDtopo® IGN 2013, Graphic Parcel Register (RPG, 2012)) to pre-classify SPOT imagery. The urban context was calculated as the proportion of impervious surfaces in a 500m buffer around each nest. Latitudes (WGS84) as proxy for temperature in the climatic gradient along the Saône and Rhône valleys (Joly et al., 2010) were log-transformed and scaled to reduce the influence of extreme values and improve model convergence.

Analyses

We used logistic linear mixed model to investigate the effect of latitudinal position and urban context on the presence of *T. immigrans* in study area using package lme4 (Bates, Maechler, Bolker & Walker, 2014) in R v. 3.3 software (R Development Core Team, 2016). This model treated species presence in the sampling site (presence = 1, n = 544; absence = 0, n = 607) as a repeated measurement of species occurrence. Sampling zones (n = 16 urban landscape gradients) were introduced in the model as a random effect. The latitude of each nest (LWGS84), the percentage of impervious area in a 500m buffer around the nest (I500), and the interaction between them (LWGS84: I500) were introduced as explanatory terms in the fixed part of the model. This model was then used for parameter estimates, the significance of each explanatory term was tested using a Wald test and looking at confidence intervals on the estimates. Homoscedasticity, independence and normality of residues were checked for each model.

To determine the number of genetically homogeneous groups using microsatellite data, we used the Bayesian clustering algorithm implemented in the software STRUCTURE v. 2.3.1 (Pritchard, Stephens & Donnelly, 2000) based on the admixture model with correlated allele frequencies and LOCPRIOR model with sampling zone as prior location of samples (Hubisz et al., 2009), and with a number of a priori unknown clusters (K) varying from K = 1 to K = 16 (i.e. the number of sampling zones), with 10 iteration runs for each K-value. Each run consisted in 500,000 replicates of the MCMC after a burn-in of 500,000 replicates. To investigate the 10 independent runs, clustering results were analyzed using CLUMPAK (Kopelman et al., 2015) based on a Markov clustering algorithm which groups sets of highly similar runs into modes and separates these distinct groups of runs to generate a consensus solution for each distinct mode. For any given K, the different runs were either consensual with a single mode or resulting in both a majority mode consisting of most of the iterations and one or more minority modes consisting in the remaining iterations. CLUMPAK was then used to identify an optimal ordering of inferred clusters across different values of K, and to define the optimal K-value using the method of Evanno, Regnaut and Goudet (2005). The mean of the logarithm of the data probabilities and associated per K variance were calculated using Structure Harvester (Earl, 2012). The most conservative value of K compatible with these different elements – concordance between runs, Evanno et al.'s (2005) method and mean lnP(K) – was retained. The membership coefficient of each individual at each of the K clusters corresponding to the consensus solution of the majority mode was selected as Q-value. At each hierarchical level, individuals were grouped assuming a membership coefficient of at least 50% to belong to a cluster (Balkenhol et al., 2014). The same process was then separately iterated hierarchically within each cluster to measure sub-structuring within the identified clusters. All parameters remained identical except for the maximum number of clusters tested which systematically corresponded to the number of sampling zones involved in the hierarchical level. We considered that a cluster was genetically homogeneous when no individuals in this cluster had a Q-value greater than 0.9 at the next hierarchical level.

We further evaluated clustering results with classical frequency-based approaches by computing Nei's distances between sampling zones using GENETIX (Belkhir, 1999), then performing a Principal Coordinates Analysis (PCoA, PAST v. 3.18; Hammer, Harper & Ryan, 2001) to graphically represent the distance matrix, with a superimposed Kruskal tree to visualize the minimum crossing path. Only the 11 sampling zones with at least 15 samples were used in pairwise distance calculations. To assess differentiation between clusters defined using hierarchical Bayesian clustering, pairwise F_{ST} were calculated between cluster pairs using GENALEX (Peakall & Smouse, 2006).

Garza-Williamson's M ratios (Garza & Williamson, 2001) were calculated in each genetically homogeneous cluster to test for bottlenecks. In cases where population have recovered and pre-bottleneck population sizes were large, using M-ratios is the most pertinent method to detect bottlenecks that have lasted for several generations (Williamson-Natesan, 2005). The M ratio is the ratio between the number of alleles and the range in allele size (number of expected alleles) observed at a microsatellite locus, averaged over loci. The observed M ratio for each population was compared to the lower 5% tail of the critical ratio (MC) distribution generated by Critical_M program (Garza & Williamson, 2001), which requires three parameters: $\theta = 4\ Ne * \mu$ (where Ne = effective population size and μ = mutation rate), Δg (mean size of non-one-step mutations) and ps (the per cent of one-step mutations). We used the parameter values $\Delta g = 3.5$ and ps = 0.2 as recommended by Garza and Williamson (2001) and following previous studies on ants (Ugelvig et al., 2008; Yang et al., 2012; Sanllorente, Ruano & Tinaut, 2015). The mutation rate was set at $\mu = 10^{-4}$/locus/generation, which is the most commonly used mutation rate for microsatellite loci in ants (Ugelvig et al., 2008). The pre-bottleneck value $\theta = 4\ Ne * \mu$ was varied from the lowest value recorded in the literature (0.1; Ugelvig et al., 2008) to the highest value recorded in the literature (10; Yang et al., 2012), therefore testing θ equal to 0.01, 0.1, 1, which correspond to effective population sizes of 25, 250, 2500 and 25000 respectively. For each sub-cluster and each set of parameters, an equilibrium population was simulated 10,000 times using these parameter settings. Hierarchical Bayesian clusters with an observed M ratio under the MC threshold were considered having experienced a recent bottleneck (i.e. over the last hundred generations; Garza and Williamson 2001). Last, mean rarefied numbers of alleles (Na_r) were calculated using HP-RAR for each cluster (Kalinowski 2005) and an analysis of variance with Tukey pairwise multiple contrasts on paired data has been used to compare genetic richness between hierarchical Bayesian clusters.

RESULTS

Occupancy in *T. immigrans* is greater in the south (logistic linear mixed model, est. = -0.7748; z = -2.085; Pr(>|z|) = 0.0371), and in urban areas (est. = 0.8741; z = 9.660; Pr(>|z|) < 2* 10^{-16}; Table 1). The interaction between latitude and percentage of impervious area in a 500m buffer around the site is also a significant factor explaining species presence (est. = 0.3024; z = -3.222; Pr(>|z|) = 0.0013). In other words, the probability of *T. immigrans* occurrence in urban areas increased with latitude.

Table 1. Parameter estimates (Estimate), Wald test results (z value and significance of each explanatory term Pr(>|z|)), confidence intervals of the estimates (2.5%; 97.5%) and results of the likelihood test (F value) of the logistic linear mixed model.

	Estimate	z value	Pr(>|z|)	2.5 %	97.5 %	F value
I500	0.8741	9.660	< 2* 10^{-16}	0.7009	1.0586	87.1897
YWGS84	-0.7748	-2.085	0.0371	-1.5759	-0.0198	3.4876
I500:YWGS84	0.3024	3.222	0.0013	0.1206	0.4925	10.7132

Bayesian clustering resulted in two hierarchical levels (Fig. 1), with two first level clusters (cluster A, n=166 and cluster B, n=378) divided respectively into two (sub-cluster A1, n=52 and sub-cluster A2, n=114) and three second-level clusters (sub-cluster B1, n=81, sub-cluster B2, n=210 and sub-cluster B3, n=57). B3 clearly corresponds to individuals with allelic frequencies intermediate between clusters A and B and with a membership coefficient close to 50% (Fig. 1), suggesting that individuals in this group do not correspond to a genetically distinct cluster. Some individuals (n = 30, on the right in Fig. 1) have maximal Q-values below 50% for all B sub-clusters, reflecting a mix between different sub-clusters.

Clusters A and B are spatially structured along the latitudinal axis, their limits being located near the 7th sampling zone (Fig. 1). Similarly, sub-clusters A1 and A2, and B1 and B2 are separated between sampling zones 3 and 4 (A1-A2 limit) and 11 and 12 (B1-B2 limit) (Fig. 1). All individuals which could not be placed in any B sub-clusters because of maximal Q values below 0.5 (right part of Fig. 1) are located at the B1-B2 limit in Montélimar and correspond to individuals with intermediate allele frequencies between B sub-clusters. The contact zone between clusters A and B corresponds to the area where sub-cluster B3 has been found (Fig.1), confirming that this cluster is made up of individuals with intermediate allele frequencies between clusters A and B.

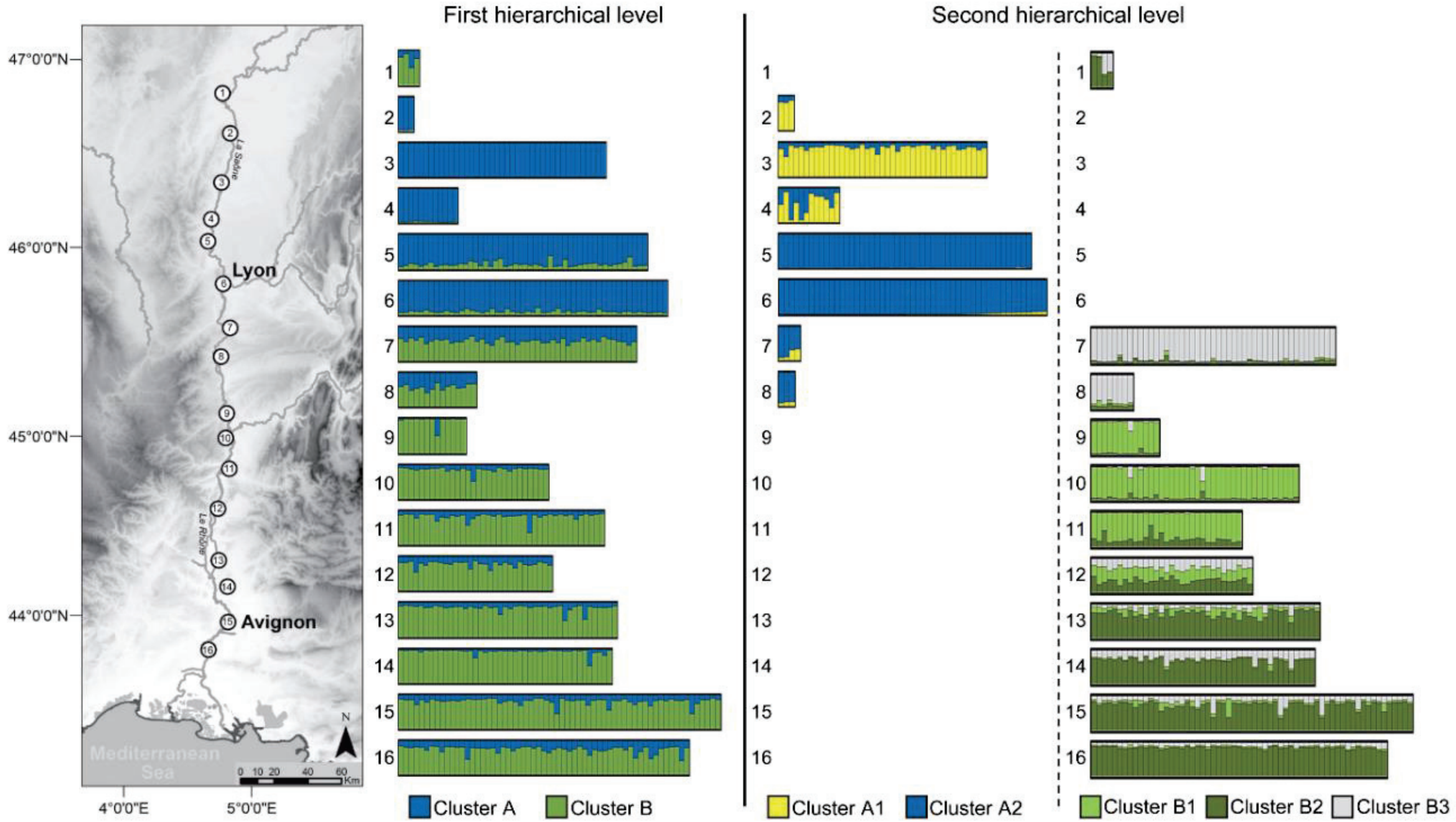

Figure 1. Left - Map of the 16 sampling zones. 1: Chalon-sur-Saône, 2: Tournus, 3: Mâcon, 4: Belleville, 5: Villefranche-sur-Saône, 6: Lyon, 7: Vienne, 8: Péage-de-Roussillon, 9: Tournon sur Rhône, 10: Valence, 11: Livron, 12: Montélimar, 13: Bollène, 14: Orange, 15: Avignon, 16: Tarascon. Main rivers are indicated in dark grey, altitude is indicated by grayscale (black = high altitude). Right - Barplots obtained at each hierarchical level from Bayesian clustering. Each vertical line corresponds to an individual. Colors indicate membership to each cluster (A in blue, n=166; B in green, n = 378) and sub-clusters (A1 in yellow, n=52; A2 in blue, n=114; B1 in light green, n=81; B2 in dark green, n=210; B3 in grey, n=5

Finally, only 4 individuals were found in the northernmost sampling zones and were associated with geographically distant sub-clusters (2 A2 individuals and 2 B1 individuals, Fig. 1); nevertheless, the low number of individuals sampled in that case precludes any reliable interpretation of these associations.

Nei's genetic distances between sampling zones strengthen the Bayesian clustering results (Fig. 2), including the intermediate position of individuals in the 7th (between A and B) and 12th (between B1 and B2) sampling zones. They also indicate that sampling zones belonging to sub-cluster A2 are genetically closer to B2 than to A1 and B1. Fixation indices (F_{ST}) between Bayesian clusters show that sub-clusters B1 and B2 are genetically close, while A2 is closer to B2 than to A1 (Table 2).

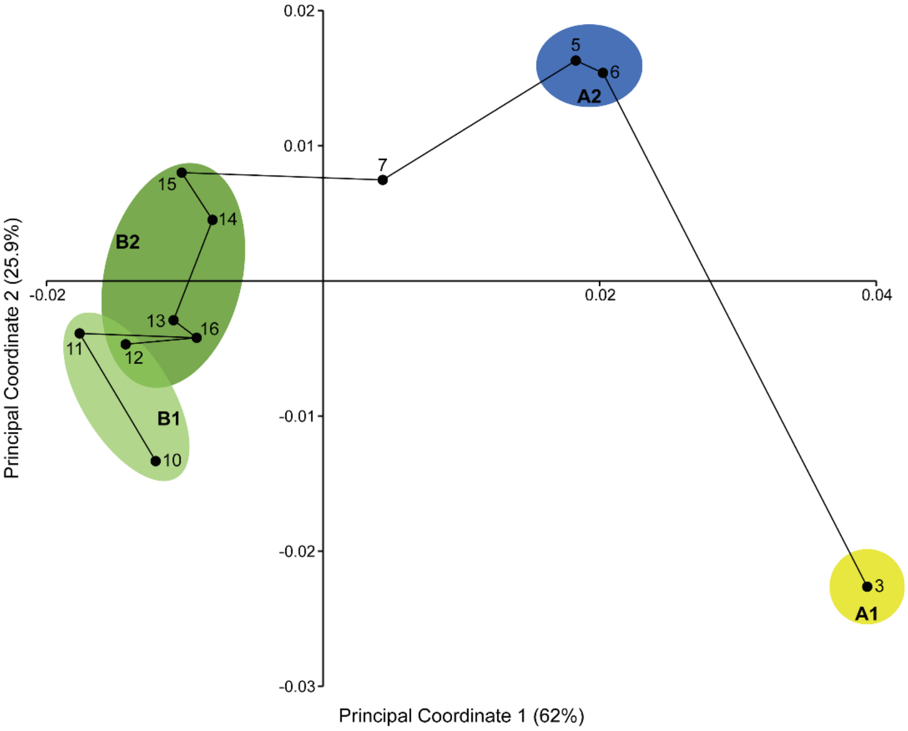

Figure 2. PcoA analysis based on Nei's distances between the 11 sampling zones with at least 15 individuals. Links corresponds to the Kruskal tree. Colored ellipses correspond to four sub-clusters identified by clustering. The two first-axes explain 88% of the total variability.

Table 2. Pairwise population F_{ST} values

	A1	A2	B1	B2
A1	-	-	-	-
A2	0.024	-	-	-
B1	0.031	0.026	-	-
B2	0.028	0.017	0.009	-

Garza-Williamson's M ratios obtained in all four sub-clusters were systematically below 0.6 while the lower 5% tail of the critical ratio always exceeds 0.6 for all effective population sizes used in simulations (Table 3), suggesting that all sub-clusters had experienced bottlenecks. M is lower in sub-clusters A1 and B1 whereas the critical value is stable in all sub-clusters, suggesting a stronger reduction in population size for A1 and B1 compared to A2 and B2. Rarefied number of alleles Na_r differed between sub-clusters ($p = 0.0052$). Na_r were higher in B1 compared to A1 sub-cluster ($p = 0.0063$) and slightly higher in B2 compared to A1 ($p = 0.019$) suggesting higher genetic diversity in southern sub-clusters compared to the northernmost sub-cluster.

Table 3. Garza-Williamson's M-ratio for each sub-cluster, with associated critical M values (Mc) for an effective size Ne between 25 and 25000. Na_r is the average rarefied number of alleles found in each sub-cluster; *sd* the associated standard deviation.

Population	M-ratio	Mc (Ne = 25)	Mc (Ne = 250)	Mc (Ne = 2500)	Mc (Ne = 25000)	Na_r
A1	0.461	0.798	0.786	0.711	0.658	11.00
A2	0.498	0.799	0.787	0.714	0.693	12.19
B1	0.457	0.799	0.790	0.714	0.679	13.33
B2	0.502	0.800	0.787	0.715	0.712	13.06

DISCUSSION

This study investigated the impact of the interaction between climate and urbanization on the expansion of *Tetramorium immigrans* using a landscape genetics approach. *Tetramorium immigrans* presence in the study area depends on climate and urban context as well as on their interactions, with a higher occurrence probability of *T. immigrans* in cities with respect to countryside in the northern part of the study area. The spatial positioning of genetic clusters obtained by STRUCTURE (Bayesian clustering) analysis showed two latitudinally distinct clusters of *T. immigrans* (A in the north, B in the south), hierarchically structured in four genetically homogeneous sub-clusters. Individuals found with intermediate maximal membership values (i.e., <0.5) for all B sub-clusters were found in a single sampling zone located between B1 and B2 clusters.

Similarly, sub-cluster B3 appear as a mix between A2 and B1 and therefore has not been considered as a genetically homogeneous population. Frequency-based approaches showed that population B1 and B2 are genetically close and that population A2 is genetically closer to B2 than to A1. Finally, the M-ratio reveal that A1, A2, B1 and B2 all suffered from bottlenecks probably caused by one or several founder effects. Lower allelic richness in northern sub-clusters compared to southern ones might indicate long term effects of stronger bottlenecks north of the distribution.

These results further evidence that the probability of occurrence of *T. immigrans* is higher in the southern part of the study area (Cordonnier et al., 2018). In addition, sites with *T. immigrans* were characterized by a higher percentage of impervious surfaces. However, no previous evidence suggested a significant interaction between these factors. Our results therefore reveal the effect of a mostly overlooked, when not simply ignored interaction between two global changes on species distribution, suggesting that in the north of its range, *T. immigrans* thrives under harsher climate by colonizing the most urbanized areas. This result is makes sense considering that most common species in heavily urbanized areas are more likely to have distributions that are skewed towards lower latitudes and, consequently, greater tolerance to dry conditions and warmer conditions than species found in more natural sites (e.g., Menke et al., 2011). The combined action of climate change and urbanization is clearly highlighted by this study and raises several conservation issues. In a perspective of accelerating global warming, the study of the colonization of urban areas could make it possible to anticipate the responses of some taxa to climate changes. Temperatures differences between urban environments and adjacent non-urban areas are often greater that the predicted global temperature change (Grimm et al., 2008). In addition, latitudinal gradients (from low to high latitudes) parallel the temperature profile in urban gradients (from urban cores to natural areas) because both low latitude and urban cores exhibit narrower temperature profiles characterized by increases in mean temperature when compared to high latitudes and natural areas respectively (Diamond et al., 2015). The originality of the present study is to consider both gradients simultaneously, a necessary perspective as the response of biodiversity to urbanization occur over regional scales, where variations in background climatic conditions play a key role (Diamond et al., 2015).

The observed pattern could reflect a relatively recent colonization of the northern part of the study area, subsequent to the 19th to 20th century urbanization at the origin of heat islands effects and increasing human trade. Following Menke et al.'s (2011) conclusions for urban ant communities in North Carolina, our results suggest that the urban heat island effect may have facilitated the establishment of *T. immigrans* in areas where climatic conditions were previously inhospitable. Introduction favoured by human activities would be the most likely hypothesis to explain this situation. That the species was almost only found in urban areas in the north of the study area strongly indicates one or more anthropogenic introductions, which are common in urbanized areas (Marzluff et al., 2001; McKinney, 2006; Cristescu, 2015; Aronson et al., 2016) as well as in ants (McGlynn, 1999; Heterick et al., 2013; Vonshak & Gordon, 2015). The landscape genetics analysis corroborates this hypothesis. Latitudinally distinct clusters and sub-clusters, with well-defined hybridization zones between clusters (i.e., in Vienne between A and B) and sub-clusters (in Montélimar between B1 and B2) do not hint at gradual isolation by distance between clusters, but more likely at different colonization histories.

Pairwise F_{ST} values calculated between sub-clusters confirmed this result as they do not follow a simple geographical pattern. In addition, reductions in population size suggested by low M-Ratios in all sub-clusters might indicated that more than one introduction occurred into the study area from one or more external sources. One likely hypothesis is that the observed pattern results from distinct introductory events followed by colonization favoured by human activities in the urban areas further north, and expansion in rural areas where climate matches preferences (Fig. 3). The opposite hypothesis of a gradual expansion northwards, e.g., resulting from a post-glacial recolonization event, would have generated a gradual signal leading to a linear differentiation pattern, unlike what is observed here. In addition, no latitudinal structuration of the M index was found, a pattern that does not fit gradual recolonization processes.

Biological introductions concomitant with urbanization (Cristescu, 2015) could result in contact zones between species capable of genetic exchanges because of a lack of pre-zygotic, geographical or ecological reproductive barriers, as shown by Crispo, Moore, Lee-Yaw, Gray and Haller (2011) in numerous taxa. Here, recent establishment of *T. immigrans* in the northernmost areas could have led to contact and hybridization with *T. caespitum*, as suggested by Wagner et al. (2017). In addition, the presence of *T.*

immigrans seems to lead to an exclusion of *T. caespitum* as *T. caespitum* is present in cities in the absence of *T. immigrans* (e.g. in Dijon or Langres; Cordonnier et al., 2018).

The observed pattern is similar to the situation described in North America, where *T. immigrans* is known to be invasive since the early nineteenth century (King & Green, 1995) and where it has been described as an urban specialist (King & Green, 1995). In the north of its distribution studied here, this species seemed to be a good urban exploiter, which raises questions about its status as a native species in Europe. Considering habitat preferences and competitive exclusion, the situation observed in south-eastern France is strongly reminiscent of the situation described in its invasive range in North America (Steiner et al., 2008a). It appears therefore likely that *T. immigrans* might not be native to the present study area. Continent-wide investigations mobilizing genetic tools to find the probable source population should make it possible to test this hypothesis.

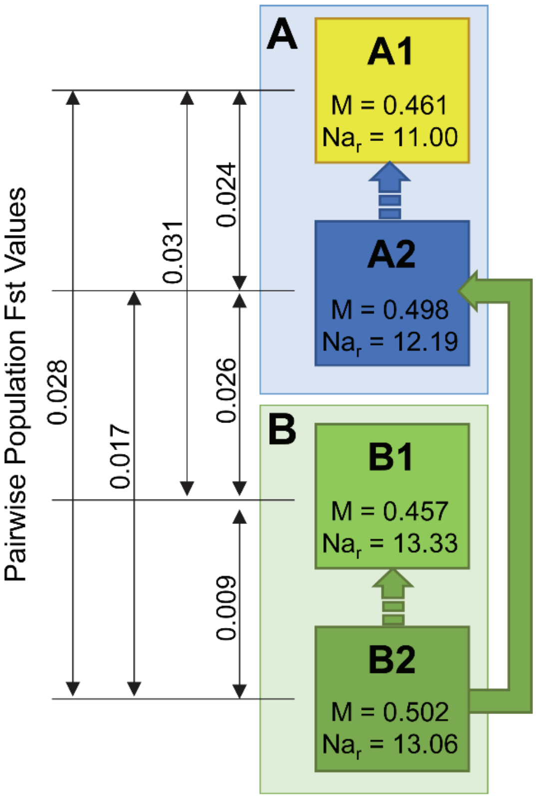

Figure 3: Compilation of the main results of the study (pairwise F_{ST} values between sub-clusters defined by Bayesian clustering, M-ratio and rarefied number of alleles for each sub-cluster) and proposition of a colonization scenario fitting the observed patterns (full arrows indicating likely introduction event, and interrupted arrows indicating possible introduction events or gene flow).

The complexity of patterns highlighted by this study questions the consequences of the establishment of *T. immigrans* as well as the risk of the species colonizing cities further north (it is for example already present in Paris, 100km North of the study zone; Wagner et al., 2017). The hybridization of *T. immigrans* with *T. caespitum* should be researched thoroughly and the long-term impact of these interspecific genetic exchanges be evaluated. The present results should allow the construction of predictive models of the species' range expansion in a warming and urbanizing world. Our study also tells of a world where global changes interact to create novel assemblages of genes and species with yet unforeseen consequences.

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SUPPORTING INFORMATION

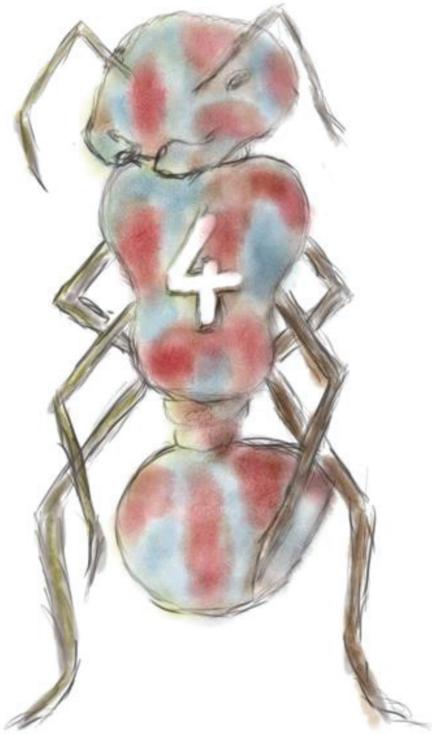
Table S1. Statistics of microsatellite markers computed using GENALEX (Peakall and Smouse, 2006) in 2 reconstructed populations defined by Bayesian clustering (Number of alleles and effective number of 3 alleles, expected and observed heterozygosity, F, Hardy-Weinberg Equilibrium HWE).

Peakall, R. O. D. and Smouse, P. E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic 7 software for teaching and research. - Molecular Ecology Resources 6: 288-295.

Locus	Cluster A1								Cluster A2							
	Na	Ne	Ho	He	F	HWE			Na	Ne	Ho	He	F	HWE		
						DF	ChiSq	Prob						DF	ChiSq	Prob
Tspe52d	11	5.328	0.769	0.812	0.053	55	58.819	0.337	15	5.353	0.868	0.813	-0.068	105	75.851	0.986
Tspe52b	14	7.144	0.904	0.860	-0.051	91	82.470	0.727	27	7.244	0.886	0.862	-0.028	351	385.026	0.102
Tspe52k	12	7.988	0.865	0.875	0.011	66	61.726	0.626	13	3.986	0.781	0.749	-0.042	78	41.911	1.000
Ttsu56d	8	3.395	0.673	0.705	0.046	28	94.411	0.000	12	3.111	0.728	0.679	-0.073	66	36.030	0.999
Ttsu55a	15	6.318	0.788	0.842	0.063	105	161.701	0.000	19	6.820	0.868	0.853	-0.018	171	229.167	0.002
Ttsu59j	8	5.809	0.827	0.828	0.001	28	31.455	0.297	9	4.829	0.825	0.793	-0.040	36	30.253	0.738
Tspe51i	15	7.804	0.827	0.872	0.052	105	116.956	0.200	17	6.033	0.841	0.834	-0.008	136	82.562	1.000
Ttsu58i	14	6.712	0.824	0.851	0.032	91	69.419	0.955	18	7.985	0.877	0.875	-0.003	153	133.604	0.869
Tspe51a	10	6.399	0.961	0.844	-0.139	45	47.602	0.367	14	5.523	0.789	0.819	0.036	91	81.703	0.747
Tspe51o	13	7.051	0.885	0.858	-0.031	78	108.640	0.012	20	9.404	0.886	0.894	0.009	190	270.261	0.000
Tspe51d	7	2.456	0.673	0.593	-0.135	21	9.276	0.987	10	3.143	0.667	0.682	0.022	45	93.083	0.000
Tspe52a	10	5.376	0.827	0.814	-0.016	45	25.620	0.991	11	5.665	0.781	0.823	0.052	55	53.039	0.550
Tspe53b	9	3.855	0.731	0.741	0.013	36	123.321	0.000	11	4.121	0.779	0.757	-0.028	55	81.108	0.013
Ttsu57l	8	3.257	0.600	0.693	0.134	28	55.799	0.001	15	2.450	0.398	0.592	0.327	105	404.151	0.000

Locus	Cluster B1								Cluster B2							
	Na	Ne	Ho	He	F	HWE			Na	Ne	Ho	He	F	HWE		
						DF	ChiSq	Prob						DF	ChiSq	Prob
Tspe52d	16	5.080	0.815	0.803	-0.015	120	77.577	0.999	14	4.340	0.771	0.770	-0.002	91	101.423	0.214
Tspe52b	21	10.398	0.914	0.904	-0.011	210	203.045	0.622	22	7.995	0.867	0.875	0.009	231	230.249	0.502
Tspe52k	11	5.723	0.802	0.825	0.028	55	71.329	0.068	14	4.501	0.790	0.778	-0.016	91	81.696	0.747
Ttsu56d	11	4.824	0.840	0.793	-0.059	55	131.370	0.000	15	3.637	0.710	0.725	0.021	105	221.119	0.000
Ttsu55a	19	8.248	0.901	0.879	-0.026	171	257.177	0.000	25	9.199	0.894	0.891	-0.003	300	235.925	0.997
Ttsu59j	15	5.586	0.790	0.821	0.038	105	124.418	0.095	18	5.508	0.837	0.818	-0.023	153	86.669	1.000
Tspe51i	19	8.599	0.864	0.884	0.022	171	174.104	0.420	28	11.006	0.900	0.909	0.010	378	285.500	1.000
Ttsu58i	15	6.309	0.827	0.841	0.017	105	76.474	0.984	19	7.058	0.848	0.858	0.012	171	184.209	0.232
Tspe51a	12	5.591	0.852	0.821	-0.037	66	75.149	0.206	14	3.965	0.714	0.748	0.045	91	218.866	0.000
Tspe51o	19	6.785	0.827	0.853	0.030	171	177.440	0.352	30	8.544	0.876	0.883	0.008	435	349.126	0.999
Tspe51d	12	7.705	0.840	0.870	0.035	66	50.344	0.924	14	6.858	0.843	0.854	0.013	91	71.999	0.929
Tspe52a	8	3.090	0.642	0.676	0.051	28	42.540	0.039	13	4.605	0.714	0.783	0.088	78	280.188	0.000
Tspe53b	9	4.931	0.825	0.797	-0.035	36	23.487	0.946	8	4.460	0.813	0.776	-0.049	28	31.987	0.275
Ttsu57l	15	5.913	0.642	0.831	0.227	105	142.949	0.008	18	4.647	0.589	0.785	0.249	153	360.896	0.000

Locus	Cluster B3							
	Na	Ne	Ho	He	F	HWE		
						DF	ChiSq	Prob
Tspe52d	13	4.635	0.789	0.784	-0.007	78	45.233	0.999
Tspe52b	18	6.113	0.825	0.836	0.014	153	153.972	0.463
Tspe52k	9	3.280	0.737	0.695	-0.060	36	91.950	0.000
Ttsu56d	9	3.630	0.632	0.725	0.128	36	57.249	0.014
Ttsu55a	16	8.963	0.965	0.888	-0.086	120	116.361	0.577
Ttsu59j	9	5.194	0.825	0.807	-0.021	36	25.253	0.910
Tspe51i	17	8.746	0.877	0.886	0.010	136	124.802	0.745
Ttsu58i	13	7.503	0.912	0.867	-0.053	78	124.047	0.001
Tspe51a	9	3.742	0.625	0.733	0.147	36	25.823	0.895
Tspe51o	20	9.556	0.895	0.895	0.001	190	189.447	0.498
Tspe51d	10	4.592	0.807	0.782	-0.032	45	85.966	0.000
Tspe52a	11	5.223	0.825	0.809	-0.020	55	42.155	0.898
Tspe53b	7	4.764	0.754	0.790	0.045	21	28.682	0.122
Ttsu57l	10	2.935	0.474	0.659	0.282	45	114.963	0.000



Chapter 4. Interspecific hybridization and introgression

> *« Hybride. Fruit d'une démarche associative. »*
>
> *Ambrose Bierce*

PAPER 4. From hybridization to introgression between two closely related sympatric ant species

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Abstract

Interspecific hybridization is becoming more frequent worldwide due to increasing global changes and translocations of organisms. For individual organisms, the most significant negative consequences are sterility or inviability of hybrid offspring. However, hybridization sometimes lead to fertile offspring, promoting introgression from one species into another. In such situations, hybridization can play a key role in evolution and speciation. Combining hypervariable DNA (microsatellites) and mitochondrial DNA markers with the use of several modelling methods allows an efficient detection of hybridization processes. The present study therefore investigates hybridization between two ant species, *Tetramorium immigrans* and *T. caespitum*, using multiple methods, and systematically comparing results with simulated data to ensure accurate identification of hybrids. Introgression was revealed both by backcross detection based on 14 nuclear microsatellite loci and by mitochondrial-nuclear discordance based on comparison with mitochondrial DNA (Cytochrome Oxidase I). Results were spatially consistent, with hybrids located at latitudes where parental species are sympatric. The causes and consequences of hybridization and introgression between *T. caespitum* and *T. immigrans* remain to be further investigated, especially because *T.immigrans* could be an invasive species in France.

Keywords: Bayesian clustering, Interspecific hybridization, Introgression, Mitochondrial DNA, Pavement ant, *Tetramorium*

INTRODUCTION

Hybridization, i.e., reproductive interactions between individuals whose lineages show some degree of evolutionary divergence (Harrison, 1990; Brennan *et al.*, 2015), has been demonstrated to be relatively common (Arnold, 1992, 2006; Mallet, 2005). Such interspecific genetic exchanges are becoming even more frequent worldwide due to increasing global changes and translocations of organisms by Humans (Brennan *et al.*, 2015; Allendorf, Leary, Spruell, & Wenburg, 2001). Hybridization can have negative impacts on species or ecosystems, through loss of biodiversity and ecosystem degradation (Brennan *et al.*, 2015) contributing directly and indirectly to the extinction of many species (Allendorf *et al.*, 2001). For individuals, the most significant negative consequences of interspecific hybridization may be sterility or inviability of hybrid offspring, explaining that despite hybridization is a common phenomenon, hybrid individuals within a population should be relatively rare (Butler, Peters & Kronauer, 2018). In some particular cases however, hybrid offsprings are fertile, and hybridization can lead to the introgression of genes from one species into another (Anderson, 1953; Patten, Carioscia & Linnen, 2015), which could provide new adaptive variations (Brennan *et al.*, 2015), and sometimes – although rarely – even lead to 'hybrid speciation' (Mallet, 2007; Schumer *et al.*, 2014, 2018). Hybridization can therefore play a key role in the evolution of many plant and animal taxa (Allendorf *et al.*, 2001; Arnold & Kunte, 2017), even if most authors agree on the negative effects of introgressions of non-indigenous into native gene pools (Mallet, 2005; Allendorf & Luikart, 2009).

In view of these consequences of hybridization, its detection is of major importance in ecology and evolution. The accurate detection of putative first-generation (F1) hybrids and backcrosses resulting from hybridization is a critical task. The use of hypervariable DNA markers (microsatellites) and new Bayesian modelling methods have dramatically improved admixture analyses and individual assignment testing (Randi *et al.*, 2008). Different approaches, implemented in software such as STRUCTURE (Pritchard, Stephens, Rosenberg & Donnelly, 2000; Falush, Stephens & Pritchard, 2003), BAPS (Corander & Marttinen, 2006) and NEWHYBRIDS (Anderson & Thompson, 2002), have been used in numerous studies to identify hybrid individuals based on simulated F1 hybrids and backcrosses (Vähä & Primmer, 2006; Sanz, Araguas, Fernández, Vera & García-Marín, 2009; Cabria *et al.*, 2011). These studies concluded that all these approaches lead to consistent identification of admixed first-generation hybrids and backcrosses. However, STRUCTURE seemed more efficient than BAPS to detect an admixed genotype and to correctly estimate an individual's ancestry composition (Bohling, Adams, & Waits, 2013). Burgarella *et al.* (2009) found that the use of STRUCTURE resulted in the highest power to detect hybrids, whereas NEWHYBRIDS provided the highest accuracy, and therefore suggested to combine these two complementary Bayesian approaches and to use simulation-based validation to gain resolution in the identification of admixed individuals. More recently, Beugin, Gayet, Pontier, Devillard, & Jombart (2018) compared the performance of the model underlying *Snapclust* with NEWHYBRIDS and suggested that although NEWHYBRIDS recovered more efficiently parental populations, *Snapclust* was not only faster but exhibited improved performances for the identification of hybrids at deeper levels of hybridization.

Because mitochondria are usually inherited from the mother, recurrent backcrossing of hybrid females with males from other lineage will ultimately lead to offspring with introgressed mitochondria, i.e., mtDNA from the maternal lineage and nuclear DNA from the paternal lineage (Darras & Aron, 2015). The sharing of mitochondrial haplotypes between sympatric, but genetically divergent lineages is therefore the signature of mitochondrial introgression. According to the review of Toews & Brelsford (2012), when foreign mtDNA haplotypes are found deep within the distribution range of a second taxon, those mtDNA haplotypes are more likely to be at a high frequency and are commonly driven by sex-biased asymmetries or adaptive introgression. Combining microsatellite markers and mtDNA should help improve discrimination between situations with only F1 hybrids and situations with introgression.

Such mtDNA and nuclear DNA marker combinations have promoted the detection of hybridization and introgression processes in various organisms. In many ant taxa, hybridization is common and sometimes leads to the evolution of reproductively isolated new lineages (Feldhaar, Foitzik & Heinze, 2008). The consequences of hybridization in social Hymenoptera differ from other species as most of the potential deleterious effects are mitigated by haplodiploidy and eusociality (Butler *et al.*, 2018). Haplodiploid sex determination appears to decrease the costs of mating with an allospecific male because viable hybrid workers help cross-mated queens to produce conspecific males from unfertilized eggs; when conspecific mates are not available, queens therefore could have a fitness interest to mate with an allospecific partner (Feldhaar *et al.*, 2008). Most often, hybridization in ants appears as an evolutionary dead-end given that fertile hybrids are rarely found (Feldhaar *et al.*, 2008; Purcell *et al.*, 2016). In such situations, hybrid zones reflect a balance between migration and selection against hybrids (Barton & Hewitt, 1989). Nevertheless, these hybrid zones can also be maintained without selection against hybrids, e.g., thanks to increased fitness of hybrids along narrow ecotones or to an equilibrium between migration and selection acting along an environmental gradient (Endler 1977; Moore, 1977). The consequences for gene exchange between species therefore make hybrid zones privileged places for studying the processes of divergence between lineages, as well as the mechanisms limiting genetic exchanges that can lead to speciation or, conversely, the meeting of differentiated entities (Harrison 1990). The few situations providing evidence of introgression, including the case studied here, may therefore provide a powerful way to investigate speciation in social insects (Purcell *et al.*, 2016).

The present study focuses on two ant species of the *Tetramorium caespitum* complex: *T. immigrans* Santschi, 1927 and *T. caespitum* (Linnaeus, 1758) (Wagner *et al.*, 2017). Wagner *et al.* (2017) suggested recent hybridization as source for mitochondrial-nuclear discordance found in two individuals with a *Tetramorium immigrans* mtDNA and which clustered with *T. caespitum* for Amplified Fragment-Length Polymorphism. Cordonnier *et al.* (2018) identified 285 individuals simultaneously associated to several species based on their genotypes, most of whom had an intermediate genotype between *T. immigrans* and *T. caespitum*. All these individuals with an intermediate genotype could not be assigned to a species in Cordonnier *et al.* (2018) and were therefore excluded from the analyses. In view of the results of Wagner et al. (2017), these individuals might be hybrids between *T. caespitum* and *T. immigrans*, which would suggest a relatively frequent hybridization between these two species. So far, hybridization between *T. caespitum* and *T. immigrans* has never been studied; it is currently suspected only through the three mitochondrial-nuclear discordances found by Wagner et al. (2017). We investigate here hybridization patterns between *T. immigrans* and *T. caespitum.* To detect potential introgression, we used both backcrosses detection based on nuclear DNA (14 microsatellites loci) and mitochondrial-nuclear discordance based on comparison with mtDNA (Cytochrome Oxidase I). To ensure the most accurate possible identification of hybrids, we combined methods implemented in STRUCTURE, NEWHYBRIDS and *Snapclust* and compared putative hybrids with simulated F1 hybrids and backcrosses. We subsequently compared the range limits of the detected hybrids to the ranges of parental species in order to validate the spatial consistency of our results.

METHODS

The present study focused on *Tetramorium immigrans* Santschi, 1927 and *T. caespitum* (Linnaeus, 1758), two cryptic species of the *Tetramorium caespitum* complex distinguishable using genetic (Cytochrome Oxidase I analysis) and morphometric characters on workers (Wagner *et al.*, 2017). *Tetramorium immigrans* is an invasive species in North America, where it was introduced in cities in the 19th century or earlier (Steiner et al. 2008) and is considered native in Europe where its status has never been investigated. However, a previous study in the Lyon urban area showed that *T. immigrans* occurred in fragmented and warm areas and questioned its status in Europe (Gippet *et al.* 2017). Ongoing works by our team reveal cues of colonization histories and founder effects, making it likely that it may not be native to Southeastern France.

Microsatellite data

Cordonnier *et al.* (2018) collected one ant worker per colony in 1690 colonies of *Tetramorium* belonging to five species (*Tetramorium semilaeve* André, 1883, *T. forte* Forel, 1904, *T. moravicum* Kratochvíl, 1941, *T. immigrans*, and *T. caespitum*). Sampling was carried out along a 460 km climatic gradient located in France, east of the Saône and Rhône Rivers, extending from the city of Langres in the North (47° 51′ 12″ N, 5° 20′ 02″ E) to the city of Tarascon in the South (43° 48′ 21″ N, 4° 39′ 37″ E). Samples were collected along a predefined path in diverse environments including urban pavements, roadsides, public parks, orchards, farmlands, fields, vineyards, meadows, riverbanks and forest, in 2015 and 2016 from April to September on non-rainy days with temperatures ranging from 16 to 28°C, with a minimum distance of 200 m between two colonies. Collected ants were stored in 96% ethanol. Samples were deposited as voucher material in the collection UCBLZ, CERESE, Université de Lyon, Université Claude Bernard Lyon1.

Cordonnier et al. (2018) successfully identified 544 workers of *T. immigrans* and 698 workers of *T. caespitum* using a two-step approach combining nuclear DNA clustering (14 microsatellite markers) and species identification by mtDNA cytochrome oxidase I sequencing. However, this sample also included 285 individuals that could not be identified because they were simultaneously associated to several species based on their genotypes, 240 of them having an intermediate genotype between *T. immigrans* and *T. caespitum* (i.e., their sum of membership coefficients for these two species was higher than 0.95). All these individuals were removed from the study by Cordonnier et al. (2018) and have therefore never been analyzed. In the present study, we randomly subsampled 150 of the 240 individuals with intermediate genotypes between *T. immigrans* and *T. caespitum*. We also randomly subsampled 150 individuals from pure parental species (membership > 0.95 in a single species) for both two species, to make sure that these individuals were representative of the entire area sampled in Cordonnier *et al.* (2018). Our final dataset included genotypes for 14 microsatellite markers (described in Cordonnier *et al.*, 2018) for this set of 150 *T. immigrans*, 150 *T. caespitum* and 150 putative hybrids. Following recommendations provided in Burgarella *et al.* (2009) and Vähä & Primmer (2006), we used simulated data to assess which method would provide the most reliable results in our experimental system. We used the computer program HYBRIDLAB 1.0 (Nielsen, Bach & Kotlicki, 2006) to simulate 150 multilocus F1 hybrid genotypes between *T. caespitum* and *T. immigrans*, and 150 multilocus first generation backcrosses (75 with each of the parental species) based on the 150 genotypes of each pure species described above. We then computed two comparative datasets combining (*i*) the 300 parental genotypes and the 150 simulated F1 hybrids, and (*ii*) the 300 parental genotypes and the 150 simulated backcrosses, respectively.

Mitochondrial DNA data

To improve discrimination between situations with only first generation hybrids and situations with backcrosses, 95 individuals (11 *T. caespitum*, 45 *T. immigrans*, and 39 hybrids) were Sanger-sequenced for a stretch of mitochondrial gene cytochrome oxidase I (COI). COI was amplified by PCR using specific primers developed from longer stretches of COI from the literature (Schlick-Steiner *et al.*, 2006) in a 30 µL total volume of 170 µM dNTPs, 0.1 µg.µL^{-1} BSA (Biolabs, B9001S), 0.16 µM of primers, 1.5 mM MgCl2, 2 µL DNA, 1.2 U Taq Polymerase (Eurobio, GAETAQ00), and 1X PCR Buffer (Eurobio, GAETAQ00). Amplifications consisted in 5 min at 94°C, then 40 cycles

(30 sec at 94°C, 30 sec at 48°C, and 30 sec at 72°C), and 5 min at 72°C. After purification, products were sequenced (service provided by BIOFIDAL on a ABI 3730xl sequencer) and compared to known sequences from Genbank using Blast-n to identify the sequenced species.

Analyses

Putative hybrid assignment based on genotypes

To determine the status of the 150 putative hybrids, we used assignment methods implemented in STRUCTURE v. 2.3.1 (Pritchard *et al.,* 2000), NEWHYBRIDS (Anderson & Thompson, 2002) and *Snapclust* (Beugin *et al.*, 2018) on genotypes of putative hybrids, simulated F1 and backcrosses. STRUCTURE was used to identify the ancestry composition of individuals, based on the admixture model with correlated allele frequencies, K = 2 clusters with ten iterations. Each run consisted in 500,000 replicates of the MCMC after a burn-in of 500,000 replicates. Clustering results were analyzed using CLUMPP v. 1.2.2 (Jakobsson & Rosenberg, 2007) to determine the proportion of individual genomes originating from each cluster (Q-values). As STRUCTURE does not allow labelling hybrids with an associated probability, we used two alternative methods to measure hybrid status. *Snapclust* relies on the combination of a geometric approach (i.e., it clusters individuals based on their distances in the genetic space spanned by the allelic data, without assuming a specific population genetics model; Jombart, Devillard & Balloux, 2010) and fast likelihood optimization to more explicitly identify the hybrids between the two parental populations (Beugin *et al.*, 2018). The results of Beugin *et al.* (2018) on simulated backcrossed individuals showed that the use of membership assignment probabilities corresponding to backcrosses were not accurate enough to categorize individuals unambiguously. We therefore looked for group membership probabilities for both parental species and potential hybrids using hybridization coefficients corresponding to F1 (0.5) (see supporting information Fig. S1 for results based on backcrosses coefficients 0.25 and 0.75). As *Snapclust* does not discriminate backcrosses unambiguously, we specifically addressed the question of current introgression using NEWHYBRIDS to estimate the posterior probabilities (q) that an individual fall into five different genotype frequency classes: two parental classes (*T. caespitum* and *T. immigrans*) and three hybrid categories (F1, backcross with *T. caespitum*, and backcross with *T. immigrans*). The analysis was performed based on ten iterations carried out using Jeffreys's prior and setting the burn-in period to 20,000, with a MCMC length of 500,000 replicates.

We used several approaches to assign the analyzed individuals to a single genetic class. First, case-specific evaluations based on comparison with simulated genotypes of known ancestry were needed, because the reliability of outputs from assignment methods depends on the type and number of markers, hybridization rate, and sampling quality. We therefore compared results obtained in the four pools of individuals (pure parental individuals, simulated backcrosses, simulated F1 hybrids, and real putative hybrids) for each three assignment methods both graphically and numerically. We used a Q-value threshold $Tq = 0.95$, above which individuals were assigned to one genetic class, i.e., pure parental, F1 hybrid or backcross (NEWHYBRIDS, *Snapclust*), allowing comparison of these assignments with the Q-values obtained in STRUCTURE. Finally, we explored the distribution of individuals of each pool (pure parental individuals, simulated backcrosses, simulated F1 hybrids, and real putative hybrids) in six ranges of Q-value (0.5-0.6, 0.6-0.7, 0.7-0.8, 0.8-0.9, 0.9-0.95, >0.95) for the three assignment methods used here. For parental individuals, these distributions were systematically calculated based on analyses of the putative parent and hybrid data set.

Spatially explicit validation of the results

Previous studies revealed that latitudinal distributions of *T. caespitum* and *T. immigrans* are partially overlapping in the sampling area, *T. immigrans* being found more to the south than *T. caespitum*, with a sympatric zone between approximately 44.8°N and 47°N (Cordonnier *et al.*, 2018). We checked the consistency of our results in terms of spatial distribution of hybrids compared to parental species to confirm that the presence of hybrids was restricted to areas where both parental species are sympatric. For this purpose, we used latitudinal locations of hybrid individuals (including F1 and backcrosses) with congruent genotypic identification for all three methods, or with at least two congruent methods and not contradictory third (i.e., where an individual was assigned to no other class). We also used latitudinal locations of all 544 workers of *T. immigrans* and 698 workers of *T. caespitum* identified in Cordonnier *et al.* (2018). We then tested differences in terms of latitudinal location between *T. immigrans*, *T. caespitum* and interspecific hybrids to confirm that hybrids are located in intermediate latitudes compared to parental species. As latitudes are not normally distributed, we used a nonparametric Kruskal-Wallis test coupled with Mann-Whitney-Wilcoxon tests for contrasts (including a simple Bonferroni correction).

RESULTS

All three assignment methods lead to very similar results whatever the pool of individuals (pure parental individuals, simulated backcrosses, simulated F1 hybrids, and real putative hybrids; Fig. 1). Graphical patterns clearly show that the set of putative hybrids neither corresponded to situations with only parental species (pure individuals, supporting information Fig. S2) nor with only F1 hybrids, and therefore necessarily included backcrosses (Fig. 1).

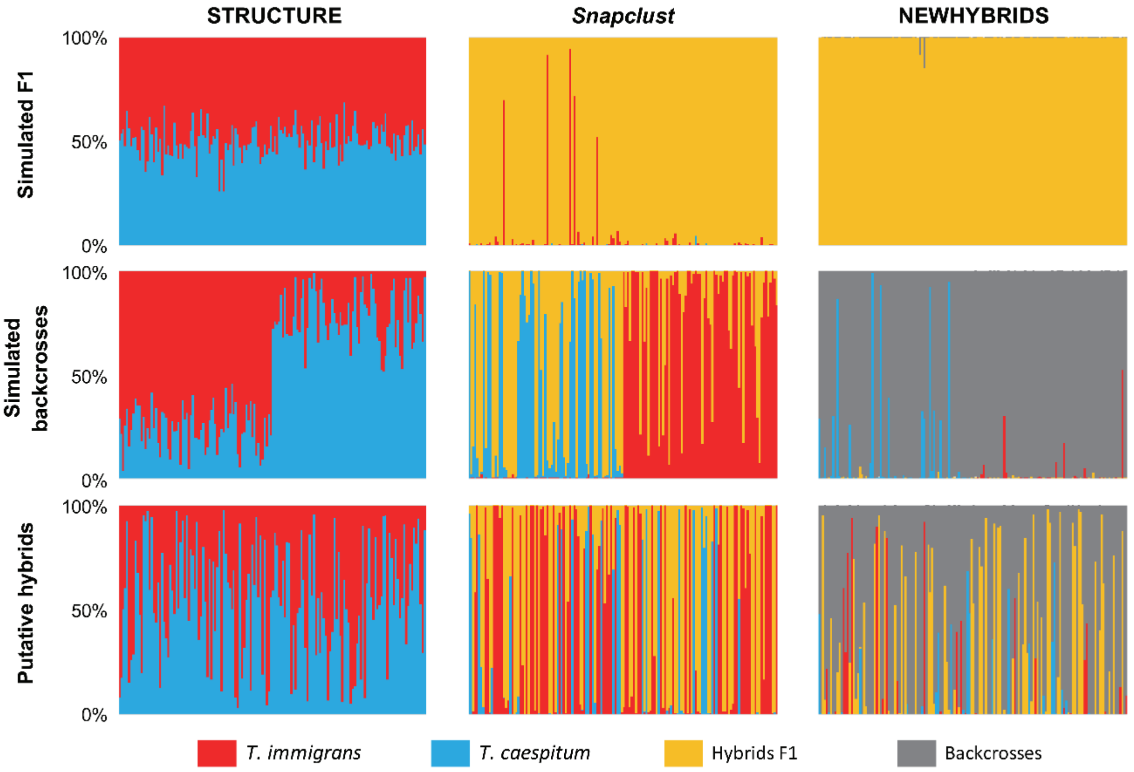

Figure 1. Barplots obtained for the three groups of hybrids (simulated F1, simulated backcrosses, and putative hybrids) and the three clustering methods (STRUCTURE, *Snapclust*, NEWHYBRIDS). Each vertical line corresponds to an individual. Colors indicate membership to each category (*T. caespitum* in red; *T. immigrans* in blue; F1 hybrids in yellow, and backcrosses in grey).

Distributions of individual Q-values of each pool (pure parental individuals, simulated backcrosses, simulated F1 hybrids, and real putative hybrids) confirm that putative hybrids were the product of F1 crosses but also backcrosses with parental species (Table 1-3).

Table 1: Distribution of individuals of each pool (pure parental individuals, simulated backcrosses, simulated F1 hybrids, and real putative hybrids) in six ranges of Q-value (0.5-0.6, 0.6-0.7, 0.7-0.8, 0.8-0.9, 0.9-0.95, >0.95) resulting from STRUCTURE assignment.

Q-values	pure parents	simulated backcrosses	simulated F1	putative hybrids
>0.95	99.0%	10.0%	0.0%	7.3%
0.9-0.95	1.0%	8.0%	0.0%	12.0%
0.8-0.9	0.0%	19.3%	0.0%	24.0%
0.7-0.8	0.0%	33.3%	1.3%	12.0%
0.6-0.7	0.0%	22.7%	16.7%	16.0%
0.5-0.6	0.0%	6.7%	81.3%	28.7%

Table 2: Distribution of individuals of each pool (pure parental individuals, simulated backcrosses, simulated F1 hybrids, and real putative hybrids) in six ranges of membership probabilities (0.5-0.6, 0.6-0.7, 0.7-0.8, 0.8-0.9, 0.9-0.95, >0.95) resulting from *Snapclust* assignment.

		pure parents	simulated backcrosses	simulated F1	putative hybrids
Parental species membership probabilities	>0.95	100.0%	36.7%	0.0%	38.7%
	0.9-0.95	0.0%	6.7%	1.3%	4.7%
	0.8-0.9	0.0%	7.3%	0.0%	7.3%
	0.7-0.8	0.0%	7.3%	0.7%	2.0%
	0.6-0.7	0.0%	4.7%	0.7%	4.0%
	0.5-0.6	0.0%	1.3%	0.7%	3.3%
F1 hybrids membership probabilities	>0.95	0.0%	19.3%	94.7%	32.7%
	0.9-0.95	0.0%	6.0%	2.0%	3.3%
	0.8-0.9	0.0%	6.7%	0.0%	0.7%
	0.7-0.8	0.0%	3.3%	0.0%	3.3%
	0.6-0.7	0.0%	0.0%	0.0%	0.0%
	0.5-0.6	0.0%	0.7%	0.0%	0.0%

Table 3: Distribution of individuals of each pool (pure parental individuals, simulated backcrosses, simulated F1 hybrids, and real putative hybrids) in six ranges of membership probabilities (0.5-0.6, 0.6-0.7, 0.7-0.8, 0.8-0.9, 0.9-0.95, >0.95) resulting from NEWHYBRIDS assignment.

		pure parents	simulated backcrosses	simulated F1	putative hybrids
Parental species membership probabilities	>0.95	95.7%	0.3%	0.0%	0.0%
	0.9-0.95	3.7%	1.0%	0.0%	0.7%
	0.8-0.9	0.3%	0.3%	0.0%	0.7%
	0.7-0.8	0.0%	0.0%	0.0%	1.3%
	0.6-0.7	0.3%	0.0%	0.0%	0.7%
	0.5-0.6	0.0%	0.3%	0.0%	0.7%
Backcrosses membership probabilities	>0.95	0.0%	83.3%	0.0%	40.7%
	0.9-0.95	0.0%	2.0%	0.0%	4.7%
	0.8-0.9	0.0%	3.3%	0.0%	4.7%
	0.7-0.8	0.0%	4.7%	0.0%	5.3%
	0.6-0.7	0.0%	2.0%	0.0%	5.3%
	0.5-0.6	0.0%	0.7%	0.0%	5.3%
F1 hybrids membership probabilities	>0.95	0.0%	0.0%	98.7%	5.3%
	0.9-0.95	0.0%	0.0%	0.7%	3.3%
	0.8-0.9	0.0%	0.0%	0.7%	6.7%
	0.7-0.8	0.0%	0.0%	0.0%	4.0%
	0.6-0.7	0.0%	0.0%	0.0%	2.7%
	0.5-0.6	0.0%	0.0%	0.0%	4.0%

Among the 150 potential hybrids tested, STRUCTURE identified 139 individuals as hybrids based on Q-value between 0.05 and 0.95. Three individuals were classified as *T. caespitum* and eight as *T. immigrans*. NEWHYBRIDS identified 61 backcross individuals, 8 F1 hybrids and 81 unclassifiable individuals based on 0.95 threshold value. Finally, using *Snapclust*, 58 individuals were considered pure parents (49 *T. immigrans* and 9 *T. caespitum*), 49 F1 hybrids, and 43 were unclassifiable, corresponding to putative backcrosses. Assignment of analyzed individuals to a single genetic class by NEWHYBRIDS and *Snapclust* based on threshold $Tq = 0.95$ corresponded to distinct ranges of Q-values obtained in STRUCTURE (Table 4).

Table 4: Range of Q-values obtained for the empirical (not simulated) data as a result of Bayesian clustering computed in STRUCTURE for each category of individuals defined by NEWHYBRIDS and *Snapclust* analyses (threshold value Tq > 0.95).

	min STRUCTURE Q-value	max STRUCTURE Q-value
Backcross [NEWHYBRIDS]	0.057	0.943
F1 Hybrids [NEWHYBRIDS]	0.393	0.607
Parental species [NEWHYBRIDS]	0.935	0.994
F1 Hybrids [*Snapclust*]	0.282	0.718
Parental species [*Snapclust*]	0.721	0.994

Snapclust favored parental species detection compared to other assignment methods (Table 2 & 4.), whereas NEWHYBRIDS detected no parental species in the putative hybrid pool (Table 3). Threshold Q-value of 0.95 in STRUCTURE appeared accurate to categorize an individual as pure species and strictly avoided inclusion of F1 hybrid individuals leading to misidentification of parental species (Table 4). Considering that an individual belongs to a pure species when Q-value is above 0.95 also limits the risks of assigning a potential backcrossed individual to parental species with a 10% error threshold (Table 1). Latitudinal locations of individuals assigned to hybrids categories were intermediate between *T. immigrans* and *T. caespitum* (Fig. 2).

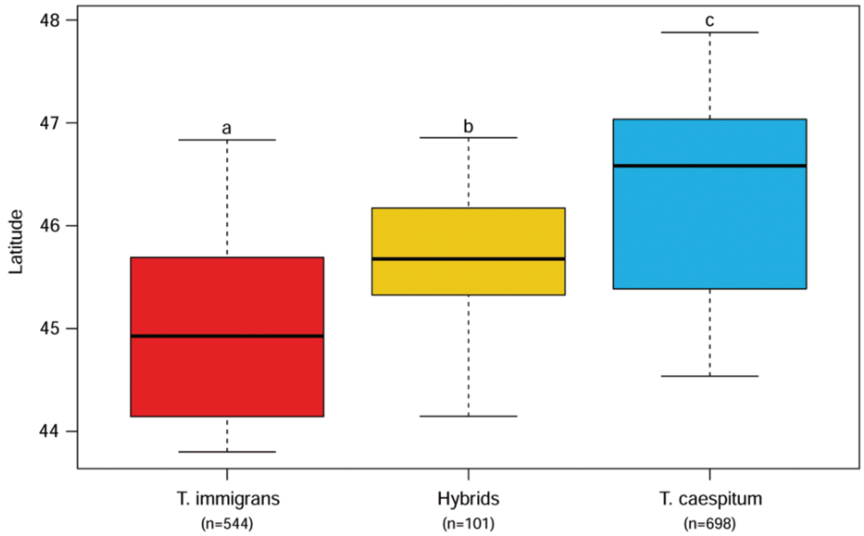

Figure 2. Latitudinal distributions of the individuals confirmed as hybrids and as pure parental species (Kruskal-Wallis chi-squared = 479.74, df = 2, p-value $< 2.2\times10^{-16}$). Thick black horizontal line: median value; box ends: first and third quartiles; whiskers: min and max values. Letters a, b, c indicate the results of the nonparametric Mann-Whitney-Wilcoxon tests (a-b: W = 41030, p-value = 3.2×10^{-15}; a-c: W = 55640, p-value $< 2.2\times10^{-16}$; b-c: W = 20032, p-value = 2.2×10^{-12}).

Finally, 11 out of the 15 F1 hybrids had haplotypes associated with *T. immigrans*, 12 of the 15 *T. immigrans* backcrosses had a *T. immigrans* haplotype and 8 of the 9 *T. caespitum* backcrosses had a *T. caespitum* haplotype (supporting information Fig. S3). Discordance frequency between nuclear assignment and mitochondrial haplotypes was similar in each parental species (respectively 3 out of 45 genotyped *T. immigrans* and 1 out of 11 genotyped *T. caespitum*), which suggests a bidirectional (in both parental species) and symmetric (with the same prevalence) mitochondrial introgression.

DISCUSSION

The present study proves the existence of hybridization processes between *Tetramorium immigrans* and *T. caespitum* and clearly evidences backcrosses of hybrids with parental species based on three methods and comparisons with simulated data. Results are spatially consistent, with hybrids located at latitudes where parental species are sympatric. In addition, mitochondrial-nuclear discordances suggest bidirectional and symmetric introgression between these species.

The 150 sampled individuals not fully assigned to *T. immigrans* or *T. caespitum* in Cordonnier *et al.* (2018) (Q-values < 0.95) thus appear to result from hybridization between these two species. The same detection methods based on a Q-value threshold have been already described for other taxa, e.g., between populations of wild and domestic cats in Italy (Randi *et al.*, 2001), between two oak species (*Quercus suber* and *Q. ilex*; Burgarella *et al.*, 2009), or between invasive sika and native red deer (*Cervus Nippon* and *C. elaphus*; Senn & Pemberton, 2009), as well as in ants, e.g., between *Formica selysi* and *F. cinerea* (Purcell *et al.*, 2016). Ito, Langenhorst & Inoue-Murayama (2015) considered that a high *qi* threshold value (> 0.95) confidently identifies pure individuals and allows the exclusion of potential hybrids. However, the reliability of assignment methods depends on the type and number of markers, the hybridization rate, and the quality of the sampling (e.g., Vähä & Primmer, 2006). Therefore, the use of a threshold of 0.95 to differentiate hybrids from parental species should be verified in each studied system, through a robust approach based on the multiplicity of methods and confirmation through simulations. Here, we confirm that the *qi* threshold value of 0.95

proposed in Cordonnier *et al.* (2018) to consider an individual as belonging to a pure species ensures that no F1 hybrid individuals are erroneously assigned to parental species. All three methods were congruent in recovering parental populations and discriminating individuals from both F1 hybrid and backcross classes. However, Snapclust tended to over-detect parental species compared to other assignment methods, whereas NEWHYBRIDS detected no parental species in the putative hybrid pool and was the most accurate at properly assigning the F1 hybrids and backcrossed individuals. Together, these findings clearly support the use of hyper variable microsatellite markers to identify F1 and backcrossed hybrids, and the use of mitochondrial markers to confirm the existence of mitochondrial introgression. The proposed marker (COI) is currently used in *T. immigrans* and *T. caespitum* for barcode identification of species (Schlick-Steiner *et al.*, 2006; Wagner *et al.*, 2017). Hybridization is sometimes incompatible with the use of DNA barcodes for species delimitation (e.g., Dupont, Porco, Symondson & Roy, 2016), more especially in haplodiploid systems (Patten *et al.*, 2015). The mito-nuclear discordances found in our study make barcode species identification within the *Tetramorium caespitum* complex questionable.

The large set of putative hybrids tested here included numerous backcrosses (more than 40% according to NEWHYBRIDS), revealing the first clues of interspecific reproductive events leading to fertile offspring. The occurrence of backcrossed individuals indicates that hybrid queens or males are fertile, which is confirmed by the presence of mitochondrial haplotypes of one species within another. The discovery of fertile hybrids is unusual in ants (Purcell *et al.*, 2016; Feldhaar *et al.*, 2008), and is particularly interesting as the numerous backcrosses compared to F1 hybrids may reveal a high fitness of hybrids, or at least a weak selection against hybrids. In such situations, hybridization sometimes results in the extirpation of one of the parental species or in the replacement of species pairs by hybrid swarms (Gilman & Behm, 2011). Interspecific hybridization may allow adaptive combinations to evolve at a higher rate (Mallet, 2005), therefore increasing the fitness of hybrids (Twyford & Ennos, 2012). Moreover, hybridization is a powerful engine for speciation, especially when hybrid lineages are ecologically or spatially divergent from the parent species (Twyford & Ennos, 2012). Previous studies about hybrid zones in ants have already provided insights into speciation.

For example, Cahan & Vinson (2003) showed that *Solenopsis xyloni* evolved a social hybridogenesis in the hybrid zone with *S. geminata*, leading to obligate hybridization for worker production, but preventing hybrids from being represented in the reproductive caste. Purcell *et al*. (2016) worked on the hybrid zone between *Formica selysi* and *F. cinerea* and showed an asymmetric distribution of hybrids skewed towards *F. cinerea*, suggesting a pattern of unidirectional nuclear gene flow from *F. selysi* into *F. cinerea*. The hybrid zone detected in the present study should therefore be studied much deeper in the future as it could provide a suitable system to investigate speciation in social insects.

Hybridization must be considered not only in its genetic but also in its ecological context. Studying the biological mechanisms facilitating interspecific mating between T. *immigrans* and T. *caespitum* should improve our understanding of hybridization processes between them. Wagner *et al.* (2017) already suggested that hybridization between these species might be facilitated by similar male genital morphology, overlapping phenology, and frequent sympatric occurrence. Since there are no anatomical barriers to mating between these species, the production of hybrid offspring may result from an absence of discrimination of the heterospecific partners. Weakly differentiated cuticular hydrocarbon profiles and diminished levels of interspecific aggression could further reveal a lack of species recognition cues (Purcell *et al.*, 2016). Studying whether hybridization is favored preferentially in males or females is also necessary, as Kulmuni & Pamilo (2014) showed that in two hybridized *Formica* species introgression is favored in diploid females but selected against in haploid males. It will be therefore necessary to measure whether hybrid queens or hybrid males are equally produced in the *T. caespitum*/*T. immigrans* hybrid system and to investigate if both sexes can produce viable offspring.

Finally, large-scale processes involved in the setting up of the hybrid zone described here should be explored. The literature associates hybridization with three main processes. (*i*) Increases in rates of hybridization and introgression are often attributed to translocations of organisms by humans (Allendorf *et al.*, 2001). When species colonize new environments, colonizing populations are often small, and opportunities for sexual reproduction may be limited by availability of mates, leading colonizing species to exploit the presence of heterospecific congeners (Hall, 2016). In addition, in contact zones, distinct lineages may hybridize, increasing genetic variation and reducing genetic constraints in newly formed hybrid populations, thereby increasing their genetic potential or adaptability (Roy, Lucek, Walter & Seehausen, 2015). Across ant taxa, Feldhaar *et al.* (2008) predicted that detailed research should reveal numerous additional cases of hybridization, in particular in those ant faunas that are characterized by the recent introduction of multiple invasive species. In addition, hybridization may play a significant role for introduced species to become invasive (Ellstrand & Schierenbeck, 2000; Allendorf & Luikart, 2007; Hall, 2016), e.g., allowing genetically admixed individuals to invade novel niches unoccupied by any of their parent species (Roy *et al.*, 2015). Mesgaran *et al.* (2016) showed for example that transient hybridization with the colonizer has probably driven the rapid replacement of the plant *Cakile edentula* by *C. maritima* over a large part of its invasive range in Northwestern America, New Zealand, and Australia. (*ii*) Habitat disturbance is also considered responsible for the increase in hybridization between species in recent years. Indeed, hybridization between naturally co-occurring species that normally do not interbreed is being documented following anthropogenic habitat modifications for an increasing number of taxa (Allendorf *et al.*, 2001; Grabenstein & Taylor, 2018). Identifying the conditions promoting hybridization in disturbed habitats, the generality of these conditions across taxa, and the taxa most affected by human-mediated changes is therefore critical for furthering our understanding of human impacts on hybridization (Grabenstein & Taylor, 2018). (*iii*) Finally, climate changes also promote hybridization or introgression. In response to climate changes, species change their distributions, leading to new contact zones between previously isolated taxa (Brennan *et al.*, 2015). For example, several studies shown that when hybridization occurs within a contact zone between two closely related bird species, interspecific interactions and climate interact in determining hybrid zone location and dynamics (Reudink, Mech, Mullen, Curry & Klicka, 2007; Taylor *et al.*, 2014; McQuillan & Rice, 2015). Garroway *et al.* (2010) recorded the first report of hybrid zone formation following a range expansion induced by contemporary climate change between two species of North American flying squirrel. In plants, the impact of climate changes has also been reported to increase opportunities for hybridization among previously isolated lineages, facilitating the breakdown of reproductive barriers and the formation of hybrids (Vallejo-Marin & Hiscock, 2016).

Given latitude overlap between the distribution of *T. caespitum* and *T. immigrans* (Cordonnier *et al.*, 2018), the potential role of urbanization-induced disturbance in habitats on the presence of *T. immigrans* (Wagner *et al.*, 2017; Gippet *et al.*, 2017; Cordonnier *et al.*, ongoing work), the fact that *T. immigrans* is invasive in North America (Steiner *et al.*, 2006, 2008) and has a questioned status in France (Gippet *et al.*, 2017) where it also could have been introduced (Cordonnier *et al.*, ongoing work), the causes and consequences of hybridization will deserve further investigation focused on the hybridization zone delineated in the present study.

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SUPPORTING INFORMATION

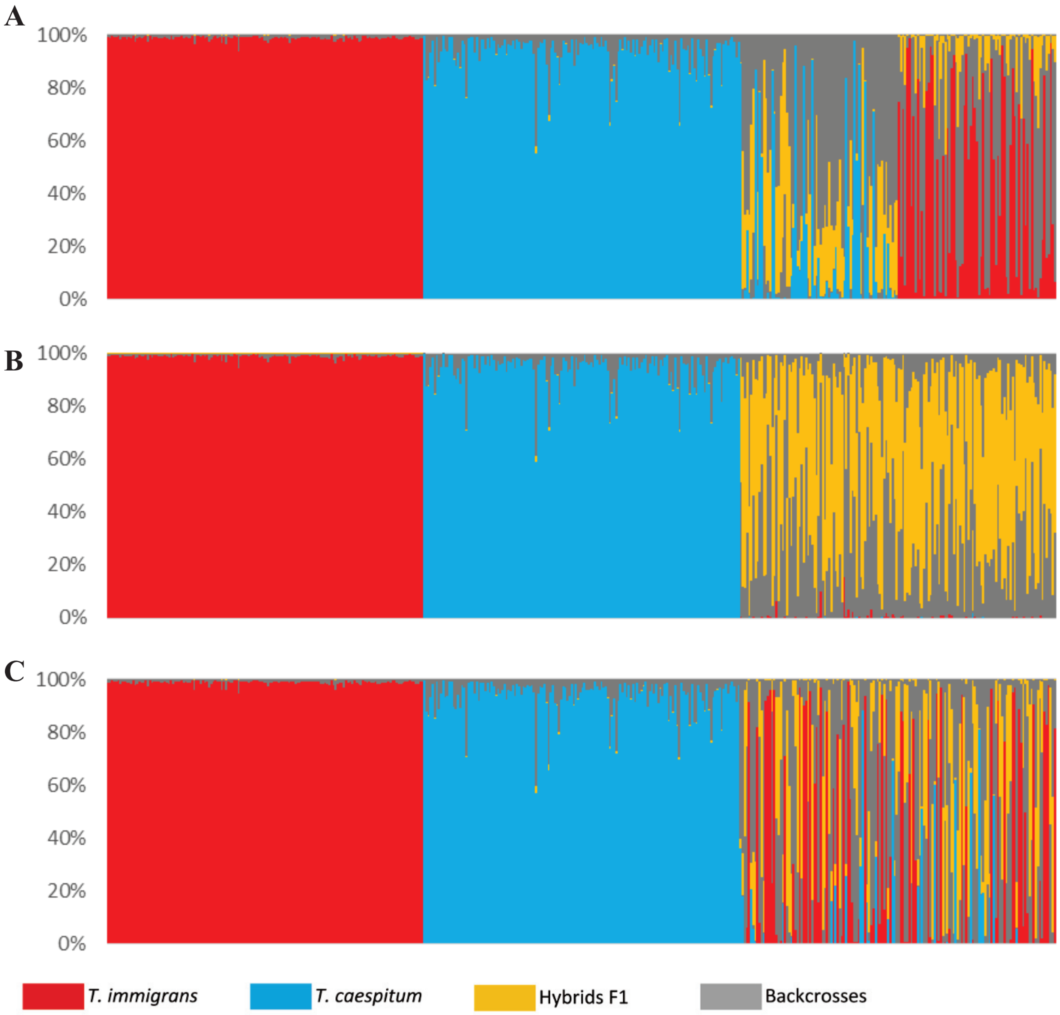


Figure S1. Barplots obtained from Snapclust with hybrid coefficients 0.25 and 0.5 (A: Parental species + simulated F1; B: Parental species + simulated backcrosses; C: parental species + putative hybrids). Each vertical line corresponds to an individual. Colors indicate membership to each category (*T. caespitum* in red, *T. immigrans* in blue, F1 hybrids in yellow, and backcrosses in grey).

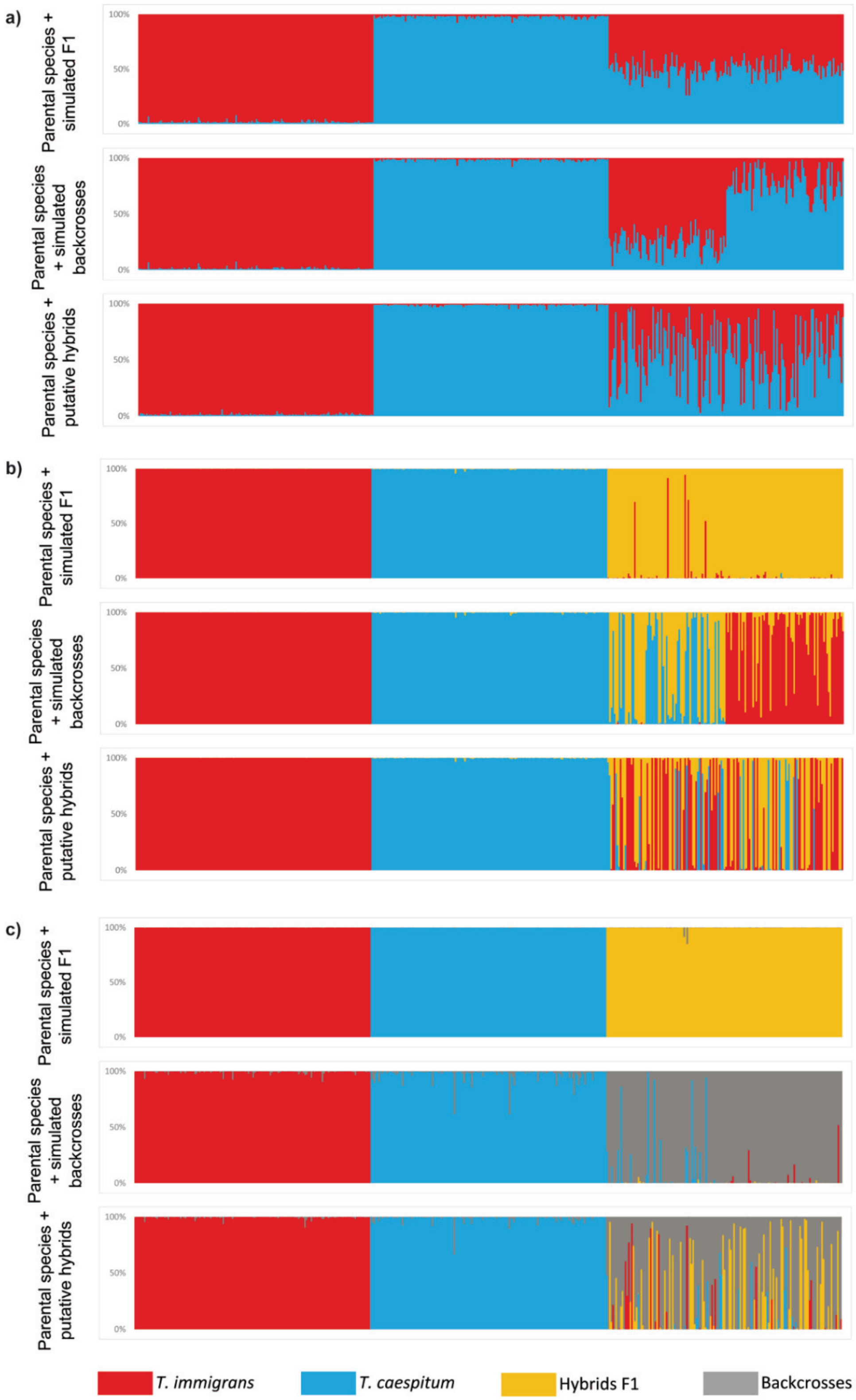
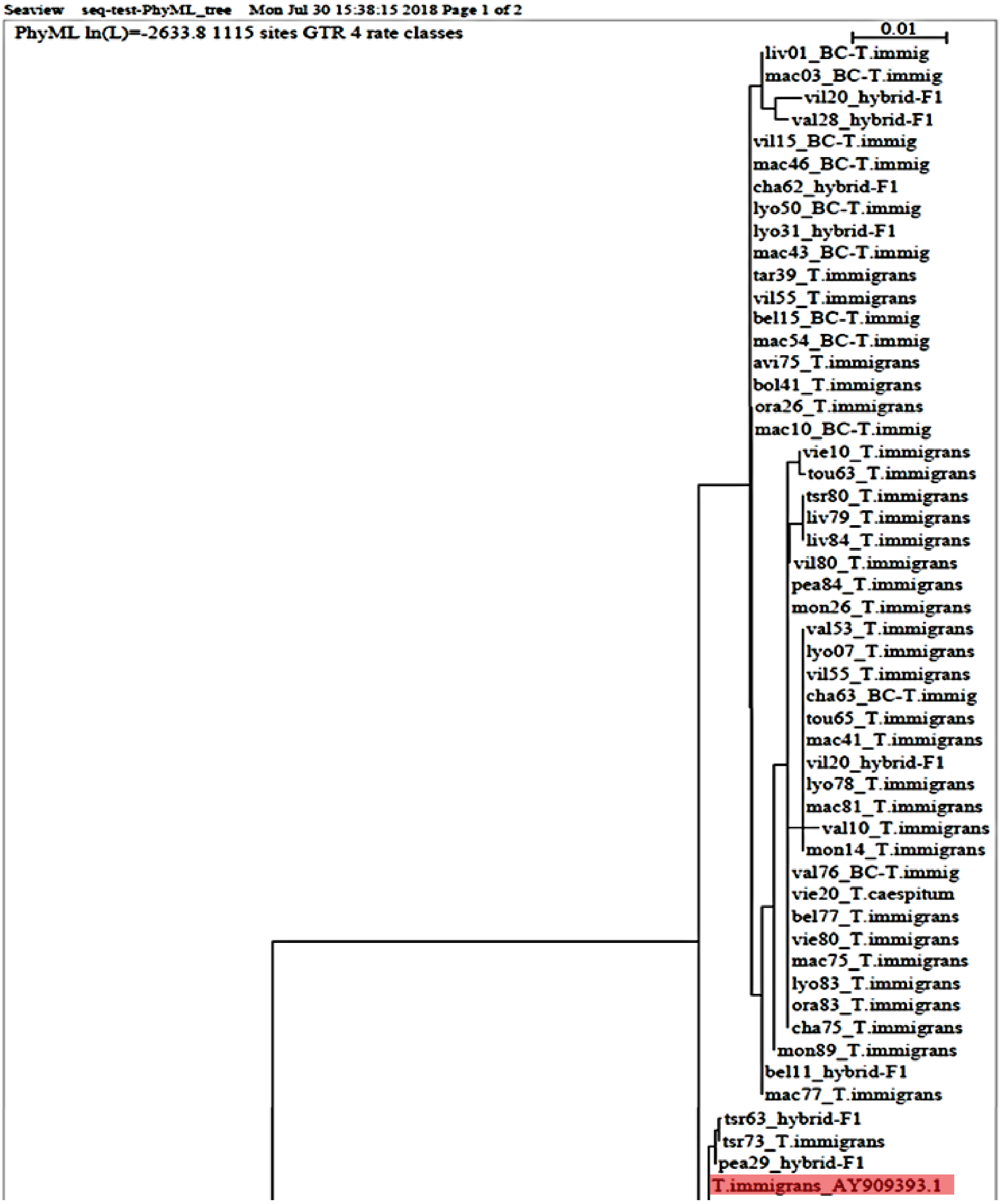


Figure S2. Barplots obtained from the three clustering methods (a: STRUCTURE; b: *Snapclust*; c: NEWHYBRIDS). Each vertical line corresponds to an individual. Colors indicate membership to each category (*T. caespitum* in red; *T. immigrans* in blue; F1 hybrids in yellow, and backcrosses in grey).


Seaview seq-test-PhyML_tree Mon Jul 30 15:38:15 2018 Page 1 of 2
PhyML ln(L)=-2633.8 1115 sites GTR 4 rate classes
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mac03_BC-T.immig
vil20_hybrid-F1
val28_hybrid-F1
vil15_BC-T.immig
mac46_BC-T.immig
cha62_hybrid-F1
lyo50_BC-T.immig
lyo31_hybrid-F1
mac43_BC-T.immig
tar39_T.immigrans
vil55_T.immigrans
bel15_BC-T.immig
mac54_BC-T.immig
avi75_T.immigrans
bol41_T.immigrans
ora26_T.immigrans
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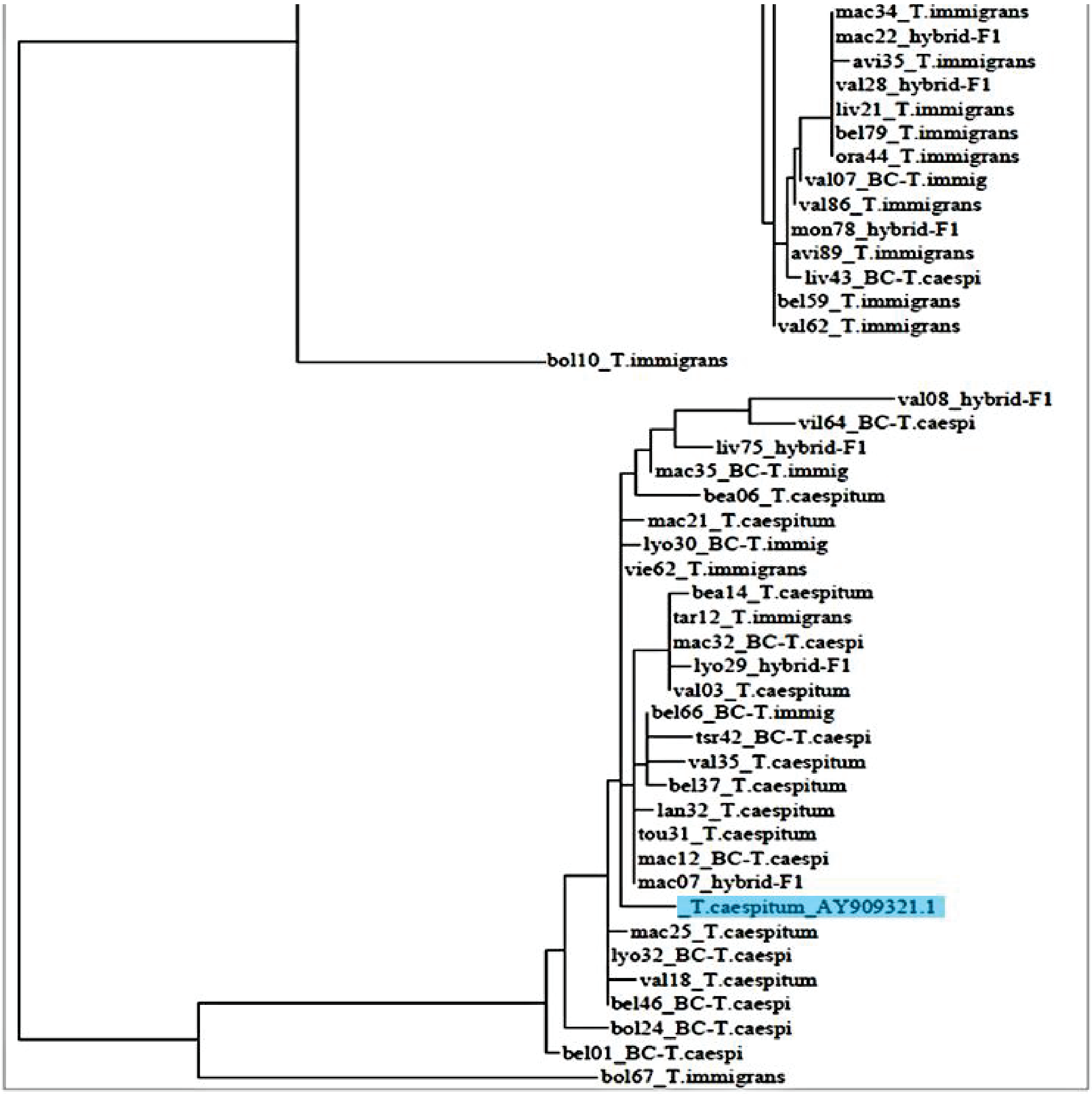

Figure S3. Global relationships of haplotypes based on COI sequences generated in this study and compared to reference sequences obtained from GenBank for *Tetramorium immigrans* (in red) and *T. caespitum* (in blue) (sequence names include species names and GenBank accession number). "T.immigrans" indicates a genotype from *Tetramorium immigrans*, "T.caespitum" indicates a genotype from *T. caespitum*, "BC" indicates a backcross and "hybrid-F1" indicates a genotype of first-generation hybrid. All sequences were aligned using the default options in MUSCLE v3.8.31 as implemented in SeaView v4.2.9. Based on these aligned sequences, the tree has been calculated using the PhyML algorithm with the GTR distance without invariable sites, optimized nucleotide equilibrium frequencies, and tree-searching operations involving best of NNI & SPR. The branch lengths are proportional to estimated divergence along each branch.

PAPER 5. Discriminate conspecifics from heterospecifics in a hybrid zone: from behavioral cues to chemical signals

This article is in prep. for a submission in Behavioral Ecology

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Abstract

Species and nestmate recognition in social insects occurs mostly through cuticular hydrocarbons acting as chemical cues. These compounds generate a colony-specific odor template depending on genetic and environmental factors. Interindividual recognition results in specific behavioral responses, regulating the level of aggressiveness towards other individuals during an interaction. Although species discrimination and recognition cues have been poorly studied in the context of interspecific hybridization, such systems offer an opportunity to further investigate the influence of heritable and environmental factors on recognition. We explore the strength of discrimination in a hybrid zone between two ant species – *Tetramorium immigrans* and *T. caespitum* – by comparing cuticular hydrocarbon profiles and measuring intra- and interspecific worker aggression in both areas of sympatry and areas of allopatry among species. Species cuticular hydrocarbon profiles is well-differentiated and interspecific aggression is high, revealing highly discriminating species recognition cues. Hybrids' cuticular hydrocarbon profiles consist in a mixture of the parental bouquets, but also exhibit hybrid-specific patterns. Behavioral assays show that *T. immigrans* is as aggressive towards hybrids as towards heterospecifics. Finally, aggression between heterospecific workers is lower when protagonists came from areas of sympatry among species than from areas of allopatry, in accordance with the "dear enemy" phenomenon. Taken as a whole, these findings paint a particularly complex picture of the recognition cues system in *T. immigrans*, *T. caespitum* and their hybrids, and highlights that hybrid zones afford a still under-explored opportunity for investigating recognition mechanisms and discrimination between species.

Keywords: Aggressive behavior, Cuticular hydrocarbons, Hybrid zone, Recognition cues, *Tetramorium*

INTRODUCTION

Species, nestmate and caste recognition in social insects occurs mostly through chemical cues, especially by means of cuticular hydrocarbons (CHC) (Hölldobler and Willson 1990). Primarily acting as a barrier against desiccation and pathogens, CHCs have been co-opted to serve as a multicomponent cue in solitary species for reproductive behavior, as well as in social species where they serve at least as signature mixtures and pheromones at various levels (individual, within colony, and between colonies) (D'Ettorre et al. 2017). CHCs are stored in the post-pharyngeal gland and exchanged between members of the colony by trophallaxis, allogrooming, and physical contact (Lenoir et al. 2001; Chapuisat et al. 2005). The resulting chemical mix contributes to the creation of a colony-specific odor template (Frizzi et al. 2015). This odor depends on (*i*) the environment, including chemical compounds coming from sources such as food, construction materials of the nest, and microorganisms associated with the colonies, (*ii*) physical contacts among nestmates, and (*iii*) genetically heritable odors resulting from metabolites produced by the ants themselves, such as hydrocarbons (reviewed in Dimarco et al. 2010). As a consequence, both genetic (Dronnet et al. 2006) and environmental factors (e.g., heavy metal pollution, Sorvari and Eeva 2010; food, Chen and Nonacs 2000; Liang and Silverman 2000) can induce changes in CHCs profiles. These factors therefore play a major role in interindividual recognition and can alter behavior, specifically generating increase or decrease in aggression towards the partner during an interaction (Frizzi et al. 2015). Such mechanisms thus allow the recognition of nestmate and limit aggressiveness between nestmates, which is crucial to regulating colony cohesion and interactions with other colonies. The "dear enemy" phenomenon (Wilson 1975) leads residents to be less aggressive towards owners of neighboring territories than towards foreigners, making ants less aggressive towards neighboring relatives, and could therefore play a significant role in interindividual recognition (Heinze et al. 1996; Thomas et al. 1999). This less aggressive behavior between neighbors could be due to the fact that geographically closer colonies are genetically closely related and therefore have a more homogeneous odor signal (Dimarco et al. 2010). As an alternative hypothesis, workers could learn the chemical profile of neighbor colonies (Gordon 1989), via a habituation phenomenon. In such situation, neighbors could recognize the cuticular compounds of the neighboring colonies as the persistent odor is experienced repetitively, independently of the genetic divergence between them (Dimarco et al. 2010), which ultimately could lead ants to modify their aggressive responses during subsequent encounters (Heinze et al. 1996; Langen et al. 2000; but see Boulay et al. 2007). This reduced aggression could also be explained by the fact that neighboring colonies share the same environmental context, including the same diet and nest material, therefore homogenizing their cuticular chemical profile (D'Ettorre and Lenoir 2010). Thus, through these mechanisms, two sympatric colonies could share more similar colonial odors and be less aggressive towards each other than towards distant colonies.

Recognition cues could be even more complex when hybridization occurs between species. Although hybridization is relatively common in ants (Feldhaar *et al.* 2008), species discrimination and recognition cues have so far been poorly studied in the context of interspecific hybridization (but see Fadamiro et al. 2009; El-Shehaby et al. 2011). Investigating the cuticular profiles of hybrids might help to disentangle the influence of genes and environment on colony odor (El-Shehaby et al. 2011). For example, in fire ants, *Solenopsis invicta* x *richteri* hybrids had cuticular hydrocarbons corresponding to a mixture of the parental bouquets (Vander Meer et al. 1985) and were more aggressive towards hybrid non-nestmates than either parental species, due to a higher variability of genetic recognition cues (Obin & Vander Meer 1989). In such hybrid complexes, not only the genetic aspect of discrimination but also the impact of environment can be investigated. For example, Purcell et al. (2016) investigated species discrimination between *Formica selysi* and *F. cinerea* and found strongly differentiated cuticular hydrocarbon profiles and heightened interspecific aggression revealing robust species recognition cues, but no significant difference in aggression between workers from areas of sympatry among species or from areas of allopatry among species. This work suggested limited influence of environmental factors on recognition. Hybrid zones consequently offer an opportunity to investigate the influence of heritable and environmental factors on recognition and to address issues relative to the competitive ability of hybrids and parental species (Fadamiro et al. 2009).

Building on these premises, we investigate here the strength of discrimination in a hybrid zone between *Tetramorium immigrans* Santschi, 1927 and *T. caespitum* (Linnaeus, 1758) by comparing cuticular hydrocarbon profiles and measuring intra- and interspecific worker aggression between workers coming from both areas of sympatry and allopatry among species. *Tetramorium immigrans* is an invasive species in North America, where it was introduced in cities in the 19th century or earlier (Steiner et al. 2006, 2008). Although usually considered native in Europe, Cordonnier et al. (submitted in Journal of Animal Ecology) concluded that it may also be invasive in Southeastern France. *Tetramorium immigrans* mainly occur in urban habitats and can hybridize with *T. caespitum* who is present only in rural habitats in latitudes where *T. immigrans* occur (Cordonnier et al. 2018). Hybrids are fertile, leading to a high frequency of hybrid colonies in sympatric populations of these species (Cordonnier et al. submitted in *JZSER*). Our main objective was to provide insight into the structure of the hybrid zone and to identify factors that may prevent these two closely related species from collapsing into a single species. We analyzed chemical recognition cues in 10 pure colonies of *T. immigrans*, 7 pure colonies of *T. caespitum* and 13 hybrid colonies, as well as behavioral discrimination patterns through 273 behavioral assays involving 14 pure colonies of *T. immigrans*, 7 pure colonies of *T. caespitum*, and 6 hybrid colonies. The study aimed to: (*i*) determine if differentiated cuticular hydrocarbon profiles and heightened interspecific aggression reveal that species recognition cues are both present and perceived; (*ii*) provide an assessment of the discrimination of hybrids by individuals from parental species through both chemical cues (do hybrids have chemical profiles corresponding to a mixture of the parental CHCs?) and behavioral assays (are the species less aggressive towards hybrids than towards heterospecifics?); and (*iii*) evaluate the dear enemy phenomenon hypothesis in an interspecific hybridization context in investigating differences of aggression between heterospecific workers from areas of sympatry versus allopatry among species.

METHODS

Biological model

The present study focuses on *Tetramorium immigrans* Santschi, 1927 and *T. caespitum* (Linnaeus, 1758) (Wagner et al. 2017). Hybridization has been shown between *T. immigrans* and *T. caespitum* (Wagner et al. 2017; Cordonnier et al. 2018, submitted in *JZSER*). These species differ in their ecological specificities – e.g., Cordonnier et al. (submitted in Landscape Ecology) indicate that *T. immigrans* is present in niches characterized by higher level of impervious areas (i.e., more urbanized) than niches occupied by *T. caespitum*. The present study is based on colonies precisely located and monitored annually, previously genetically identified as *T. immigrans*, *T. caespitum*, F1 hybrids (from male and female of different species), or hybrids backcrossed with *T. immigrans* (*T. immigrans* males mated with hybrid females) (Cordonnier et al. 2018, in prep). All colonies sampled for this study were collected in Southeastern France, in the hybrid zone described in Cordonnier et al. (submitted in *JZSER*), corresponding to the area 6 (Mâcon area), 9 (Lyon area) and 13 (Valence area) in Cordonnier et al. (2018) (Tables 1 and 2). Sampling was conducted in April 2017 on non-rainy days with temperatures ranging from 16 to 25°C. At least 20 individuals from each sampled colony were deposited as voucher material in the UCBLZ collection (CERESE, UCBL, Université de Lyon). We measured two phenotypic traits linked to species discrimination: cuticular hydrocarbon profile (Experiment 1) and aggression between non-nestmate workers (Experiment 2). Sampling was performed by teams of two persons by collection with a fine-tip flexible forceps (Experiment 1) or an entomological aspirator (Experiment 2; see sampling details below).

Experiment 1 - Cuticular hydrocarbons (CHCs)

We assessed the cuticular hydrocarbon profiles of three groups of five workers per colony from 10 *T. immigrans* colonies, 7 *T. caespitum* colonies and 13 hybrid colonies including 7 colonies containing only F1 workers and 6 with only backcrossed workers (Cordonnier et al. in prep) from three sampling zones which were at least 20 km apart (n = 96 samples; Table 1; see Table S1 for colony details).

Table 1. Location and taxonomic content of the colony samples used for the Experiment 1.

	Location	*T. immigrans* colonies	*T. caespitum* colonies	Colonies with F1 hybrids	Colonies backcrossed with *T. immigrans*
Macon area	46°2863.43″N, 4°9547.77″E	4	2	3	3
Lyon area	45°7553.93″N, 4°8525.17″E	3	2	2	2
Valence area	44°9320.99″N, 4°9200.90″E	3	3	2	1

The worker groups were weighed (Metler Toledo XP6 with a resolution of 1 µg) and frozen at -20°C. We extracted cuticular components by immersing the same 5 workers three times successively in 50 µl of hexane solution for 10 min including 1 min mechanical stirring, and by transferring all 150 µl of this hexane solution and 1 µl of internal standard (Squalane 234311 Aldrich C_{30}; 0.5 mg.ml^{-1}) to a new vial. Subsequently the solution was evaporated at 35°C for four hours and the vial was stored at 5°C. Before the Gas Chromatography – Flame Ionization Detector (GC-FID) analysis, 20 µl of hexane were added in the sample and 3 µL of the solution were injected into an Agilent® 7890 GC-FID gas chromatograph equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 mm). The gas chromatograph was operated in splitless injection mode and two-layer sandwich injection type, with helium used as the carrier gas. The oven temperature was set to 80°C and ramped up at 20°C.min^{-1} to a temperature of 150°C and then at 4°C.min^{-1} to a final temperature of 300°C for 20 min. The position and corrected height of each CHC peak were assessed in OpenLAB ChemStation Edition C.01.05. Compound were identified by Gas chromatography - Mass spectrometry (GC-MS) in the Laboratory of Experimental and Comparative Ethology (P. D'Ettore, Université Paris 13).

All analyses were conducted using R v.3.3 software (R Development Core Team 2004). Peak areas were reported to a quantity (based on peak area of Squalane corresponding to 0.5 µg) and standardized by the sample mass, then square-root transformed to reduce the influence of the most abundant variables (Clarke and Warwick 2001). The mean quantities of each compound over the three replicates in each colony were then calculated. Only peaks present in at least 10% of the samples were kept in the analysis (Table S2). We first tested the effect of taxa on chemical profile by computing the Euclidian distance between CHC profiles for each sample and compared these distances between families using nonparametric (permutational) analysis of variance (permanova; package *vegan*; Oksanen et al. 2013). We then used Principal Component Analysis (PCA; package *ade4*; Dray and Dufour 2007) to identify the peaks most contributing to the variation along each axis. We subset the peaks that contributed to 50% of the overall variability explained by the three first axes of the PCA and tested again the effect of taxa on this subset to ensure that the reduction of the number of variables did not modify the result. We also performed a discriminant analysis, using a non-parametric version of Pillai's test to evaluate the significance of the eigenvalues (package *ade4*; Dray and Dufour 2007). Peaks with above-average contributions were considered the best candidates for kin-recognition cues.

Experiment 2 - Aggression between workers

We collected at least one thousand worker ants per colony sample from 14 *T. immigrans* colonies, 7 *T. caespitum* colonies and 6 hybrid colonies including 2 colonies with only F1 workers and 4 with only backcrossed workers (identified in Cordonnier et al. in prep) from the same three sampling zones used for Experiment 1 (Table 2; see Table S3 for colonies details).

Table 2. Locations and distribution across taxa of the colony samples used for Experiment 2.

Location		*T. immigrans* colonies (from areas of sympatry)	*T. immigrans* colonies (from allopatric urban areas)	*T.caespitum* colonies	Colonies with F1 hybrids	Colonies backcrossed with *T. immigrans*
Macon area	46°2863.43″N, 4°9547.77″E	2	2	2	0	3
Lyon area	45°7553.93″N, 4°8525.17″E	2	2	2	1	1
Valence area	44°9320.99″N, 4°9200.90″E	3	3	3	1	0

To evaluate the strength of species discrimination in areas of sympatry among species and in areas of allopatry among species, *T. immigrans* nests were collected in areas where *T. caespitum* occurs (n = 7; corresponding to the hybrid zone described in Cordonnier et al. 2018; area of sympatry among species; red area in Fig. 1) and in areas where only *T. immigrans* occurs (n = 7; area of allopatry among species; green area in Fig. 1), i.e., with at least 30% of impervious areas in a 500 m buffer, and defined by classes 1.1.1, 1.1.2 and 1.2.1 in Corine Land Cover 2012 (CLC 2012©, Copernicus (https://land.copernicus.eu/); Cordonnier et al. 2018; Fig. 1). All the collected colonies were kept at 24°C in artificial plastic nests with walls brushed with Fluon® PTFE (Polytetrafluoroethylene) to prevent escape (Fig. S1) and were fed with 10% honeyed water and dead insects (*Gryllus assimilis*).

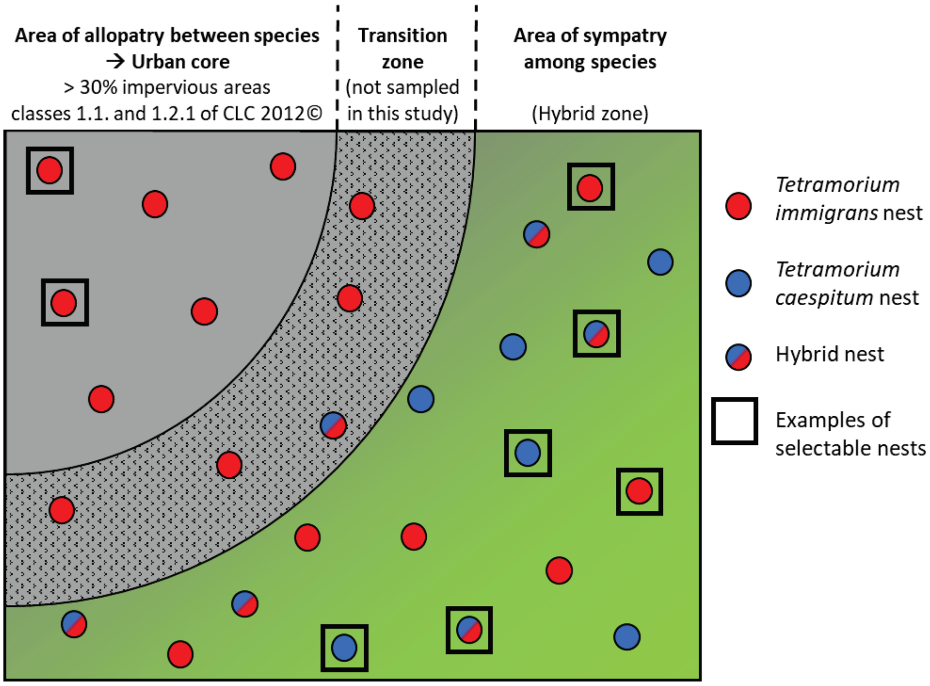

Figure 1. Schematic representation of sampling design of colonies. Sampling nests are framed. *Tetramorium immigrans* colonies (red) have been sampled in area of allopatry (grey) and in area of sympatry (green) among the two species; *T.caespitum* and hybrids colonies (resp. blue and bicolor) have been collected in the area of sympatry corresponding to the hybrid zone described in Cordonnier et al. (submitted in *JZSER*).

Ant aggressiveness was measured by monitoring five-min pairwise interactions between non nestmate workers. We tested the following combinations of workers: *T. immigrans vs. T. immigrans*, *T. immigrans vs.* Backcrossed, *T. immigrans vs.* F1 hybrids, T. immigrans *vs. T. caespitum*. For this last category, independently of geographical area, we distinguished combinations of *T. immigrans* from urban areas, i.e. areas of allopatry among species *vs. T. caespitum* and *T. immigrans* from areas of sympatry among species *vs. T. caespitum*. Pairs of ants were transferred to a Petri dish with sides lined with Fluon.

We filmed the interactions for five minutes with a Sony DCR-SX34 camera. For each interaction, the aggressiveness score was defined on the following scale (adapted from Suarez et al. 1999; Giraud et al. 2002; Boulay et al. 2007):

- "1" represents no change in direction or speed of movement and no initiation of new behavior;
- "2" represents a non-aggressive touching, usually with the antennae;
- "3" represents an avoidance behavior, such as a change in walking direction or an increase in speed after contact;
- "4" represents a temporary aggressive behavior, such as biting, pulling, mounting, or rearing;
- "5" represents prolonged fighting.

For each aggression test (n = 273), we retained the highest aggression score (Rosset et al. 2007). Scoring was carried out by two observers who were blind with respect to the origins of each combination, with a subset of cases scored by both to ensure consistency. As a control, 87 additional tests were performed to ensure the absence of intra-nest aggressiveness (n = 27 tests, the maximum score obtained was 2) and the replicability of the results over the dyad tested (n = 30 tests, involving 6 dyads representative of all the different combinations tested, with 5 replicates per dyads, 80% identity of the maximum score obtained, maximum difference of 1 with the score initially found) and over time (n = 30, same dyads tested during 10 mn, same scores obtained in all cases). To assess the statistical significance of differences in aggression within and between species and hybrids, we performed nonparametric Kruskal-Wallis test coupled with a chi-square test of independence and Mann-Whitney-Wilcoxon tests as well as independence permutation (package *coin*; Hothorn et al. 2008) for contrasts (including a simple Bonferroni correction). To assess if aggressiveness in heterospecific dyads was higher when *T. immigrans* workers came from urban areas compared to sympatric areas, we performed unilateral Mann-Whitney-Wilcoxon and independence permutation tests (package *coin*; Hothorn et al. 2008).

RESULTS

Experiment 1 - Cuticular hydrocarbons (CHCs)

Thirty compounds were identified (Fig. 2; Table S2).We first confirmed that chemical profiles depended on taxa (permanova; F = 8.41, df = 3, p-value = 0.001). The Principal Component Analysis allowed the identification of 19 peaks contributing to 50% of the overall variability explained by the three first axes (Table S2; Fig. 2).

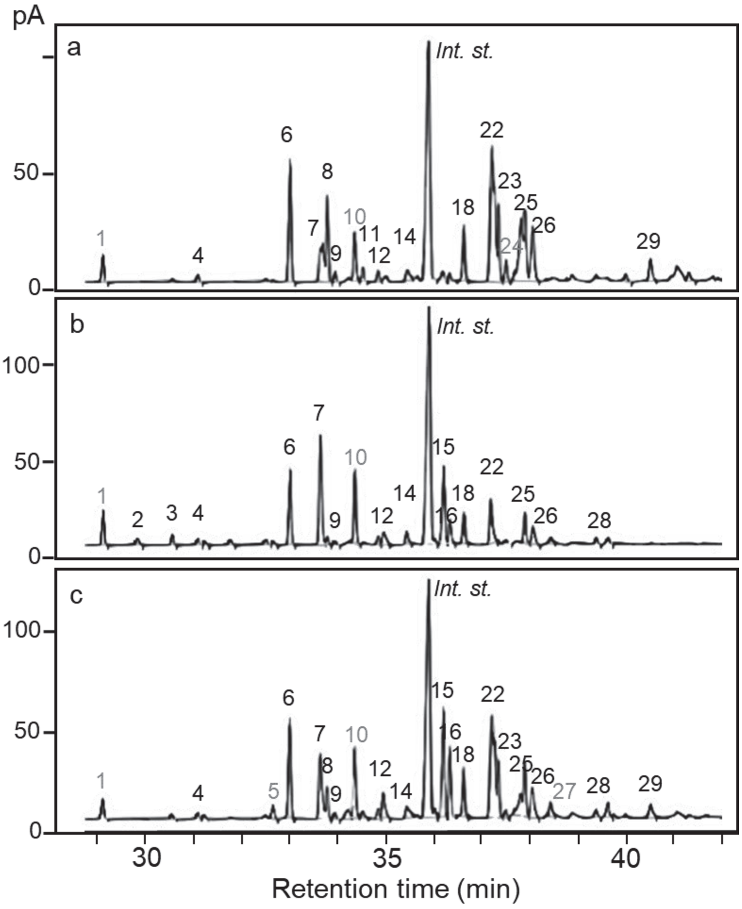

Figure 2. Examples of gas chromatograms of cuticular hydrocarbons for (a) *Tetramorium caespitum* colony (id mac_25) (b) *T. immigrans* colony (id val_10) and (c) F1 Hybrid colony (id lyo_31). The samples were extracted from 5 workers of the same colony. Peaks kept in the final analyses are identified by number in black. Peaks 13, 17, 19-21, and 30 are not identifiable in these gas chromatograms. Identified components: 1: C_{23}; 3: $3MeC_{23}$; 4: C_{24}; 6: C_{25}; 7: $13+11MeC_{25}$; 8: $7MeC_{25}$; 10: $3MeC_{25}$; 12: C_{26}; 18: C_{27}; 22: $13+11MeC_{27}$; 23: $7MeC_{27}$. *Int. st*: squalane internal standard.

The permanova on this subset confirmed that the reduction of the number of variables did not modify the chemical profile differences across taxa (F = 9.11, df = 3, p-value = 0.001). The discriminant analysis was significant (non-parametric version of Pillai's test; Obs. = 0.125, Exp. = 0.104, p-value = 0.025) and separated both species along the first axis and the hybrids along the second axis (Fig. 3).

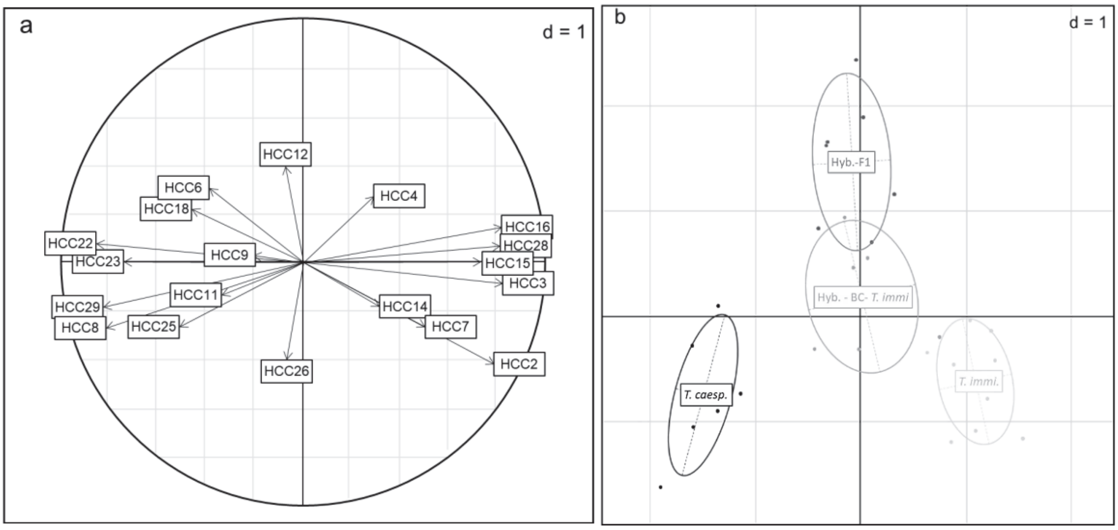

Figure 3. Discriminant analysis graphs. (a) Plot of the factorial map of a correlation circle. (b) First canonical plane (i.e., two first canonical variates) locating the sampled individuals and taxa (50% confidence ellipses).

Peaks with high contributions to axes were considered the best candidates for kin-recognition cues: CHC2, CHC3 ($3MeC_{23}$), CHC15, CHC16 and CHC28 were identified in *T. immigrans*, CHC8 ($7MeC_{25}$), CHC22 ($7MeC_{27}$), CHC23 and CHC29 in *T. caespitum*, and CHC4 (C_{24}), CHC6 (C_{25}), CHC12 (C_{26}) and CHC18 (C27) in hybrids (Figs. 2 and 3; Table 3; Table S2).

Table 3. Identification of the main cuticular hydrocarbons (peaks retained for analyses in the present study): CHC number; retention time; identity of the compound; proportion of each compound in the compounds bouquet for each taxon (*T. immigrans*, *T. caespitum*, F1 hybrid, Backcross with *T. immigrans*)

	Retention time	Identified components	*T. immigrans*	*T. caespitum*	F1 hybrid	Backcross with *T. immigrans*
2	29.82	-	1,47%	0,00%	0,14%	0,34%
3	30.55	$3MeC_{23}$	1,68%	0,07%	0,81%	0,73%
4	31.08	C_{24}	0,90%	0,56%	1,04%	0,82%
6	33.01	C_{25}	9,82%	12,20%	15,20%	13,76%
7	33.65	$13+11MeC_{25}$	18,39%	7,52%	10,91%	12,69%
8	33.78	$7MeC_{25}$	1,59%	6,94%	2,65%	3,35%
9	33.94	-	1,08%	1,18%	1,40%	1,50%
11	34.53	$3MeC_{25}$	0,50%	0,86%	0,48%	0,55%
12	34.85	C_{26}	1,17%	1,12%	1,59%	1,61%
14	35.45	-	2,44%	1,23%	1,63%	1,75%
15	36.03	-	1,21%	0,04%	0,77%	0,51%
16	36.21	-	10,86%	1,52%	7,82%	8,94%
18	36.63	C_{27}	3,89%	5,71%	6,20%	6,74%
22	37.35	$13+11MeC_{27}$	0,28%	8,90%	3,75%	4,01%
23	37.51	$7MeC_{27}$	0,36%	1,95%	1,08%	1,30%
25	37.91	-	3,99%	5,34%	4,38%	4,61%
26	38.06	-	4,40%	3,89%	3,49%	4,03%
28	39.4	-	1,45%	0,04%	0,72%	0,79%
29	40.53	-	0,05%	2,07%	0,54%	0,40%

Experiment 2 - Aggression between workers

Aggression was different between dyads involving *T. immigrans vs. T. immigrans*, *T. immigrans vs.* backcrossed, *T. immigrans vs.* F1 hybrids, and *T. immigrans vs. T. caespitum* (KW test : $\chi^2 = 28.65$, df = 3, p-value = 2.6×10^{-6}; $\chi^2 = 48.03$, df = 12, p = 3.1×10^{-6}). *T. immigrans vs. T. immigrans* resulted in a lower aggressiveness than *T. immigrans vs.* Hybrids F1 (Mann-Whitney-Wilcoxon test: W = 744, p = 0.003; permutation test: z = -3.43, p = 0.0036) and *T. immigrans vs. T. caespitum* (W = 2714.5, p = 9.2×10^{-6}; z = -4.79, p = 1.01×10^{-6}) (Fig. 4b). Aggressiveness in heterospecific dyads was significantly higher when *T. immigrans* workers come from urban areas (areas of allopatry among species) than from area of sympatry among species according to the unilateral Mann-Whitney-Wilcoxon test (W = 956, p = 0.034), but non-significantly according to the permutation test (z = -1.42, p = 0.16) (Fig. 4c).

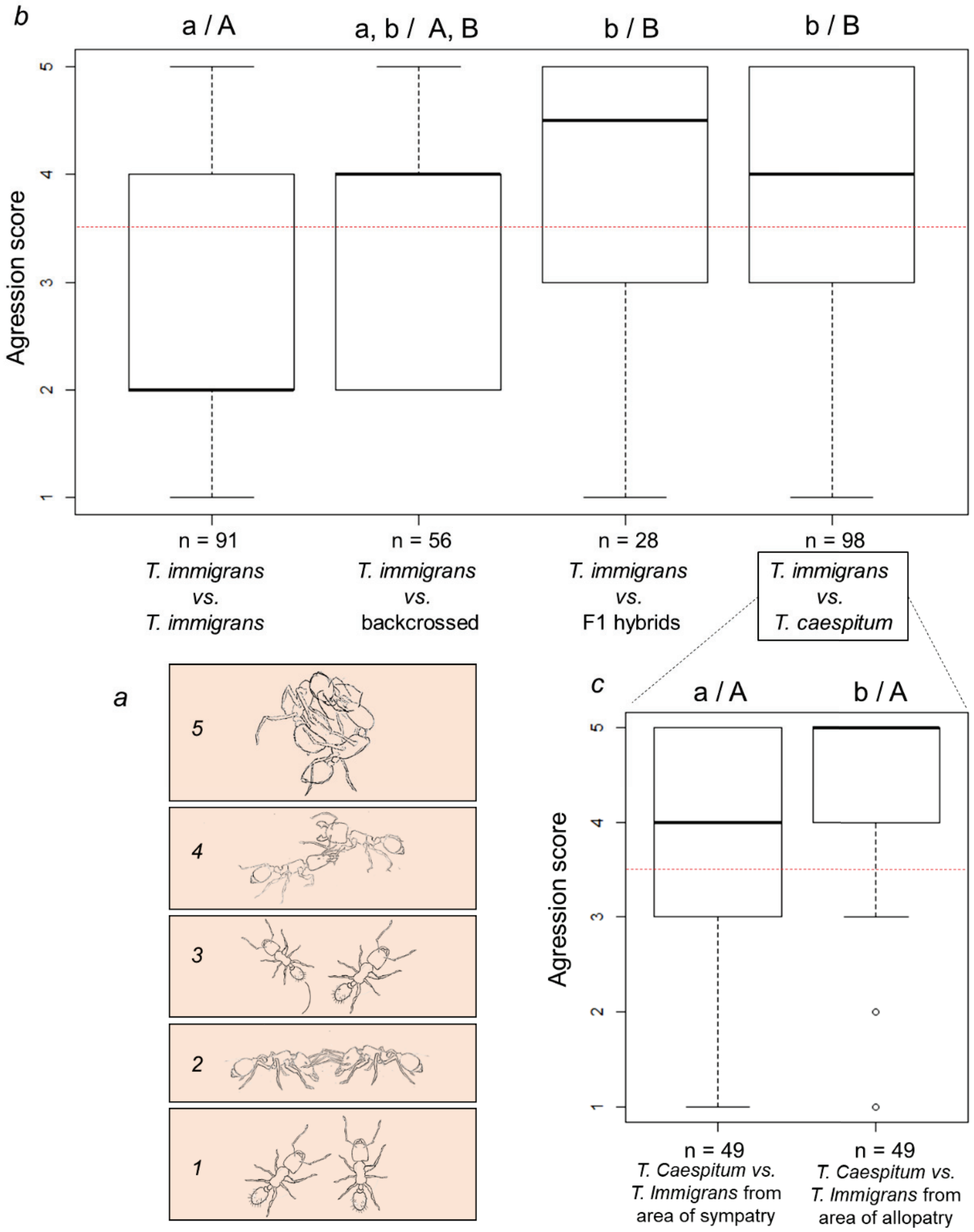

Figure 4. Results of the aggression tests carried out between *T. immigrans*, *T. caespitum*, F1 hybrids and backcrossed hybrids. (a) Aggression scores (1-5) and associated typical postures. Comparison of aggression scores (b) within *T. immigrans* and between *T. immigrans* and other taxa, and (c) between *T. caespitum* and *T. immigrans* coming from area of sympatry among species (left) or allopatry among species (right). Thick black horizontal line: median value; box ends: upper and lower quartiles; whiskers: max and min values. Horizontal dashed line represents the threshold of aggressiveness beyond which the behavior is considered antagonistic. Letters a, b indicate the results of the nonparametric Mann-Whitney-Wilcoxon tests; letters A, B indicate the results of the permutation tests (different letters when the difference of scores between the dyads is significant at the 0.05 Bonferroni-corrected level).

DISCUSSION

The study aimed to provide insights into interspecific hybridization mechanisms through the recognition system in ants, and to explore whether recognition cues may prevent closely related species from collapsing into a single species within a hybrid zone. We determined that differentiated cuticular hydrocarbon profiles and heightened interspecific aggression reveal strong species recognition cues. Moreover, hybrids had cuticular hydrocarbon profiles corresponding to a mixture of the parental bouquets, but their chemical signal also featured compounds present only in trace amounts in both parental species. Behavioral assays showed that *T. immigrans* was as aggressive towards hybrids as towards heterospecifics. Finally, the dear enemy phenomenon hypothesis was partially verified as aggression between heterospecific workers was higher when protagonists came from areas of allopatry among species than from areas of sympatry among species.

Strongly differentiated cuticular hydrocarbon profiles and heightened interspecific aggression further reveal that species recognition cues are both present and perceived between *Tetramorium immigrans* and *T. caespitum*. Steiner et al. (2002) categorized CHCs of individuals morphologically determined as *T. caespitum* but this study was performed before the discovery of rampant cryptic diversity within the genus (Schlick-Steiner et al. 2006) by Self-Organizing Maps. Schlick-Steiner et al. (2006) then re-analyzed these data with more accurate mtDNA-based species identification and showed that *T. caespitum* was different from *T. sp. E* (now called *T. immigrans*; Wagner et al. 2017) but did not identify which chemical compounds were implicated in these differences, and did not investigate the chemical profile of *T.* sp. U2 (corresponding to *T. caespitum* ants of the present study; Wagner et al. 2017). Sano et al. (2018) combined behavioural tests and CHCs assessments to investigate the use of CHCs in recognition of conspecifics *vs.* heterospecifics in a *Tetramorium* species (that they called *Tetramorium caespitum* in their paper but according to the geographical location, it is more likely *T. immigrans*, as *T. caespitum* only occurs in Europe according to the literature and because *T. immigrans* and *T. tsushimae* are the only pavement ants described so far from North America; Schlick-Steiner et al. 2006, Steiner et al. 2006, Wagner et al. 2017, Steiner et al. 2008; note that the comparison of the cuticular profiles obtained by Sano and those presented in Fig. 2 seems to corroborate this hypothesis). Our results clearly show that *T. immigrans* can strongly differentiate conspecifics from heterospecifics (both *T. caespitum* and hybrids). Quite to the contrary, *T. immigrans* (= *T. caespitum* sensu Sano et al. 2018) responded with same levels of aggression to conspecific and heterospecific non-nestmates in the study of Sano et al. (2018), suggesting that *T. immigrans* workers simply exclude all non-nestmate ants regardless of their species. However, the heterospecific species they tested (*Pogonomyrmex occidentalis* and *Camponotus modoc*) were phylogenetically very distant from *T. immigrans* compared to *T. caespitum* or with F1 hybrids in the present study. Perhaps a higher intraspecific aggression in North America than in France could explain why these authors did not detect any difference in aggression level towards con- and heterospecific non-nestmates. The non-nestmates discrimination have been suggested to be founded on the relative abundance of methyl-branched alkanes and n-alkenes (Sano et al. 2018). Guerreri et al. (2009) showed that only dimethylated alkanes were effective in eliciting aggression. It would be relevant to identify all the compounds involved in our study to compare these different systems and to measure the signal reproducibility.

In *T. immigrans* and *T. caespitum*, the increased aggression towards heterospecifics may play a role in speciation, as high levels of aggression between species may result in interspecific exclusion, biased mating and possibly reduced fitness of hybrid colonies (Purcell et al. 2016). Therefore, it could be relevant to further investigate the recognition cues in reproductives, although this may prove to be difficult as identification of these taxa is based on genetic methods that are destructive of individuals which cannot be further used for CHC extraction or behavioral assays. It would therefore be worth identifying CHCs in males and gynes. If species are able to discriminate the identity of the partner, how is interspecific hybridization maintained? The result we obtained here could be contrasted for males and females at a time of mating because inside the mating swarms, more exchanges of CHCs with other reproductives that come into physical contact could homogenize their profiles and reduce their ability to use species-specific differences (Herrmann 2016). Consequently, accurate identification of the potential mates' species before copulation could be undermined. In the absence of pre-copulatory cues, males may benefit from maximizing mating frequency as breeding with a wrong partner could have a more negligible consequence on fitness if females mate with multiple males (El-Shehaby et al. 2011, Suni and Eldakar 2011). When unable to measure in situ the chemical profile of individuals, the study of polyandry and mate choice in these species could therefore constitute a first step to resolve the hypothesis of a lack of discrimination of the species during mating.

The higher levels of aggression between *T. immigrans* and *T. caespitum* when heterospecific protagonists come from areas of allopatry among species (i.e. when *T. immigrans* come from urban core) could indicate an environmental component in the cuticular profile. Some of the recent studies about relations between environment and aggressive behavior revealed correlations (e.g., Benedek and Kobori 2014; Frizzi et al. 2015) while others did not (e.g., Langen et al. 2000; Martin et al. 2012). One of the environmental factors involved in such potential changes in aggressiveness is the modification of the cuticular compounds in highly polluted urban habitats. Exposure to heavy metals can increase or decrease aggression towards non-nestmates, for example in *Formica aquilonia* exposed to long-term pollution showing higher heavy metal concentrations and being less aggressive towards foreign unpolluted colony (Sorvari and Eeva 2010). Moreover, these urbanized areas may be less resource-rich, increasing competition for food, and thus aggressiveness (Sorvari & Hakkarainen 2004). Nevertheless, other environmental factors could also be involved. For example, in *Iridomyrmex purpureus*, non-nestmate aggression was influenced by the density of surrounding conspecific nests, inducing more aggressive behavior when nest density was higher (Thomas et al. 1999).

Behavioral assays showed here that *T. immigrans* is as aggressive towards hybrids as towards heterospecifics. On the contrary, Fadamiro et al. (2009) showed that *Solenopsis invicta* exhibited more aggression towards *S. richteri* than towards hybrids. Regarding the chemical signals, our results are consistent with the findings of El-Shehaby et al. (2011) which suggest that the CHCs profiles of *Temnothorax nylanderi* x *crassispinus* hybrids were not consistently intermediate between those of the paternal species, suggesting either non-additive interactions among the parental biosynthetic pathways or systematic differences in environment-derived odor cues. In the carpenter ant *Camponotus herculeanus*, the presence, but not the absence, of an additional hydrocarbon elicited aggression (Guerreri et al. 2009). This result is consistent with our findings that all chemical components of paternal species were found in hybrids (with variations in quantities) but one compound (CHC12, C26) was found in larger amount in hybrids and could be responsible of such a pattern.

Taken as a whole, these findings paint a particularly complex picture of the recognition system of *Tetramorium immigrans*, *T. caespitum* and their hybrids. In cases of interspecific hybridization, the present study highlights the necessity of investigating species discrimination and recognition cues in every compartment of the system, including hybrids. Although poorly studied in a context of discrimination of heterospecifics in the literature, findings of our study clearly showed that hybrid zones offer a unique opportunity to deeply investigate recognition mechanisms and discrimination between species.

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SUPPORTING INFORMATION

Table S1. List of colonies used for chemical analyses, with type, name, areas of origin (Symp: S = nest in area of sympatry among species; A = nest in area of allopatry among species), percentage of impervious areas in a buffer of 500 m around the nest, location (X, Y) and classes in Corine Land Cover 2012; CLC 2012©, Copernicus (https://land.copernicus.eu/)

Colony type	Colony name	Symp	PI500	Y	X	CLC12
T. immigrans	lyo_07	S	5.92	45.6795	5.0149	211
T.caespitum	lyo_13	S	2.45	45.6536	5.0709	122
Backcross with *T. immigrans*	lyo_30	S	0.59	45.6601	5.0445	211
F1 Hybrid	lyo_31	S	0.03	45.6490	5.0492	211
T.caespitum	lyo_32	S	0.06	45.6484	5.0591	211
Backcross with *T. immigrans*	lyo_42	S	32.68	45.6768	4.9689	121
T. immigrans	lyo_50	S	1.57	45.6476	5.0341	211
T. immigrans	lyo_83	A	37.78	45.7811	4.8675	121
F1 Hybrid	mac_07	S	8.46	46.3273	4.9075	242
F1 Hybrid	mac_10	S	4.33	46.3277	4.9355	112
T.caespitum	mac_21	S	2.12	46.3412	5.0341	231
Backcross with *T. immigrans*	mac_22	S	1.67	46.3089	4.8532	231
T.caespitum	mac_25	S	13.40	46.3141	4.8802	112
T. immigrans	mac_34	S	10.53	46.3184	4.9410	242
T. immigrans	mac_41	S	13.31	46.3134	4.9320	231
Backcross with *T. immigrans*	mac_43	S	4.31	46.3025	4.8639	112
Backcross with *T. immigrans*	mac_54	S	2.89	46.3136	4.9728	242
F1 Hybrid	mac_68	S	64.50	46.2885	4.8169	121
T. immigrans	mac_75	A	54.71	46.3091	4.8206	121
T. immigrans	mac_81	A	43.95	46.3199	4.8300	112
T.caespitum	val_03	S	2.08	44.9499	4.9548	242
F1 Hybrid	val_08	S	1.19	44.9488	5.0016	211
T. immigrans	val_10	S	2.21	44.9600	5.0231	211
T.caespitum	val_18	S	1.51	44.9776	5.0949	211
F1 Hybrid	val_28	S	0.39	44.9534	5.0049	211
T.caespitum	val_35	S	2.35	44.9608	5.0670	211
T. immigrans	val_62	A	63.12	44.9414	4.8935	112
Backcross with *T. immigrans*	val_76	S	40.34	44.9130	4.8786	112
T. immigrans	val_79	S	37.11	44.9284	4.8835	112
F1 Hybrid	vil_20	S	0.74	46.0080	4.9497	231

Table S2. List of colonies used for chemical analyses, with type, name, and quantity (µg) of each chemical compound identified in the samples.

Colony type	Colony name	HCC1	**HCC2**	**HCC3**	**HCC4**	HCC5	**HCC6**	**HCC7**	**HCC8**	**HCC9**	HCC10	**HCC11**	**HCC12**	HCC13	**HCC14**
		29.1	**29.82**	**30.55**	**31.08**	31.22	**33.01**	**33.65**	**33.78**	**33.94**	34.36	**34.53**	**34.85**	34.97	**35.45**
T. immigrans	lyo_07	0.014	**0.009**	**0.006**	**0.003**	0.012	**0.034**	**0.104**	**0.010**	**0.004**	0.037	**0.002**	**0.004**	0.034	**0.012**
T. immigrans	mac_41	0.011	**0.003**	**0.004**	**0.003**	0	**0.038**	**0.042**	**0.003**	**0.002**	0.020	**0.004**	**0.004**	0	**0.005**
T. immigrans	mac_75	0.014	**0.006**	**0.008**	**0.003**	0.003	**0.029**	**0.071**	**0.006**	**0.001**	0.035	**0**	**0.004**	0.011	**0.008**
T. immigrans	mac_81	0.009	**0.005**	**0.006**	**0.002**	0.001	**0.017**	**0.044**	**0.002**	**0.001**	0.022	**0**	**0.002**	0.006	**0.007**
T. immigrans	val_10	0.017	**0.003**	**0.005**	**0.003**	0.002	**0.040**	**0.063**	**0.005**	**0.009**	0.036	**0**	**0.005**	0.010	**0.007**
T. immigrans	val_62	0.026	**0.005**	**0.010**	**0.006**	0	**0.058**	**0.090**	**0.005**	**0.012**	0.050	**0.007**	**0.006**	0.005	**0.013**
T. immigrans	val_79	0.012	**0.007**	**0.005**	**0.003**	0.008	**0.024**	**0.066**	**0.007**	**0.002**	0.030	**0.002**	**0.003**	0.021	**0.007**
T. immigrans	mac_34	0.014	**0.004**	**0.004**	**0.003**	0	**0.026**	**0.054**	**0.004**	**0**	0.021	**0**	**0.004**	0.008	**0.009**
T. immigrans	lyo_50	0.018	**0.004**	**0.005**	**0.004**	0.006	**0.049**	**0.059**	**0.012**	**0.006**	0.036	**0.003**	**0.005**	0.019	**0.009**
T. immigrans	lyo_83	0.013	**0.003**	**0.005**	**0.003**	0.002	**0.028**	**0.051**	**0.005**	**0.003**	0.032	**0.001**	**0.004**	0.012	**0.008**
T. caespitum	mac_21	0.007	**0**	**0**	**0.001**	0	**0.037**	**0.016**	**0.020**	**0.001**	0.017	**0.001**	**0.004**	0	**0.002**
T. caespitum	val_18	0.009	**0**	**0**	**0.002**	0	**0.041**	**0.005**	**0.015**	**0.001**	0.014	**0**	**0.004**	0	**0**
T. caespitum	val_35	0.017	**0**	**0.002**	**0.003**	0	**0.055**	**0.048**	**0.041**	**0.012**	0.024	**0.007**	**0.003**	0.001	**0.013**
T. caespitum	val_03	0.008	**0**	**0.001**	**0.003**	0	**0.061**	**0.100**	**0.040**	**0.015**	0.033	**0.011**	**0.005**	0.003	**0.023**
T. caespitum	mac_25	0.011	**0**	**0**	**0.003**	0	**0.051**	**0.024**	**0.031**	**0.004**	0.017	**0.004**	**0.004**	0	**0.004**
T. caespitum	lyo_13	0.011	**0**	**0**	**0.001**	0	**0.045**	**0.042**	**0.044**	**0.008**	0.026	**0.007**	**0.004**	0	**0.005**
T. caespitum	lyo_32	0.008	**0**	**0**	**0.003**	0	**0.048**	**0.028**	**0.019**	**0.003**	0.015	**0.002**	**0.005**	0.003	**0.002**
hybrid F1	mac_68	0.014	**0**	**0.004**	**0.003**	0	**0.041**	**0.029**	**0.009**	**0.001**	0.030	**0.001**	**0.005**	0.005	**0.007**
hybrid F1	val_08	0.028	**0**	**0**	**0.005**	0	**0.093**	**0.033**	**0.019**	**0.009**	0.018	**0.001**	**0.007**	0	**0.002**
hybrid F1	val_28	0.013	**0.003**	**0.006**	**0.003**	0.008	**0.030**	**0.050**	**0.006**	**0.009**	0.047	**0.003**	**0.004**	0.030	**0.006**
hybrid F1	mac_07	0.007	**0**	**0.002**	**0.003**	0	**0.043**	**0.016**	**0.005**	**0.003**	0.022	**0**	**0.005**	0	**0.001**
hybrid F1	mac_10	0.012	**0**	**0.003**	**0.003**	0	**0.039**	**0.038**	**0.006**	**0.003**	0.023	**0.002**	**0.005**	0.006	**0.006**
hybrid F1	vil_20	0.013	**0**	**0.002**	**0.004**	0	**0.067**	**0.054**	**0.009**	**0.005**	0.032	**0.002**	**0.007**	0.012	**0.008**
hybrid F1	lyo_31	0.007	**0**	**0.001**	**0.002**	0.001	**0.040**	**0.035**	**0.010**	**0.004**	0.022	**0.002**	**0.004**	0.012	**0.007**
Backcross with *T. immigrans*	lyo_42	0.012	**0**	**0.002**	**0.002**	0.001	**0.052**	**0.050**	**0.005**	**0.003**	0.027	**0.001**	**0.006**	0.009	**0.007**
Backcross with *T. immigrans*	mac_43	0.018	**0**	**0.003**	**0.004**	0	**0.064**	**0.031**	**0.004**	**0.003**	0.035	**0**	**0.008**	0.008	**0.005**
Backcross with *T. immigrans*	mac_22	0.011	**0**	**0.003**	**0.004**	0	**0.064**	**0.042**	**0.010**	**0.004**	0.036	**0.002**	**0.007**	0.002	**0.008**
Backcross with *T. immigrans*	val_76	0.010	**0**	**0.002**	**0.002**	0	**0.032**	**0.036**	**0.008**	**0.009**	0.018	**0**	**0.004**	0.003	**0.004**
Backcross with *T. immigrans*	mac_54	0.011	**0.007**	**0.005**	**0.003**	0.003	**0.031**	**0.077**	**0.009**	**0.004**	0.026	**0.004**	**0.004**	0.012	**0.009**
Backcross with *T. immigrans*	lyo_30	0.005	**0**	**0**	**0.001**	0	**0.029**	**0.015**	**0.023**	**0.004**	0.015	**0.003**	**0.003**	0.001	**0.001**

Colony type	Colony name	**HCC15**	**HCC16**	HCC17	**HCC18**	HCC19	HCC20	HCC21	**HCC22**	**HCC23**	HCC24	**HCC25**	**HCC26**	HCC27	**HCC28**	**HCC29**	HCC30
		36.03	**36.21**	36.34	**36.63**	36.74	37.21	37.25	**37.35**	**37.51**	37.82	**37.91**	**38.06**	38.45	**39.4**	**40.53**	43.46
T. immigrans	lyo_07	**0.008**	**0.065**	0.020	**0.012**	0.003	0.035	0	**0**	**0**	0	**0.014**	**0.023**	0.010	**0.005**	**0**	0.010
T. immigrans	mac_41	**0.003**	**0.023**	0.005	**0.018**	0.001	0.027	0	**0**	**0.001**	0	**0.009**	**0.010**	0	**0.003**	**0.001**	0.007
T. immigrans	mac_75	**0.006**	**0.050**	0.002	**0.011**	0.003	0.021	0	**0**	**0**	0	**0.010**	**0.012**	0.003	**0.003**	**0**	0.007
T. immigrans	mac_81	**0.002**	**0.019**	0.005	**0.006**	0.002	0.028	0.002	**0**	**0.001**	0	**0.012**	**0.011**	0.005	**0.007**	**0**	0.007
T. immigrans	val_10	**0.002**	**0.049**	0.014	**0.016**	0.001	0.030	0	**0**	**0.001**	0	**0.016**	**0.014**	0.004	**0.005**	**0**	0.009
T. immigrans	val_62	**0.004**	**0.053**	0.007	**0.020**	0.003	0.050	0	**0**	**0.003**	0	**0.021**	**0.018**	0	**0.007**	**0**	0.009
T. immigrans	val_79	**0.005**	**0.032**	0.007	**0.009**	0	0.020	0	**0**	**0**	0	**0.010**	**0.016**	0.005	**0.003**	**0**	0.008
T. immigrans	mac_34	**0.003**	**0.027**	0.007	**0.012**	0.002	0.043	0.002	**0**	**0**	0	**0.012**	**0.015**	0.004	**0.008**	**0**	0.014
T. immigrans	lyo_50	**0.005**	**0.047**	0.011	**0.016**	0	0.043	0	**0.012**	**0.003**	0.003	**0.019**	**0.017**	0.007	**0.003**	**0**	0.005
T. immigrans	lyo_83	**0.004**	**0.024**	0.006	**0.012**	0.003	0.032	0	**0**	**0.002**	0	**0.014**	**0.016**	0.007	**0.004**	**0**	0.015
T. caespitum	mac_21	**0**	**0.001**	0.003	**0.018**	0	0.053	0.017	**0.019**	**0.006**	0.009	**0.015**	**0.011**	0	**0**	**0.008**	0.009
T. caespitum	val_18	**0**	**0.005**	0.005	**0.022**	0	0.006	0.029	**0.029**	**0.006**	0	**0.011**	**0.007**	0	**0**	**0.003**	0.006
T. caespitum	val_35	**0**	**0.004**	0.005	**0.017**	0	0.121	0.062	**0.034**	**0.012**	0.049	**0.029**	**0.032**	0	**0**	**0.014**	0.008
T. caespitum	val_03	**0**	**0.009**	0.013	**0.025**	0	0.033	0.127	**0.083**	**0.013**	0.050	**0.042**	**0.025**	0	**0**	**0.010**	0.014
T. caespitum	mac_25	**0**	**0.003**	0.003	**0.023**	0	0.080	0.008	**0.027**	**0.008**	0.027	**0.027**	**0.023**	0	**0**	**0.010**	0.012
T. caespitum	lyo_13	**0**	**0.010**	0.011	**0.018**	0	0.069	0.044	**0.052**	**0.010**	0.015	**0.020**	**0.016**	0	**0**	**0.009**	0.010
T. caespitum	lyo_32	**0.001**	**0.011**	0.005	**0.026**	0	0.049	0.023	**0.027**	**0.003**	0.012	**0.019**	**0.009**	0.001	**0.001**	**0.006**	0.005
hybrid F1	mac_68	**0**	**0.022**	0.010	**0.015**	0.001	0.050	0.004	**0.015**	**0.003**	0.003	**0.015**	**0.010**	0	**0.004**	**0.004**	0.008
hybrid F1	val_08	**0**	**0.014**	0.012	**0.040**	0	0.063	0	**0.025**	**0.008**	0.003	**0.016**	**0.010**	0	**0**	**0.005**	0.010
hybrid F1	val_28	**0.006**	**0.029**	0.007	**0.010**	0	0.018	0	**0**	**0.001**	0	**0.014**	**0.020**	0.006	**0.003**	**0**	0.007
hybrid F1	mac_07	**0**	**0.027**	0.008	**0.021**	0	0.019	0.003	**0.010**	**0.004**	0	**0.011**	**0.006**	0	**0**	**0**	0.005
hybrid F1	mac_10	**0.006**	**0.019**	0.003	**0.015**	0	0.033	0	**0.011**	**0.002**	0.005	**0.014**	**0.010**	0.001	**0.004**	**0**	0.006
hybrid F1	vil_20	**0.003**	**0.029**	0.009	**0.023**	0.001	0.048	0	**0.012**	**0.005**	0	**0.011**	**0.011**	0.003	**0.002**	**0**	0.007
hybrid F1	lyo_31	**0.003**	**0.034**	0.019	**0.019**	0	0.054	0.009	**0.015**	**0.001**	0.015	**0.019**	**0.014**	0.005	**0.003**	**0.005**	0.007
Backcross with *T. immigrans*	lyo_42	**0.001**	**0.036**	0.011	**0.023**	0	0.041	0.001	**0.007**	**0.003**	0.003	**0.011**	**0.009**	0.002	**0.001**	**0**	0.006
Backcross with *T. immigrans*	mac_43	**0.003**	**0.029**	0.003	**0.030**	0.001	0.032	0	**0.004**	**0.005**	0	**0.018**	**0.013**	0	**0.006**	**0.001**	0.006
Backcross with *T. immigrans*	mac_22	**0.001**	**0.041**	0.014	**0.033**	0.004	0.069	0	**0.019**	**0.008**	0.007	**0.023**	**0.016**	0	**0.006**	**0.003**	0.007
Backcross with *T. immigrans*	val_76	**0**	**0.035**	0.010	**0.016**	0.001	0.022	0.005	**0.013**	**0**	0	**0.011**	**0.011**	0	**0.001**	**0**	0.007
Backcross with *T. immigrans*	mac_54	**0.005**	**0.033**	0.004	**0.010**	0.004	0.033	0	**0**	**0.001**	0.003	**0.010**	**0.018**	0	**0.004**	**0**	0.011
Backcross with *T. immigrans*	lyo_30	**0**	**0.004**	0.003	**0.019**	0	0.031	0.014	**0.028**	**0.008**	0	**0.015**	**0.011**	0	**0**	**0.003**	0.007

Table S3. List of colonies used for behavioral analyses, with type, name, areas of origin (Symp: S = nest in area of sympatry among species; A = nest in area of allopatry among species), percentage of impervious areas in a buffer of 500 m around the nest, location (X, Y) and classes in Corine Land Cover 2012; CLC 2012©, Copernicus (https://land.copernicus.eu/)

Colony type	Colony name	Symp	PI500	X	Y	CLC12
T. immigrans	lyo_07	S	5.92	5.0149	45.6795	211
T.caespitum	lyo_13	S	2.45	5.0709	45.6536	122
T. immigrans	lyo_28	S	0.70	5.0276	45.6638	211
F1 Hybrid	lyo_31	S	0.03	5.0492	45.6490	211
T.caespitum	lyo_32	S	0.06	5.0591	45.6484	211
Backcross with *T. immigrans*	lyo_42	S	32.68	4.9689	45.6768	121
T. immigrans	lyo_78	A	40.52	4.9370	45.7660	121
T. immigrans	lyo_83	A	37.78	4.8675	45.7811	121
T.caespitum	mac_21	S	2.12	5.0341	46.3412	231
Backcross with *T. immigrans*	mac_22	S	1.67	4.8532	46.3089	231
T.caespitum	mac_25	S	13.40	4.8802	46.3141	112
T. immigrans	mac_34	S	10.53	4.9410	46.3184	242
T. immigrans	mac_41	S	13.31	4.9320	46.3134	231
Backcross with *T. immigrans*	mac_46	S	8.12	4.8877	46.3066	231
Backcross with *T. immigrans*	mac_54	S	2.89	4.9728	46.3136	242
T. immigrans	mac_75	A	54.71	4.8206	46.3091	121
T. immigrans	mac_77	A	40.31	4.8171	46.3207	112
T. immigrans	mac_81	A	43.95	4.8300	46.3199	112
T.caespitum	val_03	S	2.08	4.9548	44.9499	242
F1 Hybrid	val_08	S	1.19	5.0016	44.9488	211
T. immigrans	val_10	S	2.21	5.0231	44.9600	211
T.caespitum	val_18	S	1.51	5.0949	44.9776	211
T.caespitum	val_35	S	2.35	5.0670	44.9608	211
T. immigrans	val_53	S	1.14	5.0503	44.9536	211
T. immigrans	val_62	A	63.12	4.8935	44.9414	112
T. immigrans	val_79	S	37.11	4.8835	44.9284	112
T. immigrans	val_86	A	40.36	4.8998	44.9165	112

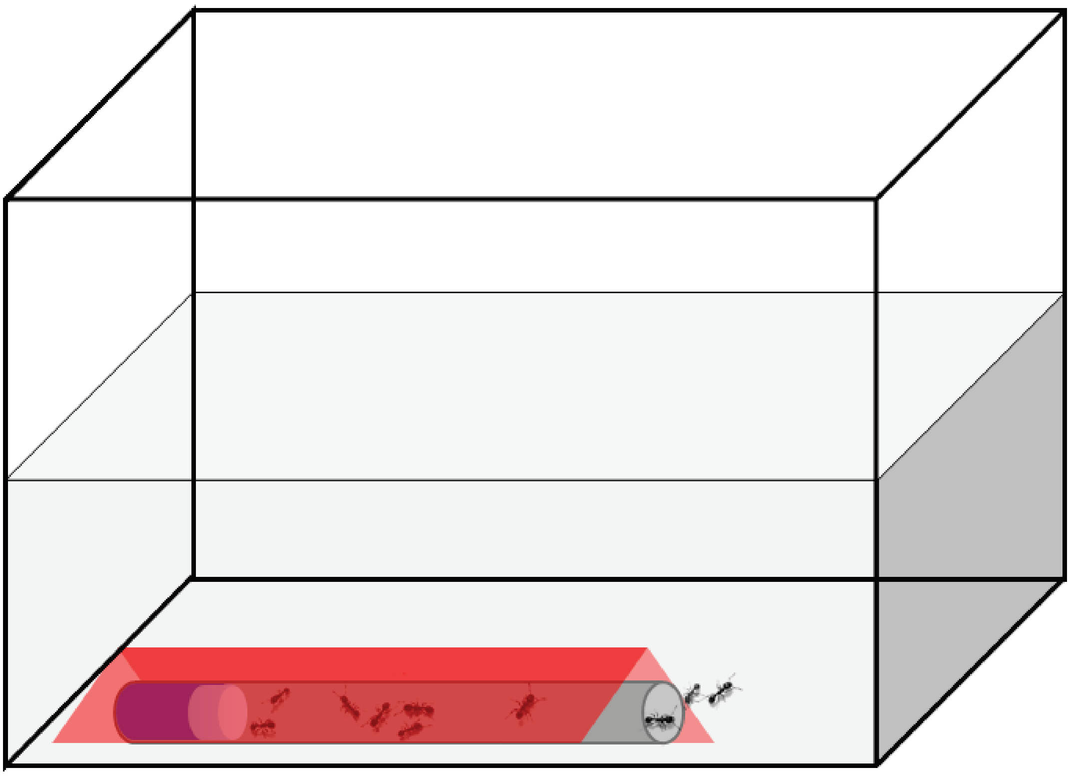

Figure S1. Schematic presentation of artificial nest boxes; walls are brushed with Fluon® PTFE (Polytetrafluoroethylene) to prevent escape (grey walls); the nest corresponds to a glass test tube covered by a red filter and a water reservoir for humidity.

PAPER 6. Multiple mating and sexual selection in the context of interspecific hybridization between two *Tetramorium* ant species

This article is in prep. for a submission in Evolution

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Abstract

In eusocial Hymenoptera, haplodiploidy and polyandry may facilitate selection for hybridization. Interspecific hybridization is common and widespread in ants and can lead to hybrid unviability as well as the formation of new species through hybrid speciation. However, in ants, polyandry is relatively uncommon. Analyzing microsatellite markers on 15 ant workers per colony, we show that the mating system of 28 pure colonies of *Tetramorium immigrans*, 15 pure colonies of *T. caespitum* and 27 hybrid colonies is a monogyne/polyandrous mating system, with a higher mating rate in *T. caespitum*. Hybrid queens, but no hybrid fathers, were deduced from workers' genotypes, in accordance to Haldane's rule extended to haplodiploid organisms, which states that the haploid sex should more often be sterile or inviable. In four colonies, hybridization and multiple mating allowed the simultaneous production of both hybrid and nonhybrid offspring. Although rare, these situations hinted at asymmetrical contributions to offspring in favor of heterospecific vs. conspecific males in colonies with a *T. caespitum* queen. Together, these findings point towards a complex and dynamic mating system in *Tetramorium immigrans* and *T. caespitum* and should contribute to better understand interspecific hybridization mechanisms and their consequences on genetic and taxonomic diversity. The study of polyandry within a hybrid zone is unprecedented and open the opportunity to investigate the selective processes involved in the evolution of multiple mating.

Keywords: Haplodiploidy, Hybridization, Introgression, Mating system, Multiple mating, Polyandry, Sexual selection, Social insects, *Tetramorium*

INTRODUCTION

Interspecific hybridization is common and widespread in ants (Feldhaar et al., 2008), and has been described for years in numerous genera, e.g., *Solenopsis* (Ross et al., 1987), *Formica* (Kulmuni et al., 2010; Purcell et al., 2016), *Lasius* (Van der Have et al., 2011), *Pogonomyrmex* (Kronauer et al., 2011) or *Tetramorium* (Steiner et al., 2010; Cordonnier et al., 2018). The impacts of hybridization can range from hybrid unviability or sterility to reverse speciation, through adaptive introgression and even the formation of new species through hybrid speciation (Beresford et al., 2017).

In ants and other eusocial Hymenoptera, combinations of mechanisms involved in reproductive behavior may facilitate selection for hybridization, including haplodiploid sex determination systems and polyandry (Anderson et al., 2008). On the one hand, the haplodiploid reproductive system, where haploid males from unfertilized eggs only inherit maternal genetic material, can mitigate outbreeding depression compared to other organisms, as male fitness is preserved at the F1 generation because diploid queens which have mated with heterospecific haploid males still produce purebred sons via arrhenotokous parthenogenesis (Feldhaar *et al.*, 2008; Kulmuni et al., 2010; Kronauer et al., 2011). When hybrids are fertile, truly hybrid males therefore only appear in backcrosses (Schilthuizen et al., 2011; Kronauer et al., 2011). Haploid males are particularly likely to show hybrid incompatibilities (Koevoets & Beukeboom, 2009), and some species have therefore evolved elaborate mechanisms to avoid producing hybrid males (e.g., Kulmuni et al., 2010; Kulmuni & Pamilo, 2014). On the other hand, eusocial Hymenoptera generally form colonies that contain a single reproductive queen and many sterile worker individuals. In ant species, gynes (virgin reproductive females) either mate only once (monandry) or several times (polyandry) (Heinze 2008). Polyandry is relatively uncommon in ants (reviewed in Strassmann 2001; Villesen et al., 2002 but see Haapaniemi & Pamilo, 2012; Hardy et al., 2008). However, Anderson et al. (2008) pointed out that polyandry can increase genetic diversity of workers, and morphological, behavioral, or physiological variation among workers and thus raise colony fitness and provide benefits from heterosis in hybrid workers, resulting in positive selection for hybridization. In eusocial insects there obviously is a pre-copulatory mate-choice phase, but it involves a very restricted set of mate quality cues (Boomsma, 2013). In cases of interspecific hybridization combined with polyandry, considering mate choice is particularly relevant. Being able to breed with a male of another species increases the probability of finding one (or more) partner(s) and could thus even prove reproductively advantageous (Rosenthal, 2013). Nonacs (2006) pointed out that when population densities are low, females may not always be able to encounter conspecific males, and hybridization may be a "best of a bad situation" strategy to gain some reproductive success. Alternatively, a lack of mate preference and high levels of polyandry should result in the production of both F1 hybrid and nonhybrid offspring within the same colony (Anderson et al., 2008).

In ants as in many species with internal fertilization, females possess a spermatheca, which is a specialized storage organ where sperm is stored between insemination and egg fertilization. Polyandry therefore results in the co-occurrence of different ejaculates in the female's reproductive tract (Jaffé et al., 2012) allowing sexual selection to operate after copulation. Mechanisms for post-copulatory sexual selection, which lead to biased paternity patterns, include sperm competition, where ejaculates from different males compete to fertilize eggs (Simmons, 2001; but see Boomsma, 2013) and cryptic female choice, where females influence which permanently stored sperm fertilize their eggs (Eberhard, 1996). In situations of hybridization between species, potentially negative consequences of hybridization can therefore be mitigated or even avoided when hybrid individuals mostly or exclusively become non-reproductive workers (Kronauer et al., 2011), or if fewer hybrid individuals are produced than purebred individuals, because of sexual selection processes, genetic caste determination (Nonacs, 2006), or fitness decrease or increase (Umphrey, 2006). In this last situation, selective processes unrelated to sexual selection can alter the viability or fitness of hybrid offspring. For example, Umphrey (2006) highlighted that if a species A queen had mated with both species A and B males, any of these mechanisms could act to select among female immatures so that a disproportionately higher share of gynes (relative to the proportion of species A sperm stored in the queen's spermatheca) are pure species A (e.g., if hybrid gynes are smaller and grow slower, they might be weeded out as larvae from becoming gynes). In addition, as both beneficial and deleterious recessive allelic combinations are selected more strongly in haploid males than diploid females, the potential differential selection between males and females (Beresford, 2017) can induce asymmetrical sex-ratio in hybrid offspring. Such selective process can also be advantageous to hybrids and affect the sterile worker caste. Indeed, one species may exhibit higher fitness for a number of traits, such as resistance to pathogens or different microhabitat niche, so that hybrid workers inheriting ameliorated traits would be of greater benefit to a queen (Umphrey, 2006), leading to survival bias favoring hybrid workers inside a colony.

The questions related to mate choice, sperm competition and cryptic female choice in polyandrous eusocial Hymenoptera therefore often differ from those addressed in non-eusocial mating systems, but their originality may allow experimental tests of the generality of sexual selection theory that cannot be performed in mating systems with re-mating promiscuity (Boomsma, 2007). Although the processes involved are complex, such questions become even more exciting when considering systems involving interspecific hybridization. The present study investigates such mechanisms in two monogynous *Tetramorium* cryptic species, namely *Tetramorium immigrans* Santschi, 1927 and *T. caespitum* (Linnaeus, 1758). *Tetramorium immigrans* and *T. caespitum* can hybridize (Wagner et al., 2017), and hybrids are fertile, leading to a high frequency of hybrid colonies in sympatric populations of these species (Cordonnier et al., submitted to *Heredity*). In the present paper, we analyze polyandry, mate choice biases and asymmetrical contributions to offspring, by investigating mating system in 28 pure colonies of *T. immigrans*, 15 pure colonies of *T. caespitum* and 27 hybrid colonies. Our main objective was to test if both hybrid queens and males reproduce. The study aimed to: (1) determine whether hybridization correlate to the polyandrous mating system of parental species; (2) provide an assessment of the bias in offspring production in hybrid colonies (e.g., capability of hybrids of both sexes to reproduce, production of both F1 hybrid and nonhybrid offspring within the same colony); and (3) verify the hypothesis of random paternity allocation between species, i.e., the random contribution of males to the offspring, opposite to the existence of potential mechanisms of post-copulatory sexual selection biasing worker paternity in colonies.

METHODS

The present study focuses on *Tetramorium immigrans* Santschi, 1927 and *T. caespitum* (Linnaeus, 1758), two cryptic species of the *Tetramorium caespitum* complex distinguishable using genetic (Cytochrome Oxidase I analysis, nuclear AFLP markers) and morphometric characters on workers (Wagner et al., 2017).

Microsatellite data

Cordonnier et al. (2018) collected workers of 544 colonies of *T. immigrans*, 698 colonies of *T. caespitum*, and 240 colonies of potential hybrids between *T. immigrans* and *T. caespitum* in southeastern France. For each colony, one worker had been identified using a two-step approach combining nuclear DNA clustering (14 microsatellite markers) and species identification by mtDNA cytochrome oxidase I sequencing. In the present study, we subsampled 75 of these colonies: 30 colonies whose worker had a genotype associated with *T. immigrans* (Qvalue >95% in Cordonnier et al., 2018), 15 whose worker had a genotype associated with *T. caespitum* (Qvalue >95% in Cordonnier et al., 2018), 15 potential F1 hybrids (genotype associated between 40% and 60% to parental species in Cordonnier et al., 2018), and 15 potential backcrosses with *T. immigrans* (genotype associated between 60% and 80% to parental species in Cordonnier et al., 2018). These colonies were randomly subsampled in 6 out of the 19 spatial areas described in Cordonnier et al. (2018) corresponding to three latitudinally distinct spatial areas (Tab. 1) covering the whole, 180 km latitudinal extent of the hybrid zone described in Cordonnier et al. (submitted to *Heredity*).

Table 1. Correspondence between the nomenclature used in this study and the sampling zones described in Cordonnier et al. (2018); mean, minimal and maximal latitudes of the North (Tournus, Mâcon), Center (Villefranche-sur-Saône, Lyon) and South (Tournon-sur-Rhône, Valence) areas.

	North	Center	South
Cordonnier *et al.* (2018: fig 1)	Sampling zones 5-6	Sampling zones 8-9	Sampling zones 12-13
Mean latitude	46.445	45.834	45.011
Latitude Range	46.280 - 46.605	45.610 - 46.010	44.910 - 45.096

For fifteen randomly selected workers per colony, DNA was extracted from whole individuals, which were crushed and then mixed with 150 µL of Chelex® 100 and 10 µL of proteinase K (15 mg.mL^{-1}) at room temperature; the solution was incubated at 55°C overnight (Casquet et al., 2012). For genotyping, 17 microsatellites developed for *T. immigrans* or T. *tsushimae* by Steiner et al. (2008b) were organized in three multiplex PCR mixes. All three mixes had a total volume of 10 µL with 1X MasterMix (kit type-it microsatellite PCR Qiagen n°206246) and 2 µL DNA. Mix1 contained 0.08 µM of tspE53a primers, and 0.2 µM of tspE52b, tspE52d, tspE52k, ttsU55a and ttsU56d primers. Mix2 contained 0.15 µM of tspE51oR3 primers, 0.2 µM of tspE51a, tspE51b, tspE51d, ttsU58i and ttsU59j primers, and 0.3 µM of tspE51i primers. Mix 3 contained 0.08 µM of ttsU54e, 0.15 µM of tspE52a, and 0.2 µM of tspE53b and ttsU57l primers. Amplifications consisted in 5 min at 95°C, then 32 cycles (30 s at 95°C, 90 s at 60°C, 30 s at 72°C), and 30 min at 60°C. All PCR products were analyzed with an ABI 3730xl sequencer (service provided by GENTYANE). Electropherograms were read and interpreted with Genemarker v.1.95 (SoftGenetics). One marker was discarded because it presented risks of misinterpretation at the reading stage. In addition, workers where alleles were not clearly legible for at least 12 markers were removed from the analysis, and colonies with less than 12 (pure *T. immigrans* or *T. caespitum*) or 14 (hybrids colonies) legible worker genotypes were discarded. Our final dataset included genotypes for 16 microsatellite markers (Tspe52d, Tspe52b, Tspe52k, Ttsu56d, Tspe53a, Ttsu55a, Ttsu59j, Tspe51i, Ttsu58i, Tspe51a, Tspe51o, Tspe51b, Tspe51d, Tspe52a, Tspe53b, Ttsu57l) for 28 putative *T. immigrans* colonies, 15 putative *T. caespitum* colonies, and 27 putative hybrid colonies (14 F1 and 13 backcrosses) with an average of 14.8 worker genotypes per colony, resulting in 1038 genotypes for 16 markers (basic statistics computed in GENALEX v.6; Peakall & Smouse, 2006; Tab. S1).

Observed number of matings

Based on the identified worker genotypes, we first “manually” inferred the genotypes of queens and their mates in each colony to minimize potential errors. At each locus two alleles shared by all the workers were assigned to the queen, while haploid fathers’ genotypes were determined by the alleles unassigned to the mother. This pattern was then iterated over the 16 markers until reaching a minimal number of sires per queen. This allowed an individual correction of all potential genotyping errors to avoid overestimating the number of potential sires. Although time-consuming, such an approach minimizes errors compared to classically used software (COLONY, Matesoft) for which data monitoring and analysis are automated, quite often without post-processes quality control. Here, scoring errors were checked and corrected one last time after identification of mothers and sires. Situations where more than one potential queen genotypes could account for the data have not been found in any of the 70 colonies tested. For each colony the observed number of matings per queen was systematically recorded. Observed number of mating differences between species and zones were investigated by nonparametric Kruskal-Wallis tests coupled with Mann-Whitney-Wilcoxon tests for contrasts (including a simple Bonferroni correction).

Bayesian admixture model

The program NEWHYBRIDS (Anderson & Thompson, 2002) was run to identify recent hybrids (F1, backcrosses) from the whole genotypic data including all workers and inferred parental genotypes. The analysis was performed based on ten iterations carried out using Jeffreys's prior and setting the burn-in period to 20,000, with a MCMC length of 500,000 replicates. Following Kronauer et al. (2007), haploid male genotypes were entered as homozygote diploids to include inferred males in the analyses. Additionally, the Bayesian clustering algorithm implemented in the software STRUCTURE v. 2.3.1 (Pritchard et al., 2000) was computed based on the admixture model with correlated allele frequencies, and with a number of clusters K = 2, running 10 iterations. Each run consisted in 500,000 replicates of the MCMC after a burn-in period of 500,000 replicates. Clustering results were analyzed using CLUMPAK (Kopelman et al., 2015) based on a Markov clustering algorithm which identifies sets of highly similar runs grouped together in modes and separates these distinct groups of runs to generate a consensus solution for each distinct mode. Based on the consensus solution of the majority mode (no minority mode was found in this analysis), we obtained 2 distinct Q-values for each individual corresponding to their membership coefficient for each cluster.

Assignment of individuals

Assignment of individuals was based on the four categories defined by NEWHYBRIDS (*T. immigrans*, *T. caespitum*, F1 and backcrossed hybrids), as this method has been demonstrated to better discriminate hybrids in our system (Cordonnier et al., submitted to *Heredity*). In case of incompatibility between worker offspring and parents' identity (observed in 13 of the 70 colonies analyzed), we used Q-values obtained from STRUCTURE to better characterize these situations.

RESULTS

The inferred genotypes of queens showed that all colonies were monogynous, as a single maternal diploid genotype was consistent with the observed worker genotypes in the 70 colonies investigated. The inferred genotypes of males revealed that polyandry occurred in both species, with an overall mean of 1.886 observed mates for the 70 colonies studied. NEWHYBRIDS allowed the identification of all 1038 analyzed workers, as well as the 70 females and 132 males inferred. In 13 colonies, worker offspring identities were incompatibles with parents' identities and Q-values obtained by STRUCTURE were used to better understand these situations. These mismatches corresponded to: (1) an over-detection of individuals belonging to parental species in situations where hybrid queens that were strongly backcrossed with *T. immigrans* mated with pure *T. immigrans* males (11/13 situations) or (2) an over-detection of F1 hybrids in situations where hybrid queens that were strongly backcrossed with *T. immigrans* mated with pure *T. caespitum* males (2/13 situations). Overall, four categories of colonies were identified: (*i*) 28 pure *T. immigrans* colonies containing only *T. immigrans* workers, queen and male(s), (*ii*) 15 pure *T. caespitum* colonies containing only *T. caespitum* workers, queen and male(s), (*iii*) 11 mixed colonies containing pure and hybrid workers but only pure parents (queen or males) and (*iv*) 16 backcrossed colonies containing hybrid workers and parents. The numbers of colonies in each category and each geographical sampling area are detailed in Table 2.

Table 2. Number of colonies in each sampling area and colony categories: pure *T. immigrans* or *T. caespitum* colonies containing only workers, queen and male(s) of pure parental species, mixed colonies containing pure and hybrid workers but only pure parents, and backcrossed colonies containing hybrid workers and parents

	North	Center	South
T. immigrans colonies	9	10	9
T. caespitum colonies	5	6	4
Mixed colonies	4	3	4
Backcrossed colonies	6	6	4

Among the 16 backcrossed colonies, five corresponded to an F1 female mated with pure males and eleven involved a backcrossed female mated with pure males. Remarkably, in all these backcrossed colonies, none of the 26 males involved were hybrids.

The observed number of mating (K_{obs}; Jaffé, 2014) was significantly higher in *T. caespitum* (K_{obs} = 2.400 ± 0.1309) than in *T. immigrans* (K_{obs} = 1.679 ± 0.1156; Mann-Whitney-Wilcoxon W = 328.5, p-value = 0.0008) (Fig. 1). Hybrid colonies had an intermediate observed number of mating (K_{obs} = 1.955 ± 0.0753) compared to parental species. Differences of observed number of mating between zones within each species suggested that polyandry possibly differed latitudinally between zones (*T. immigrans*: K-W chi-squared = 5.65, df = 2, p-value = 0.059; *T. caespitum*: K-W chi-squared = 5.25, df = 2, p-value = 0.072) with a higher level of polyandry in the south compared to the north in *T. immigrans* (Mann-Whitney-Wilcoxon W = 16.5, p-value = 0.055) (Fig. 1).

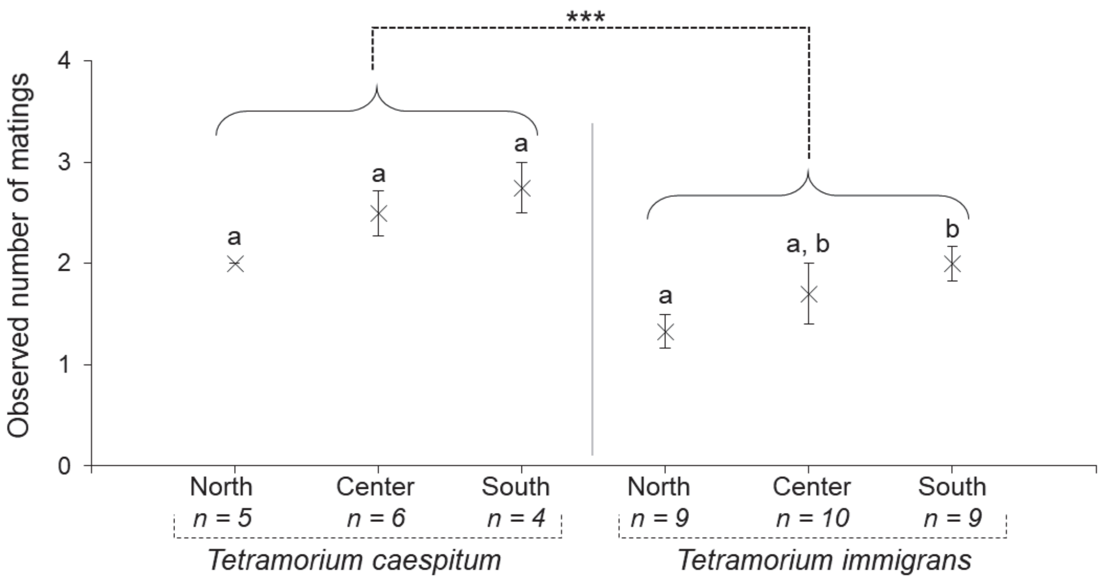

Figure 1. Observed number of mating (K_{obs}) in each pure species (*T. caespitum* and *T. immigrans*) and for each area (North, Center, South). Stars indicates a significant difference (p<0,001) between species assessed by Mann-Whitney-Wilcoxon test. Letters indicate significant differences (p<0,05) between areas assessed by Mann-Whitney-Wilcoxon test with a simple Bonferroni correction.

In mixed colonies (i.e., colonies involving heterospecific mating), the simultaneous presence of both conspecific and heterospecific males was rare (three situations involving a *T. caespitum* queen, and one situation involving a *T. immigrans* queen). In the three situations where the *T. caespitum* queen mated with both *T. caespitum* and *T. immigrans* males, worker offspring were overwhelmingly sired by heterospecific males (Tab. 3), whereas in other colonies the paternity skew was suggestively lower, with an average contribution to the worker offspring of 70.6% for the most productive of two males, falling to 49.6% for three males.

Table 3. Percent contribution of males to worker offspring for the three mixed colonies involving a *T. caespitum* queen and the simultaneous presence of both conspecific and heterospecific males

Colony ID	Male 1 (conspecific)	Male 2 (conspecific)	Male 1 (heterospecific)
Lyo29	20%	20%	60%
Tsr32	6%	-	94%
Vil47	20%	-	80%

DISCUSSION

Mating system

By investigating the mating system of 28 pure colonies of *T. immigrans*, 15 pure colonies of *T. caespitum* and 27 hybrid colonies, we demonstrated that both *T. immigrans* and *T. caespitum* displayed a monogyne/polyandrous mating system, with a higher mating rate in *T. caespitum*. To our knowledge, our study is the first to describe polyandry in these two species. The occurrence of multiple mating has raised theoretical and experimental attention to issues related to sexual selection and sexual conflict, such as the mechanisms involved in sperm competition and cryptic female choice, as well as the costs and benefits that females may incur with multiple mating (Pizzari & Wedell, 2013). In social Hymenoptera (ants, bees and wasps), the adaptive significance of polyandry is often explained by hypotheses suggesting that polyandry is advantageous to queens because the resultant increase in genetic variation within colonies leads to an increased colony performance (Keller & Reeve, 1994; but see Fjerdingstad et al., 2003 or Fournier et al., 2008). These hypotheses are also often put forward to explain the value of hybrid offspring, as increased allelic variation and transfer of adaptively important genetic variation may increase the fitness of the introgressed lineage (Twyford & Ennos, 2012; see below). Future measurements of colony performance in the species studied here (and their hybrids) as a function of their degree of polyandry should be considered, as such a multiple mating and hybridization system offers unique insight into important eco-evolutionary mechanisms, such as sexual selection and sexual conflict or species interaction and niche partitioning.

Latitudinal variation of mating frequency

In this study, the level of polyandry seemed to increase at lower latitudes although data are too scarce to confirm a latitudinal pattern. Consistent with our observations, Corley & Fjerdingstad (2011) investigated two populations of *Lasius niger* in North Europe *vs*. two populations in South Europe and concluded that multiple mating by queens was far more frequent in the two southern populations than in the two populations from Northern Europe. These authors suggested that this pattern arose because multiple mating might increase fitness for queens and colonies in Southern European climates, whereas only exceptionally good colonies can survive in harsher northern environments, thus favoring single mating *vs*. multiple paternity which could reduce variance in colony performance. As far as the two *Tetramorium* species studied here are concerned, it should be interesting to extend the present study to a broader latitudinal range, and to investigate situations outside the hybrid zone, in which other mechanisms might control reproductive mechanisms within and between these species (Umphrey, 2006; see below).

Hybrid females, but not males, produce offspring

Our results clearly showed the reproductive capability of hybrid females, but no hybrid males were detected in the paternity of workers. Introgression into females but not into males is compatible with the extended Haldane's rule for haplodiploids, which states that the haploid sex should more often be sterile or inviable (Koevoets & Beukeboom, 2009). Nevertheless, to date, this pattern has been rarely documented in ants. The only example in the literature involved hybrids between the red wood ants *Formica aquilonia* and *F. polyctena* (Kulmuni et al., 2010). In this system, two genetic groups coexist in highly polygynous nests; the alleles of one genetic group are found in the diploid individuals (i.e., females) but not the haploid males of the other genetic group, leading to hybrid females (workers and queens) but no hybrid males (Kulmuni et al., 2010). Kulmuni & Pamilo (2014) proposed two hypotheses to explain this pattern: the selection and segregation hypotheses. On the one hand, the selection hypothesis implies a strong postzygotic selection against hybrid males, because recessive incompatibilities can be masked in diploid heterozygous females but not in haploid males. In that case, hybrid males die but females can survive. On the other hand, the segregation hypothesis states that hybridization has led to the formation of two independently segregating sets of alleles, one always transmitted from the queens to their sons (Y-type) and the other to their daughters (X-type) after fertilization by sperm carrying the paternal complement (Y), leading to hybrid females but only pure males. According to Lowry & Willis (2010), this second situation could contribute to hybrid incompatibilities and lead to reproductive isolation of hybrids. In this context, it should be relevant to test this hypothesis in the present system, e.g., by monitoring genotypes at various stages of development (very young embryos, sexual larvae and pupae, worker larvae and pupae, adult females, males and workers), as was done by Kulmuni & Pamilo (2014).

Asymmetrical contributions to offspring of conspecific vs. heterospecific males

A major feature of the present study is the discovery of the coexistence of hybridization and multiple mating, allowing the simultaneous production of both hybrid and nonhybrid offspring within the colony. Nevertheless, the production of F1 hybrid and nonhybrid offspring within the same colony was rare and did not allow conclusions to be drawn on the hypothesis of a random paternity allocation between species. Our study provided a preliminary assessment of the variation of offspring production in hybrid colonies and suggested asymmetrical contributions to offspring of conspecific *vs.* heterospecific males in colonies with a *T. caespitum* queen. The potential negative effects of interspecific hybridization (see Feldhaar et al., 2008 for specific examples) can be offset by advantages conferred by hybrid workers, particularly because hybrid workers are likely to have a greater ability to use marginal habitats (Umphrey 2006). In such situations, Umphrey (2006) concluded that interspecifically mated queens could for example nest in sites where intraspecific competition is low and reach a higher probability of reproductive success. In the *Tetramorium* system studied here, there are significant differences in microhabitats and habitats between the two species since *T. immigrans* is predominantly present in urbanized habitats and micro-habitats (e.g., pavement) while *T. caespitum* occurs mainly in natural habitats and vegetated micro-habitats (Cordonnier et al., submitted to *Ecology*). The marginal habitat hypothesis, according to which the hybrid workers should mainly occur in the urbanized-environment colonies with *T. caespitum* queens, could therefore be tested. Lowe et al. (2015) suggested that spatial sorting, i.e., an evolutionary mechanism that allows gene frequencies to increase because of greater success through space rather than time, could partially explain the paradoxical spread of introgressive hybridization despite strong selection against hybrids. These authors concluded that because all the progeny of a hybrid will be hybrids and inherit genes from both parental taxa, admixture could increase even when most hybrid progeny do not survive. The coexistence of hybridization and multiple mating observed in the system described here could enhance these mechanisms and accelerate the spread of introgression.

Sexual selection, natural selection and sexual conflict

Although causality cannot be established directly from the patterns of paternity biases observed here, these patterns raise questions worthy of investigation, particularly with respect to the potential roles of post-copulatory sexual selection and sexual conflict in social evolution (Jaffé et al., 2012). As paternity skew across worker cohorts in the same colony seemed to occur, it would be worth checking whether this might simply indicate a difference in worker survival rate depending on the environment of the nest or reflect differential larval growth rather than temporary variable sperm use owing to incomplete sperm mixing (Boomsma 2013). As previously suggested by Boomsma (2013), it is tempting to speculate that this facultative multiple mating system evolved to allow females to correct suboptimal first inseminations, but considerable research effort will be required to unravel the interaction between sperm transfer and female sperm storage responses, which appears to be an ambitious goal as the species studied probably do not mate under laboratory conditions. A first step toward answering these questions could be to consider not only sterile worker offspring but also males and gynes. In ants, a fertilized egg can become a reproductive female or a sterile worker, because of different larval feeding regimes, seasonal effects, hormones pulses or even genetic determination (e.g., Nonacs, 2006). Analyzing sexual offspring instead of the sterile offspring less prone to selective pressures would ensure further insights into the consequences of sexual selection and sexual conflict in insect societies (Jaffé et al., 2012). Another way to tackle these evolutionary questions could be to go beyond post-hatching natural selection stages by studying copulation frequencies (which in our case are very difficult to observe, as mating occurs during nuptial flights) or insemination frequency obtained after genotyping the sperm content of the queen's spermatheca, as suggested by Jaffé (2014).

Hybrid detection and identification

Finally, in the context of hybrid colonies identification, our results show that lack of genetic information on reproductive individuals leads to underestimate introgression processes, as all workers in a mixed colony are not hybrids and because in hybrids colonies, backcrossed workers having allelic frequencies close to F1 hybrids or individuals belonging to parental species are not properly detected. As a consequence, introgression may be much more pervasive than observed by Cordonnier et al. (2018, submitted to *Heredity*).

CONCLUSION

Together, our findings paint the picture of a complex and dynamic mating system in *Tetramorium immigrans* and *T. caespitum*. Numerous studies have already focused on hybridization, and even more on multiple mating, but so far, few if any have described a system combining both polyandry and interspecific hybridization, making it worth investigating for several reasons. One reason is that, as far as we know, the study of polyandry within a hybrid zone is totally unprecedented. It could also be an opportunity to investigate sexual selection processes in social Hymenoptera and an ideal system to test the numerous hypotheses about selective processes involved in the evolution of multiple mating. Finally, further enquiring into the hybrid *Tetramorium* situation should contribute to better understand interspecific hybridization mechanisms and their short- to long-term consequences on genetic and taxonomic diversity in a world facing human-induced global changes.

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SUPPORTING INFORMATION

Table S1. Statistics of microsatellite markers computed using GENALEX (Peakall and Smouse, 2006): Mean and SE per family of sample size, number of alleles and effective number of alleles.

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		Tspe52d	Tspe52b	Tspe52k	Ttsu56d	Tspe53a	Ttsu55a	Ttsu59j	Tspe51i	Ttsu58i	Tspe51a	Tspe51o	Tspe51b	Tspe51d	Tspe52a	Tspe53b	Ttsu57
N	**Mean**	14,786	14,743	14,786	14,800	13,586	14,743	14,614	14,771	14,757	14,600	14,729	13,729	14,814	14,714	14,714	14,557
	SE	0,084	0,097	0,088	0,085	0,101	0,088	0,138	0,079	0,085	0,101	0,136	0,089	0,087	0,096	0,087	0,125
Na	**Mean**	3,357	3,557	3,043	3,100	2,457	3,386	3,414	3,557	3,371	3,086	3,314	3,343	2,886	3,086	3,157	3,014
	SE	0,093	0,101	0,103	0,122	0,128	0,110	0,103	0,107	0,096	0,103	0,101	0,104	0,111	0,090	0,090	0,103
Ne	**Mean**	2,836	3,024	2,557	2,611	2,116	2,840	2,912	3,014	2,917	2,601	2,896	2,827	2,408	2,633	2,705	2,538
	SE	0,084	0,092	0,101	0,107	0,109	0,103	0,098	0,089	0,101	0,096	0,097	0,091	0,094	0,085	0,085	0,099

Chapter 5. General Discussion

> « *The first law of ecology is that everything is related to everything else.*»
>
> *Barry Commoner, The Closing Circle, 1971*

5.1- SYNTHESIS OF THE RESULTS

Chapter 1 briefly reviewed the consequences of climate changes, urbanization, and biological invasions; I further described how these global changes promote genetic exchanges between species. I discussed the long-term evolutionary consequences of hybridization and introgression in a broad context and I showed that ants, especially *Tetramorium* species, and the Rhône-Saône valley are ideal systems for studying these problematics.

In **Chapter 2**, I investigated range limits of species over both urban and climatic gradients. I sampled 1690 *Tetramorium* colonies and used a two-step integrative approach combining nuclear and mitochondrial DNA to identify five *Tetramorium* species in the Rhône-Saône valley: *T. forte*, *T. moravicum*, *T. semilaeve*, *T. immigrans*, and *T. caespitum*. I showed that climate has a fundamental role as a factor limiting the species ranges at a well-known biogeographical limit between Continental and Mediterranean climates. I also highlighted the importance of considering several spatial scales simultaneously in order to study the impact of urbanization on species distribution. Results suggested a highly contrasted responses to urbanization of *T. immigrans* and *T. caespitum* which may indicate niche partitioning processes driven by urbanization.

Chapter 3 focused on *Tetramorium immigrans* to investigate the combined influence of climate and urbanization on its distribution along the Rhône-Saône valley. I showed that *T. immigrans* was probably introduced here from external sources, and that these introductions were followed by colonization favored by human activities in the northernmost urban areas. I discussed the fact *T. immigrans* is likely not native to South-eastern France, at least to the northernmost urban areas. I concluded that despite that the effect of climate-urbanization interaction on species distribution is mostly overlooked in the literature, many taxa may conform to the pattern described here for *T. immigrans*, making the combined study of global changes a necessary challenge for future studies.

Finally, in **Chapter 4** I showed that hybridization between *Tetramorium immigrans* and *T. caespitum* can lead to fertile offspring, with hybrids located at latitudes where parental species are sympatric. Differentiated cuticular hydrocarbon profiles and heightened interspecific aggression revealed clear species recognition cues. The mating system of *Tetramorium immigrans*, *T. caespitum* and hybrid colonies was monogyne and polyandrous. Hybrid males seemed to be sterile or inviable. I discussed the fact that such study of polyandry within a hybrid zone was unprecedented and open the opportunity to investigate the selective processes involved in the evolution of multiple mating.

In the following sections, I will start by identifying the filters crossed by species allowing them to hybridize with each other (**Chapter 5.2**). Then, I will discuss some hypotheses about the future of the *Tetramorium immigrans* x *caespitum* system (**Chapter 5.3**). In the third part (**Chapter 5.4**), I will stress the importance of considering the interrelationships between climate, urbanization, invasions and hybridization. Finally, a fourth part will be devoted to the conclusion and presentation of different research perspectives (**Chapter 5.5**).

5.2- HOW DOES HYBRIDIZATION BETWEEN *TETRAMORIUM IMMIGRANS* AND *T. CAESPITUM* OCCUR?

5.2.1- From species meeting to hybrid offspring

Hybridization between two sexually reproductive species can only occur after a series of filters have been passed through (Fig. 1). First, the involved species must live in sympatry. Hybridization therefore often occurs as a consequence of secondary contact between species, i.e., a geographic overlap between two genetically distinct lineages that derived from a common ancestor and underwent a phase of allopatric isolation. For instance, biological introductions concomitant with urbanization (Cristescu 2015) can result in contact zones between species capable of genetic exchanges because of a lack of pre-zygotic reproductive barriers (i.e. morphological, behavioral, geographical or ecological), as reviewed by Crispo *et al.* (2011) in numerous taxa. *Tetramorium immigrans* and *T. caespitum* live in overlapping areas in the Sâone-Rhône valley (**Papers 1 & 2**) and the probably recent establishment of *T. immigrans* in the northernmost areas (**Paper 3**) could have led to secondary contact with *T. caespitum*. After their meeting, numerous barriers to hybridization may still remain before mating, between mating and fertilization (prezygotic barriers), or after fertilization (postzygotic barriers; Fig. 1; Campbell *et al*. 1999; Coyne & Orr 2004). Habitat isolation, i.e. when species occupy different habitats within the same geographical area, could partially prevent hybridization between *T. immigrans* and *T. caespitum* according to the results of **Paper 2**. Massive nuptial flights however, might overcome this limitation as mating would take place at significant distances from the colonies.

Seasonal or temporal isolation can occur when copulation takes place at different times of the year. Wagner *et al.* (2017) indicated that winged reproductive of *T. immigrans* can be found in nests between March, 17 and September, 29, whereas reproductive alates of *T. caespitum* were in nests between May, 28 and August, 19, which corroborate the hypothesis of a phenological overlap compatible with interspecific mating. Sexual or behavioral isolation can then occur when sexual attraction between species is low. Species discrimination plays here a significant role as mating with heterospecific partners could result from an absence of discrimination of the partner's species. Cuticular hydrocarbons are important in mate choice in many insects, even playing a key role in reproductive isolation, e.g. in the hybridizing field cricket species *Gryllus campestris* and *G. bimaculatus* (Tyler *et al.* 2015). In **Paper 5**, I evidenced differentiated cuticular hydrocarbon profiles and heightened interspecific aggression in *Tetramorium immigrans*, *T. caespitum* and hybrid workers, suggesting that species recognition cues are both present and perceived. This situation could be different for males and females at mating, for instance because inside the mating swarms, increased exchanges of cuticular hydrocarbons (CHCs) with other reproductives that come into physical contact could homogenize their profiles and reduce their ability to use species-specific cues (Herrmann 2016). Therefore, it could be relevant to further investigate the recognition cues in reproductives, although difficult as the identification of these taxa is based on genetic methods that are wholly or partially destructive of individuals which cannot be further used for CHC extraction or behavioral assays. Mechanical isolation arises when anatomical incompatibilities between genitalia prevents fertilization. In ants, hybridization should be possible provided uniformity of the genital morphology within the same sex between hybridizing taxa (Feldhaar *et al.* 2008). Male genitalia in *T. immigrans* and *T. caespitum* are nearly identical (Schlick-Steiner *et al.* 2006; Wagner *et al.* 2017), suggesting an absence of anatomical barriers to mating between these species (Fig. 1). Gametic isolation can occur if the gametes of a species are unfit to survive in the genital tracts of another species. As far as I know, no study has investigated such mechanisms in the *Tetramorium caepitum* complex. Finally, three successive postzygotic barriers can prevent hybridization between species (Fig. 1): (i) reduced viability of hybrids, at egg, larval or adult stage, (ii) sterility of hybrids, when F1 hybrids of one sex, or both, are unable to produce functional gametes, and (iii) hybrids breakdown, when viability or fertility of hybrids of second generation (F2) or backcrosses is reduced. **Chapter 4** especially focused on postzygotic barriers in the *T. immigrans – T. caespitum* hybridization complex, and I confirmed that hybrid zygotes reach maturity (**Paper 4**; see also Wagner *et al.* 2017), and that hybrids can reproduce, as I detected introgression (**Paper 4**) and hybrid offspring produced by hybrid queens (**Paper 6**). However, hybrid males did not seem to be involved in the production of hybrid offspring, although the kind of postzygotic barrier involved cannot be deduced from my results (Fig. 1).

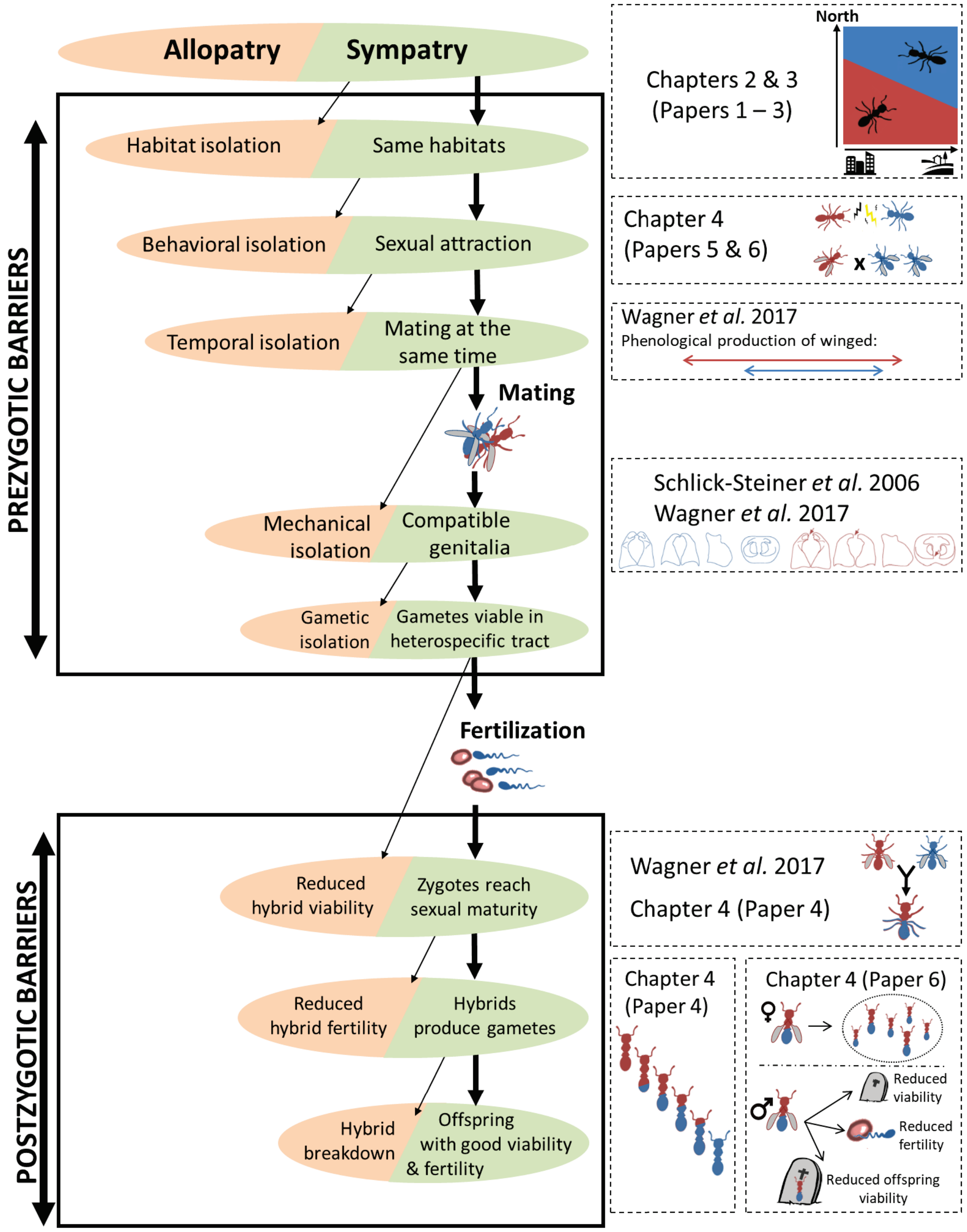
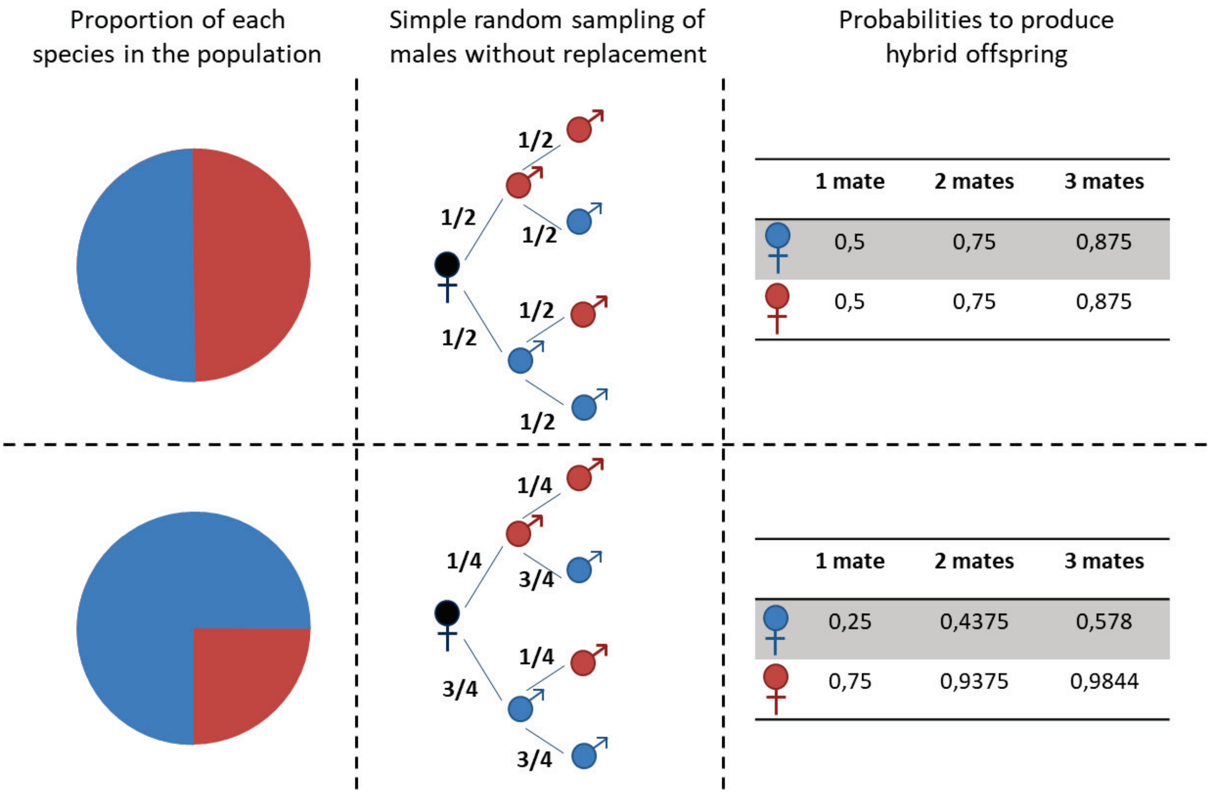


Figure 1. Left panel: Reproductive barriers to gene flow between two different species (orange) and how to overcome them (green). Prezygotic barriers occur before mating, while postzygotic do after (Adapted from Campbell *et al.* 1999 and Coyne & Orr 2004). Right panel: Synthesis of results from previous and current studies investigating these barriers in the *Tetramorium immigrans* (red) and *T. caespitum* (blue) hybridization complex. Workers are unwinged individuals.

5.2.2- When mating system and mating behavior favor the spread of hybridization

Several behavioral characters of mating system probably facilitate interspecific mating between *T. immigrans* and *T. caespitum* hence promoting hybridization. Polyandry, i.e., when females mate with multiple males, is a striking example of such as character, as such a mating system could facilitate hybridization. In **Paper 3**, I investigated the hybridization of *T. immigrans* and *T. caespitum* and evidenced the genetic introgression between these species. In **Paper 6** I showed that both *T. immigrans* and *T. caespitum* were polyandrous. In such a hybridization complex, contrary to monandrous species where each female mates with either a con- or a heterospecific male and thus produces either conspecific offspring only or hybrids only, polyandry may lead females to mate with both con- and heterospecific males and to produce varying proportions of hybrid offspring. For instance, in two theoretical situations with an initial population mixing (i) 50% of each parental species or (ii) 25% of one species and 75% of the other, the random mating of females with one, two or three potential males systematically increased probabilities of producing hybrid offspring (Fig. 2). The role of polyandry in producing hybrid zones has been rarely studied in the literature, but following previous theoretical framework, polyandry under random mating should factor positively toward the establishment of hybrid zones. According to Arnqvist *et al.* (2000), for any given degree of hybrid unviability, hybridization in monandrous systems results in a lower variance in female fitness and a lower opportunity for selection among females against hybridization under polyandry. Hartman *et al.* (2012) also showed that extra-pair copulation, which is a form of polyandry, could override assortative mating preferences and facilitate hybridization and genetic introgression.



	1 mate	2 mates	3 mates
🔵♀	0,5	0,75	0,875
🔴♀	0,5	0,75	0,875

	1 mate	2 mates	3 mates
🔵♀	0,25	0,4375	0,578
🔴♀	0,75	0,9375	0,9844

Figure 2. Statistical view of the role of polyandry showing that it should spread hybridization faster than monandry. I hypothesize here that females mate randomly with males of both species (red and blue) according to their proportions in the population (left panel). Mates per female therefore correspond to a random sampling of one or two males without replacement (central panel). More mates, whatever the female species (red or blue), systematically leads to an increase of the probability of producing hybrid offspring (right panel).

However, other mechanisms can interfere with the mating system, such as non-random mating resulting from mate choice, i.e., selection of partner dependent on the attractiveness of an individual's phenotypic or genotypic traits. Although mate choice is predicted to be lower in more polyandrous species, which would therefore be more prone to accept a heterospecific mate (Veen *et al.* 2011), the strength of preference for conspecific or heterospecific mating could mitigate the spread of hybridization due to multiple mating. Indeed, sexual conflict in mating and intersexual differences in the strength of assortative mating preferences strongly affects the ability of an alternative mating tactic to influence introgression (Hartman *et al*. 2012; Fig. 3). Studies on mating behavior and hybridization often draw a dichotomy between competitive males mating indiscriminately and choosy females limiting heterospecific mating (Lipshutz 2017). However, Lipshutz considers that this dichotomy is oversimplified in several ways. For instance, if females of two species select large males for mating, and males of one species are bigger, females of one species should prefer mating with conspecific males because heterospecific males would be smaller, but females of the other species should favor heterospecific males as these males are bigger than males of its own species. Lipshutz (2017) concluded that heterospecific preference of females occurs when heterospecific males resemble high-quality conspecifics or when ancestral preferences have not diverged, as in female orange-backed fairy wrens *Malurus melanocephalus melanocephalus* that prefer red-backed males resembling another subspecies *Malurus melanocephalus cruentatus* (Baldassarre & Webster 2013) and in female tungara frogs (*Physalaemus pustulosus* species group) that prefer call features of heterospecific males (Ryan & Rand 1993).

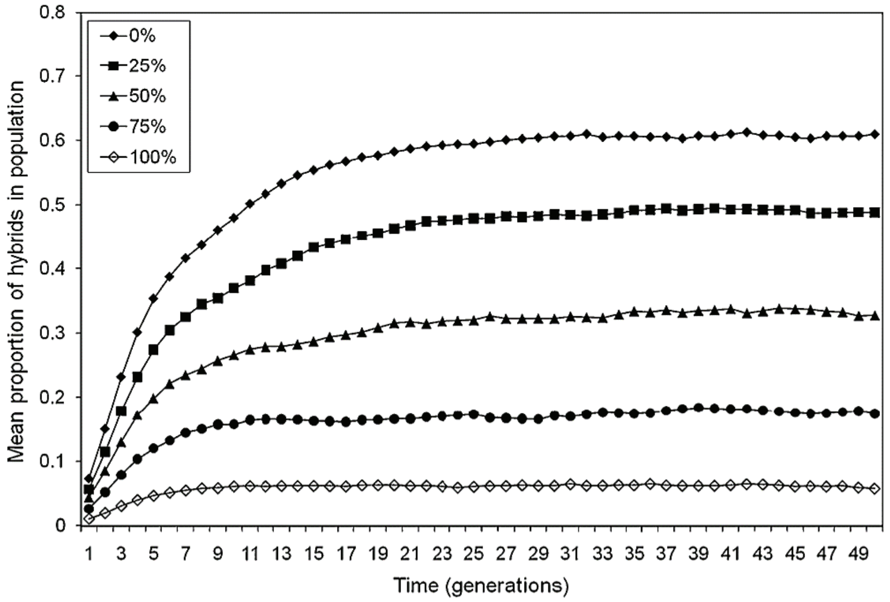


Figure 3. The mean proportion of hybrids in a population over time depends on the degree of female control over copulation and sperm competition favoring an extra-pair mate (female have complete choice (100%), or 75%, 50%, 25%, or no choice (0%; baseline value); from Hartman *et al*. 2012: fig. 4).

In the hybrid system of *T. immigrans* x *caespitum*, female mate preferences could therefore modify the outcome of both hybrids and parental species. Regrettably, in **Paper 6**, the production of F1 hybrid and nonhybrid offspring within the same colony was rare and did not allow conclusions about mate choice during nuptial flights. Another way to tackle these evolutionary questions could be to go beyond post-hatching natural selection stages by studying copulation frequencies (which in our case are very difficult to observe, as mating occurs during nuptial flights) or insemination frequency obtained after genotyping the sperm content of the queen's spermatheca, as suggested by Jaffé (2014).

5.3- THE FUTURE OF *TETRAMORIUM* SPECIES AND HYBRIDS IN FRANCE

5.3.1- *Tetramorium immigrans*, from a bottleneck to an increasingly fast spread?

Tetramorium immigrans is an invasive species in North America (Steiner *et al.* 2008) where it was introduced by the end of the 18th century, subsequently spreading to urban ecosystems of the northern USA Atlantic coast (King & Green 1995) as well as further inland. Steiner *et al.* (2008) showed that *T. immigrans* could become a global threat and spread away from its introduced range. According to its climatic niche as described in **Paper 1**, global warming could probably enhance this spread in the future decades. At a finer geographical scale, Steiner *et al.* (2008) also suggested that the spread of *T. immigrans* could be promoted by factors such as urbanization. Accordingly, we found that this species was clearly associated with urbanized areas in our study (**Paper 2**). Most urban specialist species are introduced by human-mediated activities, while only few native species seem suited to urban environments (McKinney 2006), pointing towards an anthropogenic introduction of *T. immigrans* in urban areas.

A study of 80 invasive species has shown that more than half of these species display a loss of allelic diversity greater than 20% (Dlugosh & Parker 2008). This is the case, for example, for many invasive insects, e.g., the Argentine ant *Linepithema humile* (Tsutsui *et al.* 2000), the bumblebee *Bombus terrestris* (Schmid-Hempel *et al.* 2007), and the Guatemalan potato tuber moth *Tecia solanivora* (Puillandre *et al.* 2008). Lindholm *et al.* (2005) also showed a founder effect linked to a recent bottleneck in introduced populations of guppies *Poecilia reticulata* by comparing the genetic diversity at nine microsatellite and one mitochondrial loci. The authors pointed out that genetic diversity was severely reduced in introduced compared to native populations. This reduction in genetic diversity resulting from a founder effect is likely to diminish the adaptability of exotic populations in a new environment. Invasive species therefore have to adapt to a new environment despite a generally low initial genetic diversity (Frankham *et al*. 2005). Recent studies have attempted to explain this paradox by measuring genetic diversity and testing the presence of genetic bottlenecks in introduced populations (e.g., Fonseca *et al*. 2000; Tsutsui et *al*. 2000; Zeisset & Beebee 2003; Rasner *et al*. 2004; Puillandre *et al*. 2008). The results show that even though many invasive species display a reduction in genetic diversity and signs of genetic bottlenecks (Puillandre *et al*. 2008), this decline in diversity is not unfavorable for the establishment and proliferation of the species, probably because of rapid population expansion after introduction (Cabe 1998; Zeisset & Beebee 2003). Indeed, if the rate of population growth is high, relatively high levels of heterozygosity can be maintained, even if the population has suffered a bottleneck, especially if the bottleneck has not been too marked and several individuals have been introduced (Nei *et al*. 1975). An increase in invasive success is also possible following a bottleneck. Facon *et al*. (2011) demonstrated that bottlenecks have purged populations of Asian ladybug *Harmonia axyridis* of deleterious alleles responsible for inbreeding depression and thus increased the selective value of invasive individuals. Introduced populations of Argentine Ant *Linepithema humile* have undergone a strong genetic bottleneck responsible for a very reduced genetic variability (60% decrease in the expected heterozygosity compared to the area of origin; Tsutsui *et al*. 2000), and a probable lack of variation at the loci responsible for inter-colony recognition, leading to the formation of a very large "supercolony" (Suarez *et al*. 1999; Tsutsui *et al*. 2000; Tsutsui & Suarez 2003). The loss of genetic diversity related to the introduction of a limited number of individuals therefore still allowed a successful establishment. In **Paper 3**, I confirmed multiple anthropic introductions of *T. immigrans*, and I showed that the species could have been transported from one city to another, in the same way as several house-dwelling arthropods (e.g., house cricket or cockroaches) transported all over the world by ‘‘house-to-house’’ jump dispersal as humans move (McKinney 2006). Differences in diversity and distribution between *T. immigrans* and *T. caespitum* seemed to confirm that *T. caespitum* has been present for a long time in the study area, while *T. immigrans* is closely associated with urbanization and underwent founder effects resulting from introduction. The alternative hypothesis of post-glacial recolonization from smaller or more distant glacial refugia for *T. immigrans* would generate similar diversity patterns but would explain neither genetic structure nor the strong urban preferences of *T. immigrans*. Gathering more distribution and genetic data on *T. immigrans* should improve the understanding and interpretation of the observed genetic patterns. Accurate data on the entire range of the species, and more particularly in eastern Europe, will be needed to clarify the status of this species and to describe more precisely the source location of this species in its native range, especially regarding the genetic diversity pattern described above.

Wheeler (1927) was the first to discuss the occurrence of pavement ants in North America, speculating that they came over during the colonial era (1748). However, the species had not spread across the United States from its introduction to Wheeler's study, when compared to the large spatial range known today, pointing toward a much slower spread rate at the first stage of invasion. Such lags between growth and spread of populations have been widely documented in the literature, resulting from early dynamics in the invasion process, when population sizes and area occupied are small (Crooks 2005). According to this author, the exponential growth of incipient populations undoubtedly accounts for many observed lags, combined with the fact that the area occupied is a squared function of time if populations are introduced into a small area and subsequently spread at a constant rate in all directions. A variety of supplementary mechanisms giving rise to slow initial growth of invasive populations have been proposed, such as the difficulty in finding mates when there is undercrowding (mate limitation; Veit & Lewis 1996) and increased ability to suppress natives at high invader densities (Cappuccino 2004). According to the results of **Paper 3** and **Paper 6**, these mechanisms could have play an important role in the first stages after the introduction of *T. immigrans* in urban areas of South-Eastern France. It is therefore possible that the species has endured a slow spread in the Rhône-Saône valley. In such situation, further spread likely will be faster and spatially wider in the coming decades, reinforced by "jump-dispersal" phenomena which can establish foci of invasion well ahead of the expanding front and thus rapidly increase invader range (Johnson & Carlton 1996; Johnson & Padilla 1996), as well as by the combined effects of urban sprawl promoting its establishment and climate warming favoring its propagation.

5.3.2- Is *Tetramorium immigrans* an urban exploiter in competitive interaction with *T. caespitum*?

Although my research largely focuses on *Tetramorium immigrans*, many issues regarding its interspecific interactions with *T. caespitum* are also of major interest. Together, **Papers 1, 2 and 3** depict *T. immigrans* as an efficient urban exploiter capable of colonizing cities, probably at the expense of *T. caespitum*. Indeed, the presence of *T. immigrans* seems to lead to an exclusion of *T. caespitum*, the latter being present in cities only in the absence of *T. immigrans* (e.g. in Dijon or Langres; **Papers 1 & 2**) suggesting interspecific competition.

When two species share the same resources (e.g., food, light, space, breeding site), they can interact through either exploitative or interference competition. Exploitative competition occurs where a species diminishes the availability of a limiting resource for another species by depleting it (Park 1954). Interference competition occurs where a species inhibits the accessibility to a resource for another species (Schoener 1983). Ultimately, such competition processes are considered one of the strongest drivers of community structure and functioning (Case & Gilpin 1974; Tilman 1982; Begon *et al.* 1996), even if they can be difficult to detect and quantify in natural ecosystems (Schoener 1983). In the case of *T. immigrans* and *T. caespitum*, niche partitioning processes driven by urbanization (**Paper 2**) could be due to interspecific competition. The observed pattern is similar to the situation described in North America, where *T. immigrans* is known to be invasive since the early nineteenth century and where it has been described as an urban specialist (King & Green 1995). This observed pattern could mainly result from two non-mutually exclusive situations:

- In urban areas, *T. immigrans* and *T. caespitum* could favor the same habitats, the same diet or the same nesting resources, leading to an exploitative competition underlying the observed niche partitioning displacing *T. caespitum* out of the urban environments.
- In urban areas, resources could be sufficient for the persistence of both *T. immigrans* and *T. caespitum* but the species compete for their accessibility, inducing aggressive behaviour between them and leading to interference competition, therefore displacing *T. caespitum* out of the urban environments.

Due to the lack of previous ecological studies in the species of the *T. caespitum* complex, there is little evidence to determine which situation(s) are at the origin of the observed pattern. Previous studies focusing on pavement ants showed that colonies are well known for their ant wars involving thousands of ants fighting for establishing colony boundaries (Hoover *et al.* 2016; Plowes 2008), therefore suggesting that interference competition is also likely to occur between heterospecifics. Following Shochat *et al.*'s (2006) framework, if competition happens, the competitive exclusion of the native species *T. caespitum* by the invasive species *T. immigrans* in urban areas could be due to specific traits of *T. immigrans* compared to *T. caespitum*, which would make it a better colonizer of urban areas, able to increase in abundance (i.e., an "urban exploiter"; Fig. 4). What are the ecological factors that have made this species such a successful urban invader? What are the characteristics that allow it to cross the filters of invasion stage and to spread over the landscape? Concomitantly to demographical and genetic factors, ecological traits of *T. immigrans* have probably determined its invasive success.

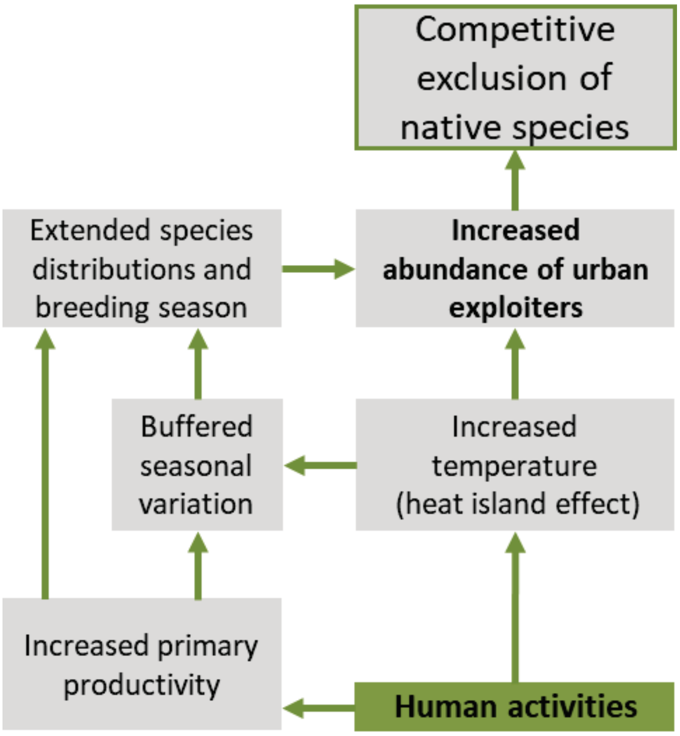


Figure 4: Schematic representation of pathways whereby human activities lead to competitive exclusion of the native species *Tetramorium caespitum* by *T. immigrans* passing through the increased abundance of invasive urban exploiter (adapted from Shochat *et al.* 2006). Grey boxes correspond to possible mechanisms underlying these patterns that remain to be further investigated.

For example, ant species in urban areas often have specific nesting habitats or food resources and survive despite increasing soil temperature and decreasing soil moisture (Philpott *et al.* 2010). They also have efficient dispersal abilities allowing founding queens to reach urban centers (Pacheco & Vasconcelos 2007). As a good urban exploiter, *T. immigrans* is therefore likely more generalist and opportunistic than *T. caespitum* (Philpott *et al.* 2010). In North America, *T. immigrans* was shown to be the prime consumer and remover of three commonly dropped food items (potato chips, cookies and hot-dogs) because of its abundance, activity levels, and dietary preferences (Youngsteadt *et al.* 2015). The results of Penick *et al.* (2015) on the abilities of *T. immigrans* to change its diet across urban habitats to shift to human foods also seem to corroborate this hypothesis. If the same pattern occurred in the Rhône-Saône valley, exploitative competition for resources between *T. immigrans* and *T. caespitum* would ensue and favor *T. immigrans* as a better forager. *Tetramorium immigrans* could also survive longer when suffering from food fasting, allowing it to have higher fitness than *T. caepistum* in urban areas where food might come in unpredictable pulses. Regarding nest habits, **Paper 2** concluded to the ability of *T. immigrans* to exploit anthropogenic resources for nesting. At the microhabitat scale, pavements turn nesting areas into warmer habitats than areas with sole vegetation cover, which could give *T. immigrans* an advantage provided such warmer temperatures actually provide a wider annual time-window than species inhabiting vegetated microhabitats such as *T. caespitum*. Urbanization indeed results in organisms exhibiting advanced phenology (Helm *et al.* 2013). Wagner *et al.* (2017) showed that winged reproductive of *T. immigrans* were produced in a wider period, which corroborate the hypothesis of a larger phenological niche for *T. immigrans*, in accordance with the pathways of Shochat *et al.* (2006; Fig. 4). Contrary to *T. immigrans*, *T. caespitum* builds soil mounds higher than 10 cm (Wagner *et al.* 2017), probably intercepting incident solar radiation, therefore maintaining ideal thermal conditions in the nest (Kasimova *et al.* 2014). The anthropized habitats of *T. immigrans* may allow this species to overcome the construction of such structures by nesting in microhabitats generating sufficient heat. Biological impacts of artificial nighttime lighting could also be involved in the niche partitioning of *T. immigrans* and *T. caespitum* as they impact individual physiology and behavior, species abundance and distribution, ecological interactions, the composition of communities, and the functioning of ecosystems (Gaston *et al.* 2017). For instance, artificial nighttime lighting has been shown to affect foraging in extending the foraging times of some otherwise diurnal animal species into the nighttime, or in changing the ways in which species forage.

Further investigations of species-level traits promoting *T. immigrans* in urban habitats should corroborate the hypothesis that this species is an urban exploiter or synanthropic species (McKinney 2006; Forman 2014). Other factors such as response to artificial light or to temperature variability, or differences in nest habits or in reproductive fitness could bring new lights on the drivers of their habitat preferences and to quantify the role of interference versus exploitative competition in their setting up.

5.3.3- The future of hybrid zones is difficult to predict but crucial to monitor

Hybridization between species is a major issue in evolutionary and conservation biology. Interspecific mating can reinforce pre-existing reproductive barriers (Dobzhansky 1937; Servedio & Noor 2003; Ortiz-Barrientos *et al*. 2009), resulting in novel gene combinations and new hybrid species (Anderson & Stebbins 1954; Arnold 2004; Seehausen 2004; Hedrick 2013; Brelsford *et al.* 2011; Hermansen *et al.* 2011; Abbott *et al.* 2013), or threatening biodiversity via the assimilation of rare species within introduced populations (Laikre *et al.* 2010) or via 'reverse speciation' of previously distinct species when ecological conditions change to favor the formation and viability of hybrids (Taylor *et al.* 2006; Seehausen *et al.* 2008). As depicted in **Chapter 1**, the outcomes of hybridization can thus span from complete species isolation to complete admixture, mainly depending on hybrids' fitness compared to parental taxa in the environmental context in which hybridization occurs. The complexity of the patterns generated by hybridization in ants makes it difficult to predict the future of hybrid zone. Nonacs (2006) showed for example that hybrid workers may allow colonies to survive and prosper in microhabitats that are hostile to pure species' worker phenotypes, or make colonies competitively superior to the parental species. On a broader context, the review of Harrison and Larson (2014) clearly illustrates how extremely complex the consequences of hybridization are, even more so in the case of introgression. For instance, in case of adaptative introgression, Harrison and Larson (2014) argue that it is possible that adaptive introgression of species A traits into species B might lead to the splitting of B into B and B′, that is, the introgression of traits from A may render some individuals of

B different enough from others that they now belong to two distinct species, as illustrated for *Heliconius* butterflies (Pardo-Diaz *et al*. 2012). Consequently, it would be illusory to describe the future of the hybrid zone between *T.immigrans and T. caespitum* based only on the current records of hybridization processes between these two taxa. However, some first clues could give a first overview of the hybrid zone structure and functionning.

Our results suggest relatively weak reproductive isolation between *T.immigrans and T. caespitum* (**Paper 4**). Due to the intermediate location of the hybrid zone between mostly urbanized habitats and more natural environments (**Papers 2 and 4**), hybrids could experience ecological conditions different from parental species, especially in the agricultural belt matching the transition between the dense urban areas and semi-natural habitats comprising woodlands and meadows. These agricultural landscapes are characterized by ecological specificities including fragmentation of remaining natural habitat or application of agrochemicals (Tscharntke *et al*. 2005). It is therefore possible that specific hybrid genotypes or phenotypes have experienced relatively strong selection in this hybrid zone. Such a distribution across landscapes could be consistent with the dispersal-independent model of bounded hybrid superiority within narrow ecotones proposed by Moore (1977) and suggested for the Great Plains hybrid zone between Red- and Yellow-shafted flickers (Moore & Buchanan 1985; Moore & Koenig 1986). Considering agricultural landscapes in the urban gradient and thoroughly evaluating the characteristics of the ecological niches of hybrids should provide key elements about the outcome of the hybrid zone and the possibility of a specialization for agricultural habitats. An approach based on population densities evaluation could also bring new insight in the understanding of this hybrid zone dynamics. Indeed, predictions from tension zone models (see **Chapter 1** for details) from well-studied hybrid zones (e.g., Barton 1979; Barton & Hewitt 1985; Hewitt 1989; Ruegg 2008) suggest that the center of the hybrid zone should correspond to an area of low population density for pure species.

Different approaches can thus be used to provide answers or to exclude some hypotheses. However, observation of hybrid zones over multiple years and spatial replicates is the most reliable method for detecting their dynamics. Buggs (2007) showed that molecular markers sometimes shed more highlight on the movement of the hybrid zone through comparative studies in space, or locally at different scales; nevertheless, only repeated sampling over time and use of historical evidence, as well as manipulative experiments on the processes acting in the hybrid zone can resolve dynamics precisely.

5.4- WHY THE INTERRELATIONSHIPS BETWEEN CLIMATE, URBANIZATION, INVASIONS AND HYBRIDIZATION SHOULD ALWAYS CONSIDERED

5.4.1- Global changes impacts on species ranges: a real bag of bones

As mentioned in **Chapter 1**, current climate changes promote latitudinal or altitudinal shifts in distributions of species of a diverse range of taxa (e.g., Hughes 2000; Walther *et al.* 2002; Parmesan & Yohe 2003; Hickling *et al.* 2006; Pearce-Higgins *et al.* 2014; Battisti *et al.* 2005; Chen *et al.* 2011; Lenoir & Svenning 2015; Lawing *et al.* 2011). Urbanization also modifies species distribution, because some species are not able to survive in urban ecosystems whereas others – especially the more generalist ones – dominate highly urbanized environments, leading to completely modified urban communities and ecosystems (e.g., Marzluff 2001; McKinney 2002, 2006 ; Kark *et al.* 2007 ; Pacheco & Vasconcelos 2007; Sanford *et al.* 2009; Philpott *et al.* 2010; Menke *et al.* 2011; Buczkowski & Richmond 2012; Müller *et al*. 2013). The results obtained in **Chapter 2** perfectly fit these patterns, as the distributions of all species strongly depended on either climate or urbanization, or both.

Nevertheless, the situation is even more complex, because the factors associated with global changes operate at different scales (e.g., the case of impervious surfaces in **Paper 2**). Moreover, in **Chapter 3** (**Paper 3**) I show that these factors interact with each other, which suggests that species distribution patterns are the result of complex and multi-scale interactions.

This notion of interaction is rarely considered in the literature. Indeed, because climate change, land-use patterns and invasive alien species are likely to occur simultaneously, we can expect that they have synergistic effects on biodiversity (Bellard *et al.* 2015). Newbold (2018) made the first global predictions of the separate and combined (additive) effects of future climate and land-use change on local vertebrate biodiversity, and Leclerc *et al.* (2018) highlighted specific associations of threats among eleven global changes such as climate changes, pollution, urbanization, cultivation, biological invasions or wildlife exploitation at different scales, showing that the analysis of each threat in isolation might be inadequate. To my knowledge, interactive effects between these global threats have never been investigated to date. However, a large number of clues of such potential interactions should have given us a hint and motivated deeper investigations, as previously suggested by Bellard *et al.* (2015). For instance, cities are often warmer compared with nearby rural habitats, with differences in the warming rate from nighttime to peak daytime temperatures, as well as spatial variations of this warming rate, being often faster in urban areas (Adams & Smith 2014). The impact of urbanization on climate is locally comparable to the ongoing global climate warming, suggesting that urbanization could strongly enhance climate change at local scales (Argüeso *et al*. 2014). My findings therefore highlight interaction effects of urbanization and climate changes.

This complexity, involving scaling effects and interaction effects, emerges even though only two global changes were partially investigated in this thesis. For instance, the impacts of pollutions have not been included in the urbanization study. The intensification of the extreme events, which is a strong component of current climate changes, have not been considered here. For example, Dale *et al*. (2001) considered that the rapid response of fire regimes to changes in climate can potentially overshadow the direct effects of climate changes on species distribution and migration. Again, these other factors probably act and interact with other ones at different spatial scales. And what about other global changes such as agriculture or deforestation, composed themselves of a multitude of factors? Clearly, a simple causality study involving a partially described global change on the distribution of a species cannot lead to a convincing understanding of the processes underlying species distributions. Further studies about the impacts of global changes should thus use more inclusive approaches combining several factors, scales and global changes.

5.4.2- The role of global changes on biological invasions

As shown in **Chapter 1**, global changes can increase the risks of biological invasions (Walther *et al.* 2009; Bradley *et al.* 2012). For instance, climate change can exacerbate biological invasions (Dukes & Mooney 1999; Sala *et al.* 2000; Hellmann *et al.* 2008) because the distribution of many invasive species is currently restricted by thermal barriers and climate change might enable them to invade higher latitudes. For example, in France, most of the invasive ant species investigated by Bertelsmeier and

Courchamp (2014) were predicted to increase their potential range in response to climate change. Extreme climatic events, such as intense heat waves, hurricanes, floods, and droughts, may facilitate biological invasions through increased movements of non-native species and decreased biotic resistance of native communities to invader establishment (Diez *et al*. 2009). Urbanization can also induce the introduction of non-native species (Marshall & Shortle 2005), leading numerous studies to show a dominance of species introduced by humans in cities (Marzluff 2001; McKinney 2006; Heterick *et al.* 2013; Vonshak & Gordon 2015; Cadotte *et al.* 2017). The results shown in **Paper 3** confirm that urbanization and climate change are likely to promote the invasion of *Tetramorium immigrans* in France. Other global changes increase the risk of invasion, such as the expansion of transport networks, technological advancements, landscape transformation, or geopolitical events (Early *et al*. 2016; Seebens *et al*. 2015), e.g., through the growth of internet-based trade in living organisms creating unique and difficult to regulate pathways of invasion (Lenda *et al.* 2014). According to Ricciardi *et al*. (2017), the changing agricultural practices and more specifically the efforts to develop new commercially farmed species and the industrial use of mutualistic organisms to increase crop yields should promote a new suite of invasive taxa.

5.4.3- Consequences on genetic exchanges between species

New human-induced hybridization opportunities have occurred in recent years and are expected to increase in the near future (Quilodrán *et al*. 2018), e.g., due to climate changes (Taylor *et al*. 2015; Gómez *et al*. 2015), the modification of natural habitats (Arnold & Martin 2008), the translocation of invasive species (Fitzpatrick *et al*. 2012), or domesticated animals (Leonard *et al.* 2014; Nussberger *et al*. 2014).

Anthropogenic species translocations and habitat modifications have caused a dramatic increase in hybridization rates worldwide, contributing to the extinction of numerous populations and species (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). Grabenstein & Taylor (2018) even spoke of "human-mediated hybridization" to characterize hybridization induced by anthropogenic habitat disturbances. Anthropogenic hybridization can directly result from human actions, e.g., when hybridization is induced by the release of exotic individuals (e.g., Casas *et al.* 2016 for the partridges *Alectoris* *spp.*). As species are transported around the world with increasing intensity, barriers to gene flow between once geographically separated species are reduced and new hybrids between introduced and resident species will probably emerge more frequently (Thomas 2013). When species colonize new environments, founding populations are often small, and opportunities for sexual reproduction may be limited by the availability of mates, leading colonizing species to exploit the presence of heterospecific congeners (Hall 2016). In addition, distinct lineages may hybridize in contact zones, increasing genetic variation and reducing genetic constraints in newly formed hybrid populations, thereby increasing their genetic potential or adaptability (Roy *et al.* 2015). Across ant taxa, Feldhaar *et al.* (2008) predicted that detailed research should reveal numerous additional cases of hybridization, in particular in those ant faunas that are characterized by the recent introduction of multiple invasive species. In addition, hybridization may play a significant role for introduced species to become invasive (Ellstrand & Schierenbeck 2000; Allendorf & Luikart 2007; Hall 2016), for instance by allowing genetically admixed individuals to invade novel niches unoccupied by any of their parent species (Roy *et al.* 2015). Mesgaran *et al.* (2016) showed for example that transient hybridization has probably driven the rapid replacement of the plant earlier colonizer *Cakile edentula* by the outcrosser *C. maritima* over a large part of its invasive range in Northwestern America, New Zealand, and Australia.

Global changes also promote hybridization or introgression. Climate-induced ranges shifts increase sympatry between previously isolated species, potentially resulting in hybridization in these new contact zones (Walther *et al.* 2002; Garroway *et al.* 2010; Becker *et al.* 2013; Brennan *et al.* 2015; Sánchez-Guillén *et al.* 2016). For example, several studies shown that when hybridization occurs within a contact zone between two closely related bird species, interspecific interactions and climate interact in determining hybrid zone location and dynamics (Reudink 2007; Taylor *et al.* 2014; McQuillan & Rice 2015). Garroway *et al.* (2010) recorded the first report of hybrid zone formation between two species of North American flying squirrels following a range expansion induced by contemporary climate change. In plants, the impact of climate changes has also been reported to increase opportunities for hybridization among previously isolated lineages, facilitating the breakdown of reproductive barriers and the formation of hybrids (Vallejo-Marin & Hiscock 2016). Urbanization also create new opportunities of encounters

(Crispo *et al*. 2011), especially as urbanized areas have a greater heterogeneity than natural environments, creating ecotones acting as new contact areas between species with widely differing ecological preferences (Brennan *et al.* 2015), inducing potential hybridization. Results of **Papers 1-3** suggest that anthropogenic species translocations and habitat modifications as well as climate and urbanization could all have promoted hybridization and introgression between *Tetramorium immigrans* and *T. caespitum*.

According to Taylor *et al*. (2015), hybrid zones should not be viewed as equilibrium situations. Long-term, quantitative experimental studies of hybrid zones are needed to investigate the consequences and outcomes of hybridization. Patterns of variation in hybrid zones could provide exciting opportunities to investigate consequences of global changes. Moving hybrid zones may indeed represent sensitive indicators for anthropogenic climate change (McQuillan & Rice 2015; Taylor *et al*. 2015). For instance, McQuillan & Rice (2015) reviewed several good examples linking climate change to hybrid zone movement (e.g., Britch *et al*. 2001; Scriber 2011). Such monitoring, although time consuming and expensive, would be highly relevant in identifying the factors responsible for the hybridization between *Tetramorium immigrans* and *T. caespitum*, the underlying mechanisms and the future of the hybrid zone.

5.4.4- When hybridization promotes biological invasions

During the spread phase of a biological invasion, hybridization may play an important role in making introduced species invasive (Allendorf & Luikart 2007), allowing genetically admixed individuals to invade novel niches that were not typically occupied by any of their parent species (Roy *et al.* 2015). Mesgaran *et al.*'s (2016) study illustrated how colonizing species can exploit the presence of congeners to overcome the problems associated with low numbers through hybridization and the subsequent reemergence of colonizer genotypes. These authors speculated that such hybridization might play a role in facilitating colonization success, perhaps even in the displacement of Neanderthals by *Homo sapiens*. Hall (2016) schematized how hybridization influences colonizer establishment by comparing how the relative frequencies of the colonizing and resident species change through time, with or without hybridization and introgression (Fig. 5). According to the results obtained in **Papers 1-4**, such a system could correspond to the situation of *Tetramorium immigrans* and *T. caespitum*. Indeed, hybridization between these two species could promote invasiveness in *T. immigrans* away from urban core areas if hybridization leads to introgression by *T. caespitum*, for instance by integrating genes providing better adaptation to local climate or to semi-natural habitats.

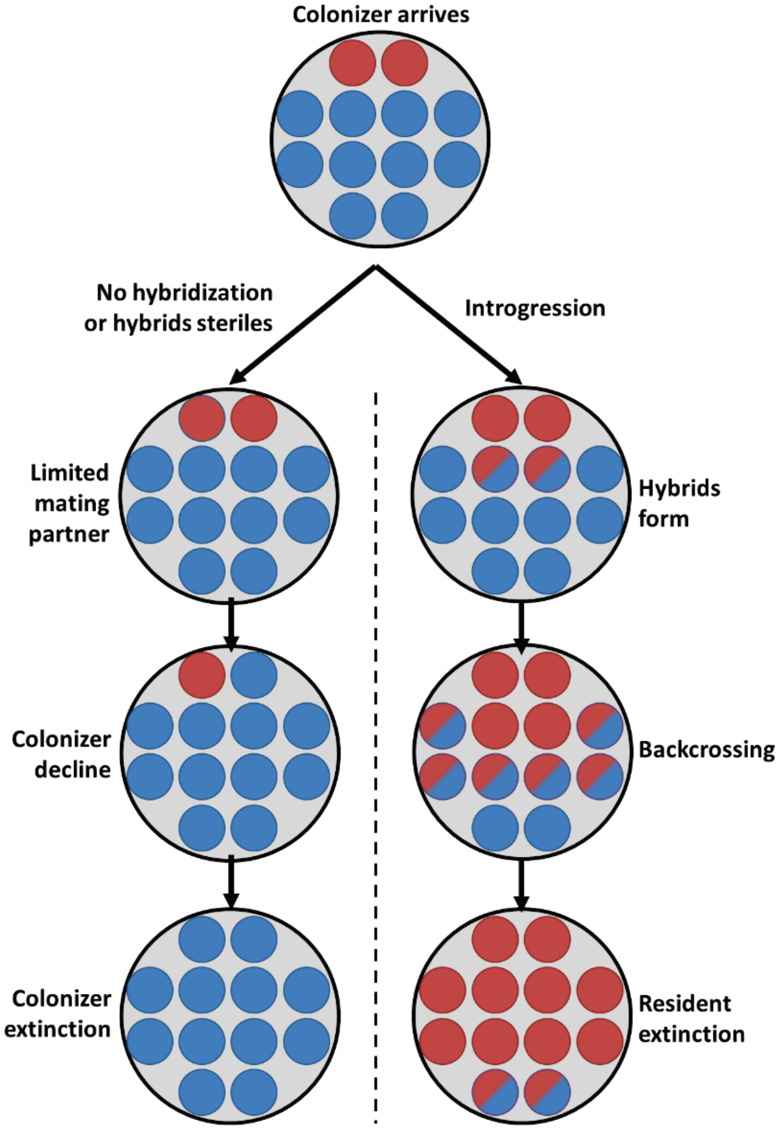


Figure 5. Fate of a small number of colonizers (red circles) interacting with a related resident species (blue circles). In the absence of hybridization (left), the rare colonizers are swamped, e.g., due to genetic incompatibility when mating with the resident or because not enough conspecific partners are available, resulting in low fecundity. Colonizers decline to vanishingly small frequency as they are replaced by resident seedlings. When hybridization and introgression occur (right), colonizer genes persist in hybrids (bicolor circles) and colonizer genotypes reassemble through backcrossing among colonizer-like individuals (Adapted from Hall 2016: fig. 1).

Interspecific hybrids may also become especially successful invaders (Hovick & Whitney 2014). Ellstrand & Schierenbeck (2000) proposed that hybridization between species may serve as a stimulus for the evolution of invasiveness through four genetic mechanisms: (i) hybridization may result in evolutionary novelty by the production of novel genotypes and phenotypes that occur in none of the parental taxa, (ii) an increase in the amount of genetic variation in hybrid populations may provide greater opportunity for natural selection to induce adaptive evolutionary changes, (iii) certain genetic or reproductive mechanisms (e.g., clonal reproduction) may stabilize first generation hybridization and fix genotypes that demonstrate heterosis, and (iv) hybridization may lead to a reduction in mutational genetic load, increasing fitness sufficiently to sustain invasiveness. Ellstrand & Schierenbeck (2000) identified 28 situations into 12 plants families where invasiveness was preceded by hybridization (see Allendorf & Luikart 2007; Huxel 1999; Senn & Pemberton 2009; or Facon *et al.* 2005 for animal examples). The possible reasons for hybrid success include increased phenotypic or genotypic variability, phenotypic novelty arising from transgressive segregation or adaptive introgression, and heterosis effects (Prentis *et al.* 2008). As a consequence, invasive hybridization is now seen as a fundamental problem in conservation biology because of its negative impact on worldwide biodiversity (Lowe *et al.* 2016).

In the *Tetramorium immigrans* x *caespitum* hybridization system, such possibilities of future invasions by hybrids should be investigated. As stated above, I suggest to first explore hybrids' ecological niches to provide key elements about the possibility of a specialization for agricultural habitats. Additionally, surveying the hybrid zone over multiple years and spatial replicates should allow characterizing hybrid zone dynamics and detecting a possible invasion by hybrid taxa.

5.4.5- A much more complex final pattern than expected

Following this discussion and in view of the different elements discussed above, it is clear that the situation fleshed out in the introduction is actually much more complex than initially supposed. Indeed, urbanization, climate and biological invasions do not act separately and individually on *Tetramorium* species distributions, and *Tetramorium* species distributions are probably not the only cause of gene flow and interspecific hybridization (dotted arrows in Fig. 6; see original assumptions in Chapter 1). In the new, more complex picture depicted by my research (Fig. 6), urbanization has been shown to directly affect gene flow and hybridization through habitat modification and the creation of ecotonal areas. Furthermore, the newly described interspecific hybridization probably played a role in the biological invasion of *T. immigrans*, as much as urbanization processes and climate factors. Urbanization and climate have been shown to act synergistically on the distribution of *Tetramorium* species, especially for *T. immigrans*. Such results provide an accurate picture of the complexity to understand drivers of genetic exchanges within and between species and point to the need to multiply the studied drivers when studying such ecological questions.

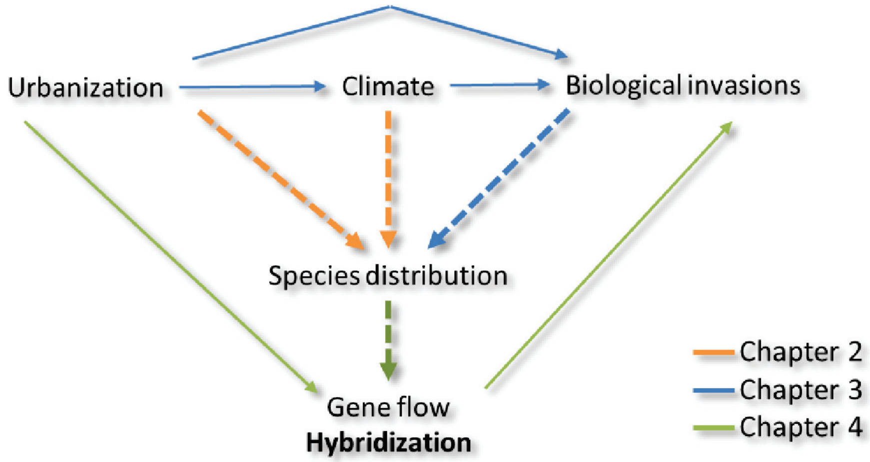


Figure 6. Review of the main relationships highlighted in the present work between global changes, invasions, species distribution and intra- and inter-specific genetic exchanges. The dashed arrows correspond to the initial postulates, the solid arrows correspond to the contributions of the research carried out in this thesis to the understanding of the processes inducing gene flow between populations and interspecific hybridization. Colors indicate associated chapters.

So far, few studies have made the effort to integrate the study of different global changes (but see Menke *et al.* 2011; Bellard *et al.* 2015; Leclerc *et al.* 2018 and Newbold 2018), and when investigating issues related to interspecific hybridization, no study has proposed a theoretical framework as complete as the one proposed here. However, this framework remains rather far from exhaustive, and other factors not yet considered (e.g., pollution linked to urbanization) or other global changes such as agriculture intensification are probably partly causal for the observed patterns and processes. Further studies should therefore be encouraged, both in this hybridization system and in others, to take into account interactions between global changes and to include the exploration of numerous factors at multiple scales to better understand processes that generate patterns of genetic exchanges.

5.5- CONCLUSION AND PERSPECTIVES

5.5.1- Concluding remarks: what is new in this study

Understanding the dynamics and consequences of natural and anthropogenic hybridization is a priority for biodiversity conservation (Allendorf *et al.* 2010). Indeed, hybridization may in some cases threaten the conservation of local biodiversity, for example by modifying the genetic integrity of native species through introgression (Taylor *et al.* 2015). Interspecific hybridization is becoming more and more common around the world, favored by human-induced translocations of organisms and habitat modification, and therefore often associated with urbanization. As a consequence, the acceleration of urbanization on a global scale makes it urgent to study hybridization in order to understand the consequences of the erosion of reproductive barriers between distinct evolutionary lineages (Vallejo-Marín & Hiscock 2016).

However, such studies about interspecific hybridization have rarely encompassed concepts like global changes or biological invasions. These concepts themselves are rarely studied simultaneously (but see Bellard *et al.* 2015; Leclerc *et al.* 2018; Newbold 2018), and their interactive effects are totally overlooked in the current literature. The whole findings of the present research therefore point towards new understanding of the relationships between biological invasions, urbanization and hybridization as a whole.

The present work is pioneering for several reasons. The first, and probably most obvious, relates to the fact that the species on which I worked have been recently redescribed, and therefore remained unstudied. It may be relevant to note here that the present study now provides an effective method to discriminate species in a complex admixture situation including both interspecific hybridization and cryptic biodiversity, while dealing with problems such as hierarchical cluster structuring due to uneven sampling across species, which constitutes also a significant progress.

Nevertheless, I would like to emphasize that the theoretical framework including the interactions between global changes, invasions and hybridization compartments constitutes a true scientific advance. Although still far from a comprehensive framework, taking into account interactions between urbanization, climate and invasions and including the exploration of multiple scales appears essential to better understand processes that generate patterns of genetic exchanges. The study of polyandry, recognition mechanisms and discrimination between ant species within a hybrid zone is also unprecedented and should provide the opportunity to investigate the selective processes involved in the evolution of interspecific mating. My research should therefore contribute to a better understanding of interspecific hybridization mechanisms and their consequences on genetic and taxonomic diversity.

5.5.2- What is still to be learned from the T. immigrans x caesptium system. An overview

Although a number of research perspectives have been developed or at least discussed throughout this chapter, the complexity of the system and the diversity of issues I have raised make it necessary to present in a more synthetic way the issues that should be addressed in future studies. I have therefore chosen a thematic approach combining four objectives formed by the main questions that remain open to discussion:

(i) Where did *Tetramorium immigrans* come from?

(ii) Why is *Tetramorium immigrans* a good urban exploiter?

(iii) How do *Tetramorium immigrans* and *T. caespitum* break down the barriers against hybridization?

(iv) What do the current data tell us about the future of their hybrid zone?

Where did Tetramorium immigrans come from?

Today, *Tetramorium immigrans* is known only from opportunistically sampled localities all over Europe (Fig. 7). Fine-scale data (e.g., a sampling pressure of 1/10,000 ha) are therefore needed to characterize the distribution of *T. immigrans* and is crucial to clear out the status of this species in Europe, as well as pinpoint its source area within its natural range. Using this extensive sampling, by combining microsatellite data and mitochondrial DNA, spatial genetic analysis should lead to identifying the source populations and native areas of *T. immigrans*, as it was done by Ascunce *et al.* (2011).

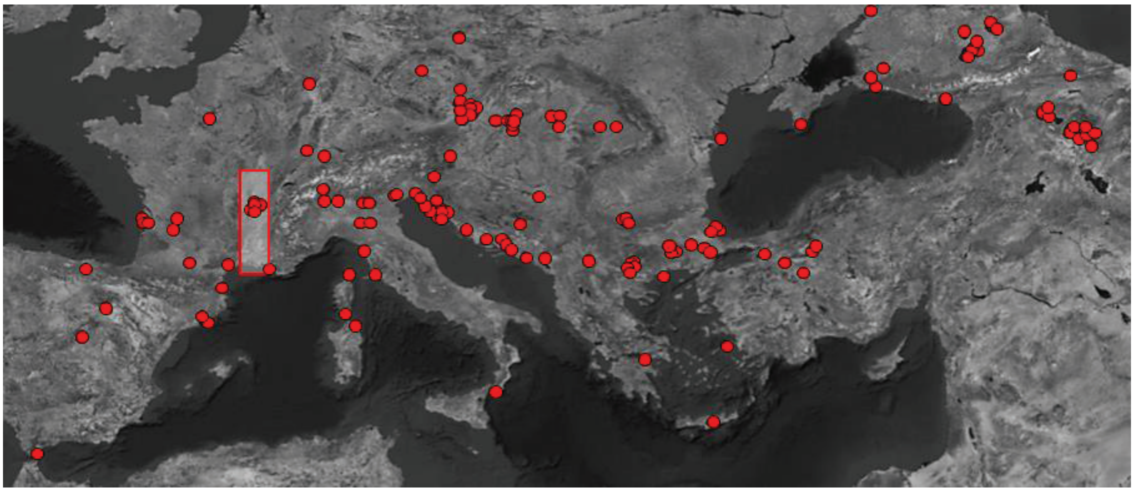


Figure 7. Location of all *Tetramorium immigrans* samples recorded so far in Europe (red points; including 219 samples of *T. immigrans* obtained from Wagner *et al.* 2017 on a sampling zone corresponding approximately to 1800 x 4500 km) and of the sampling area investigated in the present study (red rectangle; including more than five hundred samples on a sampling zone corresponding approximately to 450 x 30 km).

Why is Tetramorium immigrans a good urban exploiter?

As suggested before, global changes and more particularly urbanization may have an impact on competitive interactions, for instance through the modification of temperature or primary productivity leading to increase abundance of urban exploiters (Shochat *et al.* 2006). The exploration of characteristics of *Tetramorium immigrans* making it a good urban exploiter is therefore of prime interest as it could enhance the understanding of the ecology of this species and bring new clues about mechanisms involved in competitive exclusion of *T. caespitum* in cities. Furthermore, so far the ecological and functional traits of urban exploiters have been intensely studied in birds (Ortega-Álvarez & MacGregor-Fors 2009; Angelier *et al.* 2016; Jokimäki *et al.* 2017) but rarely in arthropods (Lowe *et al.* 2016; Zhang & Evans 2017). I therefore suggest that further studies take into account the role of factors such as stress tolerance (e.g., resistance to extreme temperatures and/or to dry conditions) in *T. immigrans'* abilities to dominate urban areas. Further investigations of species-level traits promoting this species in urban habitats should corroborate the hypothesis that this species is an urban exploiter or synanthropic species (McKinney 2006; Forman 2014). Response to artificial light or to temperature variability, differences in nest habits or in traits linked to nuptial flight allowing to overcome constraints on reproductive fitness in cities could bring new lights on the drivers of their habitat preferences. Exploration of community composition could also contribute some new elements to the discussion as urban exploiters are expected to be dominant species in urban habitats. Such investigations are of necessary to understand how some arthropods become urban exploiters.

How do Tetramorium immigrans *and* T. caespitum *break down the barriers against hybridization?*

Hybridization between *Tetramorium immigrans* and *T. caespitum* species only occur after a series of filters have been passed (see the first section of this chapter for a detailed discussion). Although some of these filters have been partially explored in my research or in other studies, others have never been investigated or remain unstudied. First, global changes could favor overlapping phenology at the time of mating flight, promoting opportunities to mate with heterospecifics. Wagner *et al.* (2017) showed that winged reproductives of these species were found in nests at overlapping periods, but considering only 7 nests of *T. caespitum* and 12 of *T. immigrans* whose localities are not known. Overlapping phenology inside the hybrid zone should be investigated as it could be promoted by the ecological context provided by the interaction between climate and urbanization. For instance, because hybrid zones are located in the southernmost part of the distribution of *T. caespitum*, the warm climate could accelerate the pupal stage of *T. caespitum* reproductives compared to the north and promote a nuptial flight simultaneous with *T. immigrans*.

The next filter corresponds to the dichotomy between behavioral isolation *vs*. sexual attraction. Regarding this filter, it could be relevant to investigate the recognition cues in reproductive at mating. Indeed, in all species where workers are highly aggressive towards conspecific, mating is still possible between reproductive individuals coming from other nests. Species recognition patterns between heterospecific reproductives may therefore not be the same than between heterospecific workers, e.g., if fertility signal homogenizes their profiles and reduce their ability to use species-specific differences, undermining identification of the potential mates' species before copulation. It could thus be relevant to further investigate the recognition cues in reproductives, both based on CHC extraction or behavioral assays. Although relatively easy to practice when winged reproductive are sampled in nests before nuptial flights, such analyses could be difficult to carry out during the nuptial flight. The detection and location of these flights is difficult and identification of individual species is based on genetic methods that are difficult to combine with chemical or behavioral assays.

Finally, most of the postzygotic mechanisms promoting hybridization between these species remain unknown. The exploration of postzygotic barriers through the comparisons between copulation frequencies (which in our case are very difficult to observe, as mating occurs during nuptial flights; see above) or insemination frequencies obtained after genotyping the sperm content of queens’ spermatheca, and the offspring observed at different stages (egg, larvae, pupae and adult) could bring new light on the natural and sexual selection processes involved and could allow a better understanding of the viability and fertility of both hybrids and their offspring. Analyzing reproductive offspring (i.e. males and gynes) instead of the worker offspring which are less affected by individual selective pressures would ensure further insight into the consequences of sexual selection and sexual conflict in insect societies.

What do the current data tell us about the future of the hybrid zone?

Hybrid zones structure can be broadly categorized as either clinal or mosaic (Taylor *et al.* 2015). The clinal pattern of the *Tetramorium immigrans* x *caespitum* hybrid zone could be maintained by endogenous selection against hybrid genotypes (tension zones; Barton & Hewitt 1985; see Chapter 1 for details), by selection favoring different parental types at each end of an environmental gradient (Endler 1977), or by selection in intermediate habitats favoring individuals of mixed ancestry (bounded hybrid superiority model; Moore 1977) (Taylor *et al.* 2015). It is difficult to provide elements regarding the functioning of the hybrid zone based on the data from the present study. Potential changes in the species interaction networks in response to invasion and hybridization could be especially complex, leading to different potential outcomes of invasion and hybridization: invasion outcomes range from invasion failure to establishment of the new species, co-existence, hybridization, or species replacement. Hybrids could therefore persist alone or in co-existence with one or both parental species (Brennan *et al.* 2015).

Although surveying the hybrid zone over multiple years and spatial replicates is the best way to accurately investigate hybrid zone dynamics, some current characteristics of the hybrid zone between *T. immigrans* and *T. caespitum* could provide insight into the mechanisms that generate the persistence of the hybrid zone in time and space. For instance, predictions from the tension zone model suggest that the center of the hybrid zone should correspond to an area of low population density where limited mating occasions promote heterospecific copulating, inducing a hybrid zone. When hybridizing species are distributed along an environmental gradient, hybridization can narrow the region in which both species co-occur, due to ecological and/or reproductive character displacement across the hybridization zone (Goldberg & Lande 2006, 2007), therefore inducing the same pattern of low population density of parental species in the hybrid zone. This prediction could be confirmed in the *T. immigrans* x *caespitum* system by investigating the densities of pure species in the hybrid zone compared to their densities in areas where they do not co-occur (Fig. 8a). Considering agricultural landscapes in the urban gradient and evaluating the characteristics of ecological niches of hybrids should also provide key elements about the outcome of the hybrid zone and the possibility of a specialization for agricultural habitats. In this case, ecological niches of hybrids should be different from the niches of parental species, either because pure species have a lower fitness in the hybrids' habitat or because hybrids' fitness is higher in these habitats. Such data should provide key elements about the possibility of a specialization for agricultural habitats (bounded hybrid superiority model; Fig. 8b) and detect a possible invasion by hybrid taxa.

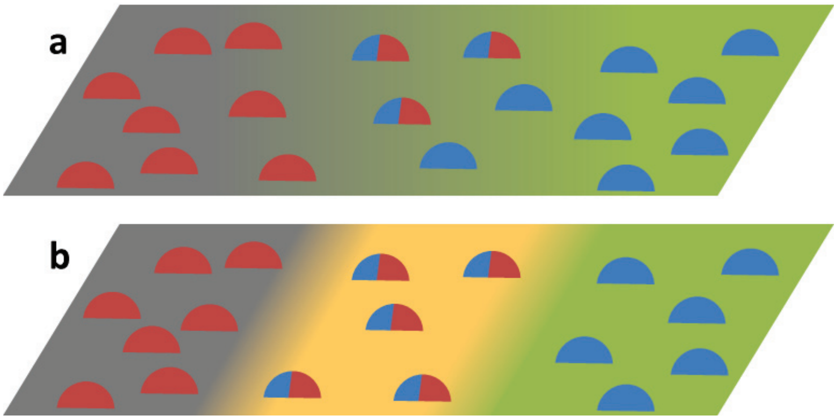


Figure 8. Potential mechanisms generating clinal hybrid zone between *Tetramorium immigrans* and *T. caespitum*. Domes correspond to *T. immigrans* (red) and *T. caespitum* (blue) colonies; mixed red-blue domes correspond to hybrid colonies. a) Pattern resulting from endogenous selection against hybrid genotypes (tension zone model) or selection favoring different parental types at either end of an environmental gradient. b) Pattern resulting from selection in intermediate habitats favoring individuals of mixed ancestry (bounded hybrid superiority model).

Questions about hybrid males remain, as no hybrid father has contributed to any sampled offspring in the present researches. So, are hybrid males an evolutive dead-end? To evaluate the inviability of hybrid males (Fig. 9.1), a first step could be to follow the development of male larvae and pupae in nests with hybrid queen in order to and evaluate their mortality and emergence rates compared to females. In cases where hybrid males are viable, their sterility (Fig. 9.2) could be evaluated by collecting sperm and artificially inseminating gynes (as done by Ball *et al.* 1983 with the smaller species *Solenospsis invicta*) and then evaluate the viability of offspring (Fig. 9.3).

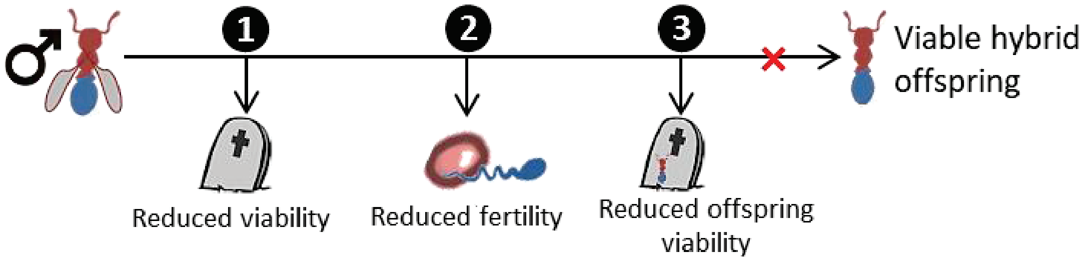


Figure 9. Schematic view of the potential mechanisms that generate the lack of hybrid males contribution to hybrid offspring obtained in the present researches, including the lethality of the hybrid males (1), their sterility (2), and the lethality of offspring (3).

5.5.3- Transposability and generalization to other spatial and biological systems

The results and perspectives of my thesis are not limited to the *T. immigrans* x *caespitum* biological system. First, although the hybrid zone presented in this study is particularly well suited to the issues addressed, it would be relevant to replicate such study in other areas of hybridization between *T. immigrans* and *T. caespitum* (provided such hybrid zones exist). Above all, these issues may however be exported to other hybridization systems and invasive species to evaluate the robustness of the theoretical framework and to test the strength of the interactions between global changes, hybridization and invasion across taxa. Such synergistic effects can affect many taxa, for instance insects (e.g., hybrid speciation in *Papilio* was catalyzed by climate warming-induced interspecific introgression; Scriber 2011), plants (e.g., hybridization between *Pericallis cruenta* and *P. echinata,* and between *P. cruenta* and *P. tussilaginis* is likely promoted by secondary contact due to human disturbance and road construction; van Hengstum *et al.* 2012), birds (e.g., climate-mediated shifts in the distribution of the Carolina chickadee *Poecile carolinensis* may indirectly lead to range retraction in the Black-capped chickadee *P. atricapillus* and affect the hybrid zone location; McQuillan & Rice 2015), and mammals (e.g., hybridization between sympatric *Glaucomys sabrinus* and *G. Volans* following a range expansion induced by the ongoing climate change; Garroway *et al.* 2010). Thus, the scope of my results should be viewed as a case-study that will have to be confirmed and replicated in various taxa. I would therefore conclude this Ph.D. thesis by illustrating how the integration of global changes, biological invasions and hybridization could be worth investigating in some other systems, and why my work could lay the foundations for a more comprehensive framework.

A number of recent studies clearly suggest that these issues are highly topical. The Asian tiger mosquito *Aedes albopictus* is currently the most invasive mosquito in the world. Sherpa *et al.* (2018) showed that long-distance human-assisted transport is probably its main dispersal mechanism in Europe. Their results also suggest that populations from invaded areas in the tropics likely failed to successfully establish in Europe, maybe due to differential adaptation to climate constraints. Such human-mediated transport increases the probability of introducing the species in urban areas where temperature is warmer than outside the cities. In such a system, urbanization may therefore help invasion of *Ae. albopictus* across Europe in the future, and especially promote gene flow between tropical invaded areas and European populations. Li *et al.* (2014) showed that in Guangzhou (China), urbanization had a significant impact on the ecology of *Ae. Albopictus*, with more larval habitats, shorter larval development time, higher adult emergence rate, and longer lifespan in urban habitats, and therefore suggested that urbanization could increase the potential for this important vector of dengue and zika viruses to colonize other parts of the world. The major role of urbanization combined with the invasive potential of this species make it a good candidate to test the strength of the interactive framework between climate, urbanization and invasion.

The processionary moths *Thaumetopoea pityocampa-wilkinsoni* complex seems a perfect model to investigate the interaction between global changes and invasion, as well as their impacts on interspecific hybridization. Petrucco-Toffolo *et al.* (2018) recently showed that although only a low frequency of hybrids has been observed in the putative contact zone between *Th. pityocampa* and *Th. wilkinsoni*, no reproductive barriers against hybridization and even introgression could be found. The range expansion of *Th. pityocampa* due to both climate changes (Battisti *et al.* 2005) and human introductions (Robinet *et al.* 2012; Avtzis *et al.* 2016) could promote new contact zones between the two species, inducing more opportunities of heterospecific mating. As suggested by Petrucco-Toffolo *et al.* (2018), such hybridization could facilitate the colonization success of expanding or invasive species through adaptive introgression or promote the emergence of new and more aggressive pest species. Again, I see this biological model as a great opportunity to enhance our understanding of complex relations between global changes, invasion and hybridization, with the opportunity of being proactive in establishing hybridization patterns and improving the development of conservation plans if hybridization become a concern.

My examples have hitherto focused on insects; however, these emerging issues can obviously concern other taxa. For instance, Heath *et al.* (2010) showed that hybridization levels between the coastal cutthroat trout *Oncorhynchus clarki clarki* and rainbow trout *Oncorhynchus mykiss* depend on anthropogenic disturbance such as logging activity or urban infrastructure development. Few years later, Muhlfeld *et al.* (2014) showed that climatic drivers and human-mediated introductions of the rainbow trout interact to increase invasive hybridization between *O. mykiss* and the threatened native *O. clarkii lewisi*. However, as far as I know, no study has investigated the combined effects of climate and urbanization in this system. One could ask, e.g., if the creation of infrastructure combined with climate warming increased the risk of invasion by the rainbow trout, or if these anthropogenic modifications affected the establishment of hybridization patterns with the closely related species.

Non-native lineages of the common wall lizard *Podarcis muralis* are frequently introduced in cities where they hybridize with native populations. Beninde *et al.* (2018) showed that the cityscape structure and invasion history of cities determine future evolutionary pathways in these novel hybrid zones where elevated genetic diversity could relate to adaptive traits, giving hybrid swarms a high potential to adapt to ecological conditions, both to the specific conditions of urban habitats or to new conditions different from those experienced by parental species. They therefore concluded that cities are likely to become major playgrounds for hybridization where human-mediated introductions have repeatedly led to the emergence of novel hybrid zones and these novel admixture processes will determine future evolutionary pathways of urban lizards. In such context, although never investigated, climate changes could interact with urbanization to generate hybridization patterns, e.g., in promoting the establishment of *Podarcis muralis* in northern cities, accelerating its invasion.

A number of other examples found in the recent literature also reveal how it might be worth expanding this framework to include other global changes. For instance, Paffetti *et al.* (2018) show that the global deforestation leading to the removal of a large number of tree rows and shrubs along rivers and in agricultural landscapes, eradicates natural barriers crucial to mitigate hybridization between *Populus species*, because such hybridization is facilitated by wind intensity favoring the spread of pollen. Champagnon *et al.* (2013) found significant rates of hybridization between wild and captive-bred mallards and suggested that long-term releases of captive-bred mallards, if carried on a large scale, could irreversibly compromise the genetic structure and composition of European mallards. Such examples highlight the value of taking into account the indirect effects of global changes and illustrate the need to simultaneously investigate a large number of factors. However, considering too many effects simultaneously presents serious risks because this generates difficulties in interpreting highly complex models. It would therefore be relevant to first identify the major global changes that control invasion and hybridization, and then focus on these changes to characterize the underlying mechanisms.

To conclude, although I have selected the preceding examples to illustrate my point, I am aware that many other systems are likely to be concerned by similar issues and should be investigated in the light of the results presented and discussed here. Numerous studies have already highlighted the need to investigate global changes, biological invasion or interspecific hybridization at different scales, or have shown that the isolated analysis of each process or factor may not be sufficient to accurately understand the system. The global climate changes combined with the increase in the size of cities (such as the megalopolis of Beijing which has doubled in size since 2000), the increase of air, sea and road traffic, of trade between countries (e.g., food, raw material) of tourism, or the changing human habits (e.g., the fashion for new pets) emphasize how important and urgent it is to address these issues.

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> *« The work on ants has profoundly affected*
> *the way I think about humans.»*
> *Edward O. Wilson*