Système de reproduction et adaptation à la toxicité du sol chez la Brassicacée pseudo-métallophyte Noccaea caerulescens

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THÈSE

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1) Thèse ?

Quand on s'apprête à finir, on éprouve parfois le besoin de se retourner et de regarder en arrière. Pourquoi cette thèse ? C'est une bonne question : pourquoi fait-on une thèse de doctorat ? Dans mon cas, je crois que je souffre d'une *curiositite aigüe*, avec complication biologique.

Cette tendance déplorable à poser trop de questions a été encouragée par un certain nombre de membres de l'Éducation Nationale. Professeur(e)s qui avez su attiser ma curiosité, je ne peux pas tous vous nommer, mais merci de m'avoir poussée vers l'avant et d'avoir cultivé ma soif de savoir. Mention spéciale à M.C. Giraudet de m'avoir convaincue que c'était fantastique d'apprendre une chose nouvelle tous les jours ; à C. Puychafray et Sylvie qui m'ont fait découvrir les merveilles de la biologie et de la géologie et en ont souffert les conséquences (« mais alors, pourquoi… ? ») ; au duo de choc des Agnès, qui en ont fait autant avec l'art et m'ont aidé à transformer la terreur brute que j'éprouvais à l'idée de parler devant un public en un trac sournois mais modéré (j'ai eu une pensée pleine de gratitude pour vous une minute avant le début de ma soutenance de thèse) ; et enfin aux deux professeurs de SVT les plus opposés qu'il soit, l'un autant structuré que l'autre s'éparpillait, Dut et Pschitt, pour avoir transformé cet intérêt pour la biologie en envie de la découvrir par moi-même.

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Et là, frappée par un épisode particulièrement fort de curiositite aigue, j'ai décidé de faire un second master. Ne riez pas, cela pourrait aussi arriver à vos enfants. Je voudrais remercier toute l'équipe administrative et scientifique qui a mis sur pied ce master, et en particulier Isabelle Olivieri, pour son soutien pendant toutes ces années. Ça a été une très belle expérience, qui a satisfait autant qu'attisé ma soif de savoir, mais je n'ai pas la place de trop détailler, sinon j'y suis encore dans dix pages.

1 Un des bons côtés : moyennant quelques moqueries, il est possible de faire la sieste en début d'après-midi au labo…

2) Anti-taise

Pour résumer l'histoire, je me suis retrouvée en thèse dans cette grande famille scientifique qu'est l'ISEM… J'ai eu deux mamans, une grand-mère, une sœur de thèse qui m'a supportée (en français comme en anglais) et a rendu la vie de thésarde plus sympathique, des frères, des cousines, des cousins, des oncles, une tante et pas mal de petits frères et sœurs. Tout ce petit monde aime manger ensemble le midi, ça rigole, ça débat, ça déconne, et c'est bien agréable. Merci à vous pour l'ambiance chaleureuse et le soutien moral. Merci de m'avoir accueillie dans vos bureaux ou même chez vous quand j'avais des questions ou juste envie de causer. Merci à Cassandra, Christophe, Eric (et Béatrice et Cortex), Sandrine, Yoann, Guillaume, Juliette, Sophie, Yoann, Asma, Ethan, Armine, Claire, Josselin, Adeline, Cécile, et tous les autres que je n'ai pas la place de nommer.

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2 Le gobelet de café, par exemple

3 Si je trouve des cartes postales intéressantes, je te les enverrai.

Myriam, Angelica, etc. La plupart d'entre vous étaient au loin, mais un merci spécial aux HEURES de la liste RATON-LAVEUR, et en particulier à Jonas, Hélène, Laetitia, Pierre, Irène, Mickaël, et les autres, pour avoir parcouru un bout de chemin avec moi. Une pensée aussi aux membres des divers groupes d'impro de l'université : on a vraiment bien rigolé. Et une pour les aïkidokas de Grabels, rien de tel pour se changer les idées que d'essayer de mettre un coup de poing à quelqu'un… et de se faire envoyer sur le tatami.

3) Saint-thèse

Merci à mon jury de s'être déplacé de plus ou moins loin pour assister à ma soutenance, merci à mes rapporteurs qui ont pris le temps de relire mon manuscrit et de le commenter.

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4 Anecdote : en déménageant, j'ai retrouvé un carnet de notes de l'été 2012. J'y avais écrit « Plus que le sujet, c'est des gens que j'ai choisis. Des gens ouverts, qui pensent vraiment à l'intérêt de leurs étudiants, des gens avec qui je peux vraiment parler ».

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CONTENU

À ma famille,

“So much universe, and so little time”

Terry Pratchett

RÉSUMÉ

Le système de reproduction a une influence majeure sur l'évolution des populations, car il affecte les flux de gènes, la dérive et la sélection. C'est donc un des déterminants à prendre en compte lors de l'étude de l'adaptation à des environnements hétérogènes en présence de flux de gènes. Dans cette thèse, j'ai étudié le système de reproduction d'une Brassicacée tolérante et hyperaccumulatrice de métaux lourds, *Noccaea caerulescens*. Cette espèce vit sur des sols hautement contaminés en éléments traces tels que le plomb, le zinc, ou le cadmium, mais aussi sur des sols non contaminés. Les deux types de populations (écotypes) sont trouvés à quelques kilomètres l'un de l'autre dans la région du Nord de Montpellier.

Dans un premier temps, j'ai caractérisé la variation du système de reproduction en populations naturelles. En utilisant des marqueurs microsatellites, j'ai montré que les populations vivant dans les mines avaient un taux d'autofécondation plus faible que les populations vivant sur sol non pollué. En outre, je n'ai pas trouvé de variation temporelle du taux d'autofécondation.

Pour essayer de comprendre ce qui peut expliquer cette variation entre écotypes, j'ai ensuite étudié l'effet de la densité sur le taux d'autofécondation, ce facteur étant connu pour influencer le comportement des pollinisateurs et donc le système de reproduction. Je n'ai pas détecté d'effet de la densité sur le taux d'autofécondation à l'intérieur de deux mines, en utilisant deux méthodes différentes, et en variant les échelles spatiales.

Enfin, je me suis intéressée à la dépression de consanguinité, autre acteur majeur de l'évolution du taux d'autofécondation. J'ai testé des prédictions sur l'effet de l'histoire d'adaptation à un environnement sur l'expression de la dépression de consanguinité, dans cet environnement ou dans un nouvel environnement (potentiellement stressant). J'ai trouvé que la dépression de consanguinité chez *N. caerulescens* est, en jardin commun, faible à modérée. Les plantes métallicoles étaient mieux adaptées au sol pollué que les plantes non métallicoles, et souffraient parfois d'une plus forte dépression de consanguinité. La toxicité du sol ne semblait que très peu influencer l'expression de la dépression de consanguinité, et ceci de la même manière chez les plantes adaptées ou non à la pollution.

N. caerulescens a donc un système de reproduction mixte, qui n'est pas contraint par une forte dépression de consanguinité (en conditions expérimentales). Ce système de reproduction devrait faciliter la sélection tout en n'augmentant pas trop la dérive génétique, et donc confère de bonnes capacités d'adaptation à de nouveaux environnements.

ABSTRACT

Mating systems deeply influence the evolution of populations, as they affect gene flow, genetic drift, and selection. They are thus important to study in the context of adaptation to heterogeneous selection pressures in the presence of gene flow. I studied the mating system of a tolerant Brassicaceae, *Noccaea caerulescens*, which grows on both normal and highly toxic soils, such as former mine wastes, contaminated with Lead, Zinc or Cadmium. One can find the two types of populations (ecotypes) separated by a couple of kilometres in the region north of Montpellier.

I first characterized mating system variation in natural conditions in populations of the South of France. I found that self-fertilisation rates were higher in non-metallicolous populations than in metallicolous populations but did not detect temporal variation.

To better understand these differences between ecotypes, I investigated the effect of the density of flowering plants on self-fertilisation, as density often influences pollinators behaviour and thus, mating system. I found no effect of density on self-fertilisation rates, in the two studied populations and using two different methods and different spatial scales.

Finally, I estimated inbreeding depression, which is a major factor of mating system evolution. I used the two ecotypes of *N. caerulescens* to test whether the history of adaptation to an environment affects the expression of inbreeding depression in this environment and in new, potentially stressing environments. I found weak to moderate inbreeding depression in *N. caerulescens* in experimental conditions. Metallicolous populations were better adapted to soil toxicity than non metallicolous populations, and sometimes had higher inbreeding depression. There was little effect of toxicity on inbreeding depression, similarly for populations adapted or not to toxic soils.

Noccaea caerulescens has a mixed mating system, which does not seem to be highly constrained by inbreeding depression. This mixed mating system is expected to have beneficial effects on selection, while not increasing genetic drift too much. This may provide good capacities to adapt to new environments.

INTRODUCTION

Préambule

Différents processus affectent l'évolution des fréquences alléliques dans les populations : la sélection, la mutation, la migration et les changements stochastiques (la dérive génétique). La sélection et la dérive agissent sur les allèles qui ségrégent dans la population ; la migration et la mutation peuvent apporter de nouveaux allèles dans une population, pour le meilleur et pour le pire. Les facteurs qui modulent les flux de gènes à l'intérieur des populations et entre elles sont d'une importance capitale pour comprendre l'adaptation. Le système de reproduction est l'un d'entre eux. Par exemple, la reproduction sexuée crée de nouvelles combinaisons d'allèles, défait des combinaisons délétères mais aussi bénéfiques, et permet à des allèles arrivant d'une population non-adaptée aux conditions locales de participer aux futures générations.

L'importance du système de reproduction est illustrée par les travaux de Janis Antonovics et de ses collègues, qui ont étudié expérimentalement et théoriquement les barrières aux flux de gènes entre une mine et les champs adjacents non contaminés (Antonovics 1968, 2006; McNeilly and Antonovics 1968; Caisse and Antonovics 1978). Outre des différences persistantes de phénologie (McNeilly and Antonovics 1968; Antonovics 2006) chez les espèces *Agrostis tenuis* Sibth. et *Anthoxanthum odoratum* L., les plantes vivant dans la mine étaient plus autofécondantes que celles dans le champ adjacent (Antonovics 1968). Ces travaux ont suscité beaucoup d'intérêt pour l'effet du taux d'autofécondation sur l'adaptation à des environnements hétérogènes. Les anciens remblais de mine qui contiennent de hautes concentrations en métaux lourds imposent une sélection extrêmement forte mais souvent limitée spatialement sur les

organismes qui y vivent. Les organismes qui sont capables de vivre dans ces conditions extrêmes mais aussi sur des sols non contaminés sont d'excellents modèles pour étudier comment le système de reproduction interagit avec l'adaptation. J'ai utilisé un de ces organismes, *Noccaea caerulescens* J. Presl & C. Presl F.K. Mey, pour tester différents attendus théoriques de l'interaction entre adaptation locale et taux d'autofécondation en tirant parti de l'existence de différentes populations évoluant sur des sols très différents.

Je vais d'abord décrire les conséquences de l'autofécondation sur les processus évolutifs en développant notamment l'effet sur la sélection, la dérive et les flux de gènes. Puis j'aborderai l'évolution des systèmes de reproduction et les facteurs susceptibles de l'influencer. Enfin, je décrirai le modèle fascinant[1] sur lequel j'ai travaillé au long de cette thèse, *Noccaea caerulescens*.

I. Conséquences de l'autofécondation sur la sélection, la dérive et le flux de gènes

L'autofécondation est la fusion d'un gamète mâle avec un gamète femelle produit par le même individu. C'est la forme la plus extrême de consanguinité et elle s'oppose à l'allofécondation, la reproduction entre individus différents de la même espèce. Dans ce qui suit, je vais décrire comment l'autofécondation et la consanguinité en général influencent plusieurs processus évolutifs.

Augmentation de l'homozygotie

Dans une lignée qui s'autoféconde (lignée autogame), la proportion d'hétérozygotes à un locus donné est divisée par deux à chaque génération, ce qui conduit à une augmentation progressive de l'homozygotie dans tout le génome. Les conséquences de cette réduction sont importantes et dépendent de l'effet des mutations et des interactions entre allèles à un même locus (leur dominance).

[1] Qui ne travaille pas sur un modèle fascinant ?

Quand les hétérozygotes ont une plus grande valeur sélective

Pour des locus super-dominants où les hétérozygotes ont une meilleure valeur sélective que les homozygotes, une diminution de la proportion d'hétérozygotes induit naturellement une diminution de la valeur sélective des individus. Cet effet fâcheux est l'un des mécanismes qui génèrent de la dépression de consanguinité, la diminution relative de la valeur sélective des individus issus de l'autofécondation (ou de croisements consanguins) par rapport aux individus issus de l'allofécondation, et sur laquelle je reviendrai prochainement.

Quand des allèles sont récessifs

L'autofécondation, en augmentant l'homozygotie en général, augmente donc l'expression des allèles récessifs. Cela permet à la sélection de les « voir », autrement dit, d'agir sur ces allèles. Cette exposition a des effets différents selon que l'allèle est délétère ou bénéfique.

Si l'allèle récessif est délétère, son expression accrue dans les individus issus de l'autofécondation décroît la valeur sélective de ces individus par rapport aux individus issus de l'allogamie. De nombreux allèles récessifs et délétères ségrégent à faible fréquence dans les populations et forment le fardeau génétique. L'expression de ce fardeau chez les individus consanguins est considérée comme l'un des principaux mécanismes générant de la dépression de consanguinité (voir Charlesworth and Willis 2009 pour revue). L'autofécondation peut aider à purger ce fardeau (Willis 1999; Crnokrak and Barrett 2002; Fox *et al.* 2008; Charlesworth and Willis 2009).

Si, en revanche, les allèles récessifs ont un effet bénéfique, l'augmentation de leur expression chez les individus consanguins augmente l'efficacité de la sélection. Cela favorise donc leur augmentation en fréquence dans la population et diminue le risque qu'ils soient perdus de manière stochastique du fait de la dérive génétique. Cet effet est bien illustré dans l'étude théorique de Glémin et Ronfort (2013), qui modélise l'adaptation à un changement environnemental. Ce modèle montre que si l'adaptation se fait à partir de nouvelles mutations récessives à un locus à deux allèles, l'adaptation est plus probable chez les populations autogames que chez les populations allogames (**Figure 1A**). De plus, si le nouvel allèle arrive à envahir la population, il le fait toujours plus rapidement chez les populations autogames qu'allogames. Si en revanche l'adaptation se fait à partir de variation génétique préexistante dans la population, c'est une autre histoire, et j'y reviendrai bientôt (**Figure 1B** et voir page - 16 -).

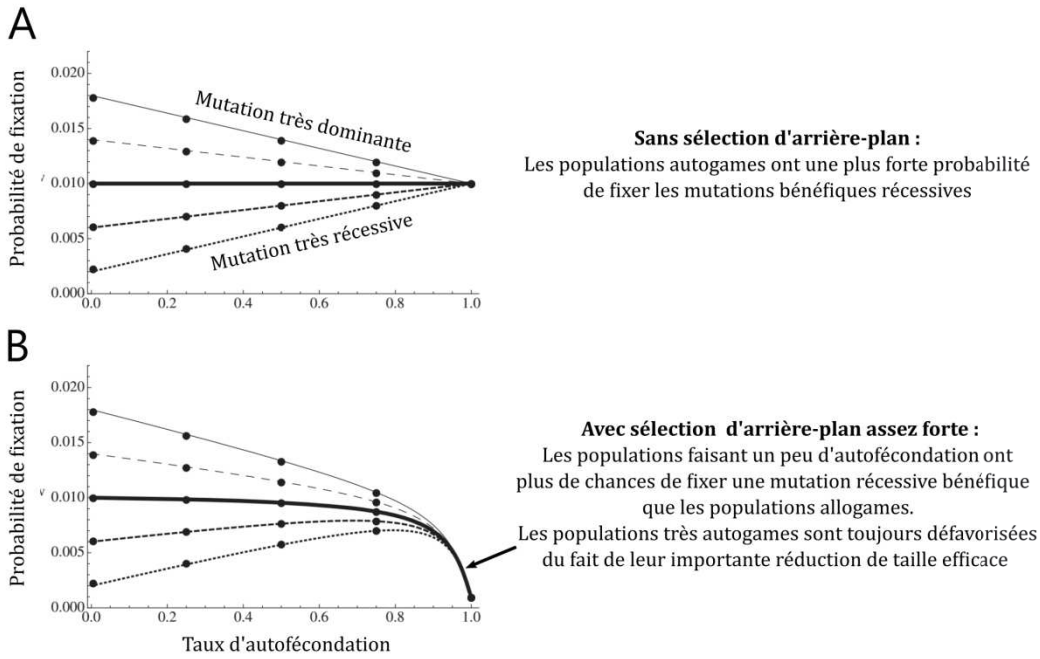

Figure 1. Probabilité de fixation d'un allèle bénéfique en fonction du taux d'autofécondation et de la récessivité. Lignes pointillées : mutations récessives ; lignes pleines et tirets : mutations dominantes ; ligne en gras : mutations codominantes. Les auteurs ont considéré l'adaptation à un locus avec deux allèles dans une population subissant un changement environnemental. Ils ont étudié la probabilité de fixation d'une nouvelle mutation bénéfique dans le nouvel environnement, avec des effets plus ou moins forts du taux d'autofécondation sur la dérive. A) Diminution de la taille efficace seulement due à l'échantillonnage non aléatoire des gamètes et B) Diminution de la taille efficace plus drastique, visant à mimer les effets de la réduction de recombinaison efficace quand le taux d'autofécondation augmente, qui crée de la sélection d'arrière-plan. Figure adaptée de Glémin et Ronfort (2013).

Baisse de la recombinaison efficace

Une autre conséquence de l'augmentation de l'homozygotie est que l'autofécondation diminue le taux de recombinaison efficace (Nordborg 2000). Cela peut également réduire l'efficacité de la sélection. Par exemple, si une mutation bénéfique apparait à côté de mutations délétères et est donc « génétiquement liée » à elles, cela peut aboutir à deux situations. Premièrement, la sélection de la mutation bénéfique entraine l'augmentation en fréquence d'allèles faiblement délétères par effet « d'auto-stop génétique ». Alternativement, si les mutations sont trop délétères ou trop nombreuses, cela rend la sélection sur la mutation bénéfique moins efficace (« sélection d'arrière-plan »).

Hartfield et Glémin (2014) montrent dans un modèle théorique que pour des mutations bénéfiques même partiellement récessives, la présence de mutations délétères dans l'arrière-plan génétique rend l'autofécondation défavorable. Par contre, si l'on considère les simulations où l'allèle bénéfique a réussi à augmenter en fréquence *a posteriori*, l'autofécondation augmente la probabilité de fixation de cet allèle par rapport à l'allofécondation, comme dans le modèle décrit précédemment. Pour certaines combinaisons de paramètres, un système mixte (avec une fréquence modérée d'autofécondation) permet d'avoir suffisamment de recombinaison pour séparer les allèles délétères de l'allèle bénéfique tout en conservant le bénéfice de l'exposition de ce dernier à la sélection.

Diminution de la taille efficace de la population

Dans les modèles que je viens de mentionner, l'autofécondation peut être désavantageuse car elle diminue la taille efficace de la population sur le long terme. La taille efficace est un concept de génétique des populations qui représente la taille d'une population sans mutation, sans sélection, sans migration, avec une reproduction panmictique dans laquelle les variations stochastiques des fréquences alléliques seraient équivalentes à celles dans la population réelle étudiée[2].

La taille efficace est réduite par la consanguinité et *a fortiori*, par l'autofécondation, de différentes manières. Tout d'abord, lors de croisements consanguins, l'échantillonnage des gamètes n'est par définition pas indépendant. Dans le cas extrême de l'autofécondation totale, cela divise la taille efficace de la population par deux. Ensuite, la diminution de la recombinaison efficace mentionnée ci-dessus réduit également la taille efficace. Enfin, un individu autofécondant est théoriquement capable de fonder une population à lui tout seul lors d'un évènement de colonisation. Cette population aura alors subi un goulot d'étranglement génétique extrême, dont les effets se feront ressentir pendant de nombreuses générations. Si les populations autogames ont plus tendance à subir des goulots d'étranglement génétiques, cela devrait également réduire leur taille efficace. Cette réduction a de nombreuses conséquences, aux effets parfois contradictoires.

[2] En d'autres termes, c'est la taille d'une population idéale qui dériverait autant que la population réelle.

Réduction de la diversité génétique

Une petite taille efficace implique de fortes fluctuations stochastiques des fréquences alléliques, ce qui conduit à une perte graduelle de diversité génétique. Par exemple, Burgarella *et al.* (2015) ont analysé la diversité génomique de deux anciennes lignées d'escargots hermaphrodites, l'une allofécondante et l'autre autofécondante, et ont trouvé un polymorphisme cinq fois plus fort chez l'espèce allofécondante que chez l'espèce autofécondante. Cette perte de diversité a deux conséquences.

Tout d'abord, elle contraint l'adaptation, car elle réduit la variation sur laquelle la sélection agit. Le modèle de Glémin et Ronfort (2013) illustre bien cet effet : si l'adaptation se fait par sélection d'un allèle existant déjà dans la population avant le changement environnemental (« variation pré-existante »), les populations plus allofécondantes ont une plus grande probabilité de s'adapter que les populations autofécondantes, et ce pour un grand nombre de combinaisons de paramètres. Cela se comprend intuitivement : la perte de diversité chez les populations autogames fait que les mutations qui seront plus tard intéressantes sont souvent perdues par dérive. De plus, si les mutations bénéfiques dans l'environnement après le changement environnemental étaient délétères avant ce dernier (effet antagoniste des mutations), les populations autofécondantes ont plus facilement purgé ces mutations.

Par ailleurs, si la perte de diversité génétique due à la dérive affecte des locus dominants et super-dominants, cela aboutit à une diminution de la dépression de consanguinité. En effet, si tous les individus sont homozygotes à un de ces locus, les individus issus de l'allofécondation et de l'autofécondation sont tous aussi mauvais les uns que les autres (pour ce locus).

La sélection est moins efficace

Une seconde grande conséquence de la dérive est que l'augmentation des variations stochastiques des fréquences alléliques « bruite » la sélection, ce qui la rend moins efficace.

Dans le cas de mutations bénéfiques, cet effet est particulièrement critique pour les nouvelles mutations, qui apparaissent souvent en une seule copie et peuvent donc très facilement être perdues dans les générations qui suivent son apparition. Le modèle de Glémin et Ronfort (2013) inclut la diminution de taille efficace due à l'échantillonnage non aléatoire des gamètes et prend en compte les autres effets en diminuant plus ou moins fortement cette taille efficace déjà réduite. On peut voir que si la taille efficace est fortement diminuée par

l'autofécondation, les populations très autofécondantes perdent leur avantage sur la fixation des allèles bénéfiques récessifs (y compris pour des allèles très récessifs) et s'adaptent moins bien que des populations allogames ou avec des taux d'autofécondation intermédiaires (**Figure 1B**).

De plus, la sélection moins efficace laisse des mutations délétères augmenter en fréquence dans la population, ce qui génère le fardeau génétique « ségrégeant » (Kimura *et al.* 1963; Bataillon and Kirkpatrick 2000). Par exemple, Burgarella *et al.* (2015) ont trouvé plus de mutations délétères non synonymes dans l'espèce d'escargots autofécondante que dans l'espèce allofécondante. Si ces mutations sont récessives, elles seront plus exprimées chez les autogames que chez les allogames et génèreront donc de la dépression de consanguinité.

Si la dérive est suffisamment forte, cela peut aboutir à la fixation de certaines de ces mutations, ce qui crée le fardeau génétique fixé. Contrairement au fardeau ségrégeant, ce fardeau fixé est constitutivement exprimé dans tous les individus de la population, qu'ils aient été produits par autofécondation ou allofécondation. Il ne participe donc pas à la dépression de consanguinité (**Figure 2**). Cela explique pourquoi la dépression de consanguinité est souvent plus faible dans les petites populations et pourquoi le croisement de deux lignées consanguines peut donner des descendants avec une meilleure valeur sélective que les parents (phénomène d'hétérosis bien connu des agronomes). En effet, les deux lignées n'ont pas nécessairement fixé les mêmes mutations délétères récessives aux mêmes locus et leur croisement permet de cacher un certain nombre de ces mutations. Par exemple, plus la taille efficace de populations de daphnies est faible, plus l'hétérosis est grande et plus la dépression de consanguinité est faible (**Figure 3**, Lohr and Haag 2015). Ici, comme pour la perte de diversité à des locus super-dominants, l'accumulation de mutations dans les petites populations diminue la valeur sélective de ces populations au cours du temps, augmentant leur risque d'extinction. Les données concernant cette accumulation commencent à voir le jour grâce à l'avancée des techniques de séquençage. Néanmoins, les résultats ne sont pas univoques (Glémin and Muyle 2014; Glémin and Ronfort 2013; Hartfield 2016), peut-être parce que les lignées autofécondantes sont souvent trop jeunes pour que l'accumulation des mutations soit détectable. Burgarella *et al.* (2015), qui ont étudié cette question chez de vieilles lignées, ont en effet trouvé une accumulation de mutations délétères chez l'espèce autogame.

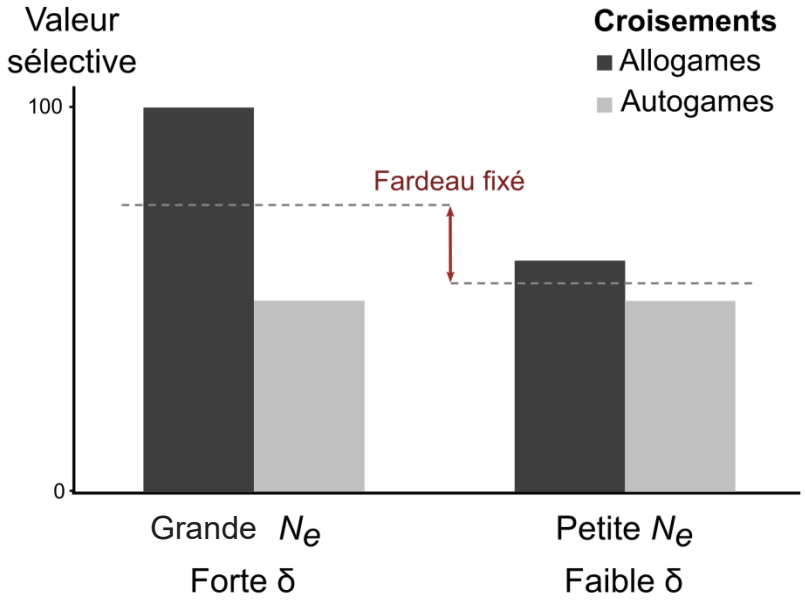

Figure 2. Prédictions qualitatives concernant le fardeau dans des populations de petite et grande tailles efficaces (N_e). Gris foncé : valeur sélective moyenne des individus issus de l'allofécondation. Gris clair : valeur sélective moyenne des individus issus de l'autofécondation. La dépression est ici plus forte dans la population de grande taille (δ=0.5) que dans la petite (δ=0.2).

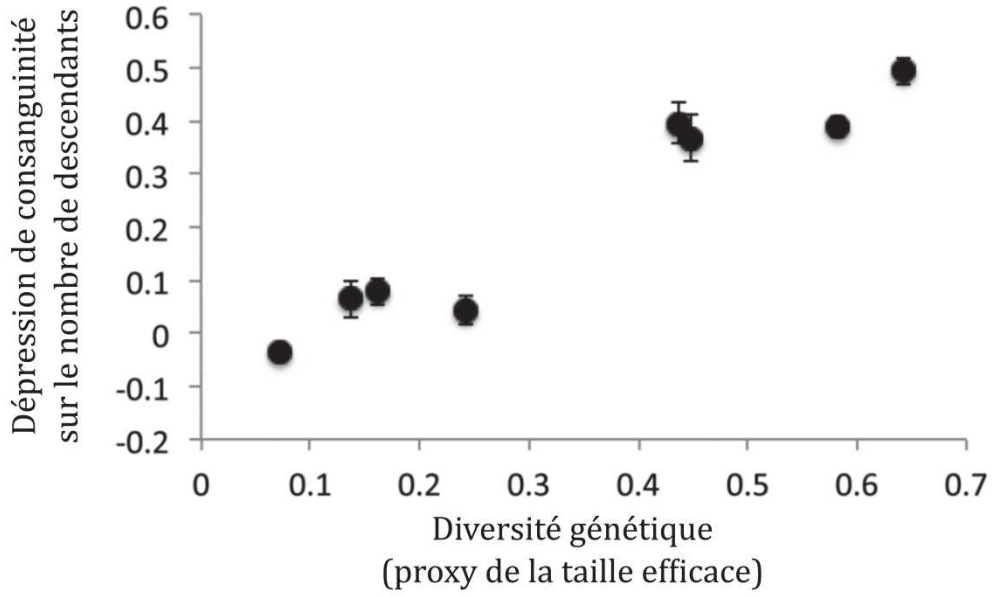

Figure 3. Dépression de consanguinité en fonction de la taille efficace, approximée par la diversité génétique. Figure adaptée de Lohr et Haag (2015).

Effet sur les traits quantitatifs

Les modèles de génétique quantitative prédisent une augmentation temporaire de la variance après une augmentation brutale de la consanguinité et avant que les effets de perte de diversité ne se fassent sentir. L'autofécondation crée en effet des corrélations entre allèles à chaque locus (Lande 1977) et, pour les traits à architecture complexe, peut convertir de la variance épistatique ou de dominance en variance additive (par exemple Cheverud *et al.* 1999). Cette augmentation de variance, bien que limitée dans le temps, peut ponctuellement stimuler l'adaptation.

Porcher et Lande (2015) ont spécifiquement étudié l'effet du système de reproduction sur la variance génétique des traits quantitatifs. Ils montrent que la variance génétique des populations à système de reproduction mixte reste relativement similaire à celle des populations allogames, jusqu'à une valeur seuil de taux d'autofécondation au-delà de laquelle la variation est perdue. Ce seuil dépend de l'architecture génétique du trait étudié (nombre de locus, nombre d'allèles etc.). Cela suggère que les populations allofécondantes ou partiellement autogames ont un potentiel adaptatif similaire, plus élevé que celui des populations autogames, en termes de diversité génétique en tout cas. Similairement, Peterson et Kay (2015) ont simulé la colonisation d'un nouvel environnement par des organismes dont le taux d'autofécondation change plastiquement à l'arrivée dans le nouvel environnement. L'expression d'un système de reproduction partiellement ou complètement autogame dans le nouvel environnement était associée à une augmentation de la variation génétique. Cette augmentation favorisait les populations avec un système de reproduction mixte après le changement environnemental, dont l'adaptation était plus rapide et le risque d'extinction plus faible (**Figure 4**). En revanche, pour les populations complètement autofécondantes, la variation génétique diminuait très rapidement et ces populations avaient un risque d'extinction plus fort que les autres (**Figure 4**).

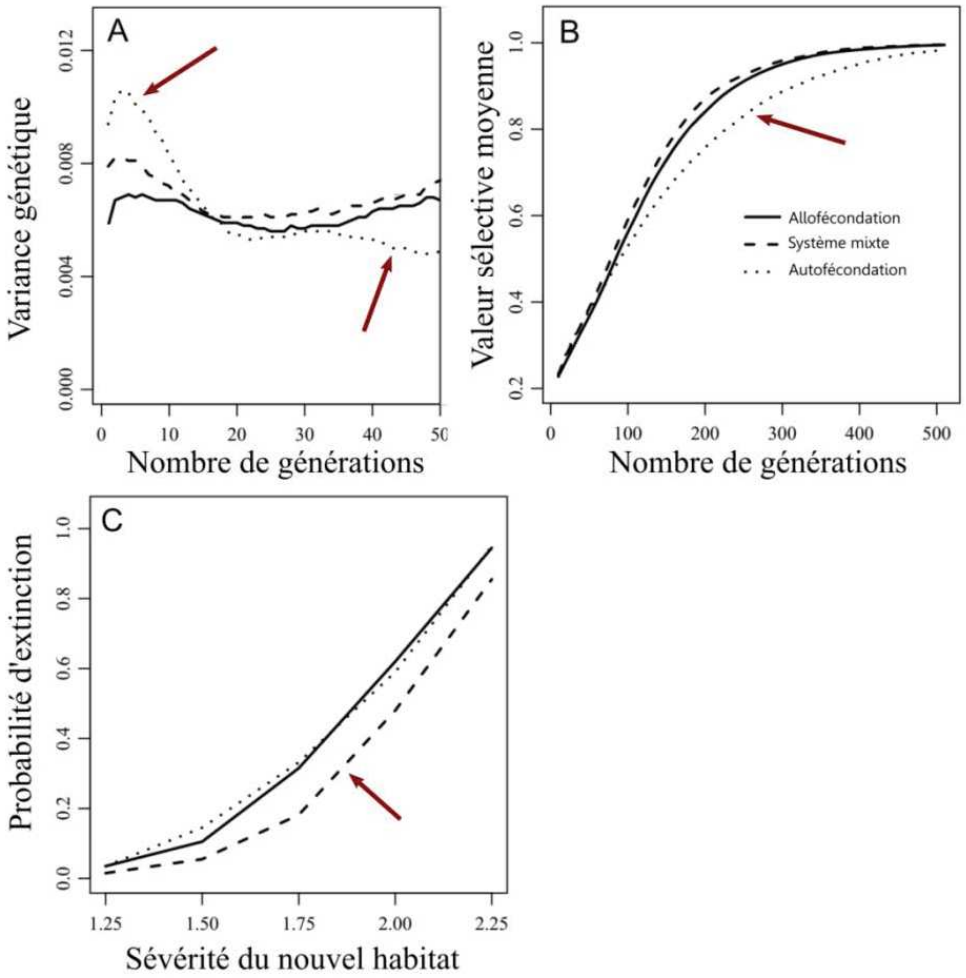

Figure 4. Effet de l'autofécondation sur l'évolution de la niche suivant la colonisation d'un nouvel habitat. Les lignes représentent les moyennes sur 200 simulations. Pointillés : autofécondation complète ; ligne discontinue : système mixte avec 50% d'autofécondation ; ligne pleine : allofécondation complète. A) Évolution de la variance génétique pendant les 50 premières générations suivant la colonisation au cours du temps (le patron à la 500^{ième} génération est essentiellement le même qu'à la 50^{ième}). B) Valeur sélective moyenne de la nouvelle population au cours du temps, suivant la colonisation. C) Proportion des populations qui se sont éteintes dans les 1000 premières générations suivant la colonisation, en fonction de la sévérité de l'habitat. Les flèches attirent l'attention sur des différences importantes entre les différents modes de reproduction. Figure adaptée de Peterson et Kay (2015)

L'autofécondation comme mécanisme d'isolement reproducteur

Dans un environnement hétérogène, l'apport de gènes mal adaptés en provenance d'une autre population peut être un frein à l'adaptation locale (Lenormand 2002). Les modèles théoriques qui se sont intéressés aux flux de gènes via le pollen et les graines suggèrent que ces

deux types de dispersion ne sont pas équivalents. Par exemple, Lopez *et al.* (2008) trouvent que lorsque l'habitat est fortement hétérogène, l'arrivée de pollen génère un fardeau génétique, dit de migration, plus fort que l'arrivée de graines. En effet, les allèles délétères dans le nouvel environnement sont souvent à l'état homozygote dans les graines et donc facilement purgés. En revanche, les allèles introduits par le pollen se retrouvent à l'état hétérozygote face à des allèles locaux plus adaptés et sont donc plus difficiles à purger. Similairement, en modélisant la colonisation d'une nouvelle population à partir d'une population source, Aguilée *et al.* (2013) ont montré que les flux de graines favorisent l'adaptation au nouvel habitat, car ils contribuent à augmenter la taille de la population dans ce nouvel habitat, et à éviter les effets Allee. Mais dans beaucoup de cas, les flux de pollen ralentissent l'adaptation car ils créent du fardeau de migration.

Dans ce contexte, l'autofécondation est avantageuse car elle diminue l'intégration de gènes mal adaptés dans la population via le pollen. Antonovics (1968) prédisait que l'autofécondation réduisait le fardeau de migration quand la migration était forte. Plus récemment, Peterson and Kay (2015) ont montré que des taux d'autofécondation intermédiaires sont avantageux du fait de l'isolement reproducteur conféré par l'autofécondation. De plus, lorsque l'afflux de pollen est extrêmement fort, l'autofécondation complète devient également plus avantageuse que l'allofécondation, malgré la baisse de potentiel adaptatif précédemment décrite.

L'autofécondation, un cul-de-sac évolutif ?

La perte graduelle de diversité chez les lignées autofécondantes compromettant potentiellement l'adaptation a conduit Stebbins (1957) à qualifier l'autofécondation de cul-de-sac évolutif : « *In one sense, therefore, self-fertilization is an evolutionary "blind alley", since it apparently closes the door to the elaboration of radically new adaptive devices* ». L'accumulation progressive des mutations délétères due à la diminution de taille efficace vient conforter cette idée (Takebayashi and Morrell 2001; Igic and Busch 2013).

Les analyses de macroévolution s'intéressant à l'évolution du système de reproduction dans les phylogénies vont dans le sens de la prédiction de Stebbins. Dans la famille des Solanacées, Goldberg *et al*. (2010) montrent que les lignées auto-compatibles (et donc susceptible de s'autoféconder) ont des taux de diversification mais aussi d'extinction plus hauts que les lignées auto-incompatibles. Le taux de diversification net, qui prend en compte diversification et extinction, est néanmoins plus fort pour les lignées auto-incompatibles. Une

analyse complémentaire trouve que les transitions depuis l'auto-incompatibilité vers l'auto-compatibilité sont plus souvent associées avec un évènement de spéciation (changement cladogénétique) qu'avec une transition simple à l'intérieur d'une lignée (changement anagénétique), ce qui indique que la transition vers un système auto-compatible est plus souvent associée avec un évènement de spéciation, et donc de l'isolement reproducteur.

Dans l'ensemble, ces résultats illustrent une relation complexe entre le système de reproduction et l'adaptation, la spéciation et l'extinction, qui est compréhensible vu les effets qu'a l'autofécondation sur diverses forces évolutives. Les effets de l'autofécondation sur l'adaptation, en particulier, pourraient se compenser. Cela pourrait expliquer en partie les résultats de deux méta-analyses récentes qui ne trouvent pas d'effet du système de reproduction sur l'adaptation locale. Leimu et Fischer (2008) ne l'ont pas détecté dans trente-cinq expériences de transplantations réciproques concernant 32 espèces. Leur classification du système de reproduction était simple (auto-compatible ou auto-incompatible) car ils n'avaient la plupart du temps pas accès aux taux d'autofécondation réalisés dans les populations étudiées. Le fait que les lignées auto-compatibles puissent avoir des taux d'autofécondation extrêmement variables pourrait expliquer les intervalles de confiance beaucoup plus larges pour le groupe auto-compatible. Hereford (2010) n'a pas non plus trouvé d'effet du système de reproduction (auto-compatible ou auto-incompatible; autofécondant ou allofécondant) sur l'adaptation locale. L'auteur suggère que cet effet est dû à des données imprécises (taux d'autofécondation et adaptation locale mesurés dans des populations différentes) ou à des effets antagonistes du taux d'autofécondation sur l'adaptation locale.

En conclusion, le système de reproduction a des effets complexes et multiples sur les processus évolutifs. Les avancées théoriques et expérimentales récentes continuent d'apporter de nouvelles données et de nouveaux points de vue à une question ancienne mais encore loin d'être résolue.

II. Variation du système de reproduction

Dans la partie précédente, j'ai décrit comment le système de reproduction affecte les processus évolutifs. La plupart des modèles que j'ai présentés le traitaient comme constant. Pourtant, le système de reproduction est basé sur un ensemble de traits souvent labiles. Et il semble évident qu'au vu de son effet sur les processus évolutifs, il soit lui-même soumis à

l'action de la sélection. Dans les paragraphes suivants, je vais vous présenter brièvement la variation des modes de reproduction et les principales forces qui agissent sur leur évolution.

Autant de modes de reproduction que d'espèces ?

Le terme « système de reproduction » recouvre une très large diversité de fonctionnement chez les êtres vivants. Il existe en effet de nombreux moyens d'engendrer de nouveaux organismes. Certains organismes se reproduisent essentiellement par multiplication asexuée, c'est-à-dire sans l'aide d'un autre individu, ou sans fusion de deux gamètes de sexe opposé. C'est un mode très courant chez les organismes unicellulaires, qu'ils soient Procaryotes ou Eucaryotes (les bactéries et les paramécies par exemple). Néanmoins, c'est un type de reproduction que l'on peut également trouver chez les cnidaires, les annélides ou les planaires. Bien qu'il existe quelques organismes qui ne se reproduisent que de manière asexuée, tels que certaines lignées de Daphnies ou quelques espèces de reptiles, ou que de manière sexuée, tels que les mammifères, de nombreux organismes alternent ou combinent les deux types de reproduction. Par exemple, de nombreuses plantes à fleurs ont, outre leur reproduction sexuée, la capacité de se reproduire de manière asexuée (dite « végétative »), via des organes plus ou moins spécialisés (stolons, bulbille, rhizomes ou fragments non spécialisés). Mais l'on peut également citer un certain nombre d'insectes hyménoptères, tels que les fourmis, les guêpes parasitoïdes ou les abeilles, qui effectuent la parthénogénèse et se reproduisent de manière sexuée.

L'extrême variation visible sur le gradient allant des organismes sexués aux organismes asexués est également présente au niveau des types de reproduction sexuée, chez les animaux comme chez les plantes. Je vais me focaliser sur ces dernières, non pas que les animaux ne soient pas intéressants, mais parce que les plantes suffisent à illustrer la fantastique diversité de modes reproductifs. Les individus peuvent ne porter des organes que d'un seul sexe (dioécie, comme beaucoup de gymnospermes, ou comme le *Ginkgo biloba*) ou des deux sexes (monoécie). Il existe des populations dont certains individus portent les organes mâles et femelles et d'autres individus uniquement les organes femelles (gynodioécie). Plus rarement, des populations contiennent des individus portant les organes mâles et femelles et des individus mâles (androdioécie). Parfois, il peut y avoir coexistence d'individus mâles, femelles et hermaphrodites dans la population (trioécie).

Au sein des populations monoïques, la diversité est également au rendez-vous : les organes mâles et femelles peuvent être séparés au sein d'un individu (monoécie stricte) ou,

réunis dans la même fleur, dite hermaphrodite. Chez certaines espèces, les individus peuvent porter des fleurs femelles et des fleurs hermaphrodites (espèces gynomonoïques) mais chez d'autres espèces, c'est le contraire : les individus portent des fleurs males et hermaphrodites (espèces andromonoïques).

Un intérêt de la monoécie et en particulier de l'hermaphrodisme est que ces systèmes permettent l'autofécondation. Ils ne la rendent toutefois pas automatique, et de nombreuses caractéristiques peuvent diminuer la probabilité qu'elle ait lieu. Les organes sexuels mâles (les étamines) peuvent être spatialement éloignés des structures sexuelles femelles (le pistil) et l'on parle alors d'herkogamie. Parfois, ces organes sont matures à des temps différents (dichogamie). Parfois, un système d'auto-incompatibilité empêche les individus de s'autoféconder. Il arrive que plusieurs morphes floraux existent dans une population, avec différentes architectures florales (pistil et étamines de différentes tailles), encourageant les échanges de pollen entre morphes. Il est fréquent qu'un système d'incompatibilité empêche les individus d'un même morphe de se reproduire entre eux. Et comment ne pas citer, pour le plaisir des systèmes étranges, l'hétérodichogamie, où certaines plantes sont fonctionnellement femelles en premier, puis mâles, tandis que d'autres plantes de la population sont d'abord mâles (par exemple, dans le genre *Alpina* (Li *et al.* 2001).

Variation du taux d'autofécondation

Si l'on s'intéresse à la capacité à s'autoféconder, on trouve ici encore une incroyable variation. Différentes espèces de plantes, y compris phylogénétiquement proches, peuvent avoir des taux d'autofécondation très différents. On peut par exemple citer *Arabidopsis thaliana*, qui fait de l'autofécondation à plus de 95% et son espèce sœur *Arabidopsis lyrata*, qui n'est pas compatible avec elle-même. Schemske et Lande (1985) ont recensé les connaissances de l'époque sur la variation des taux d'autofécondation entre espèces. Depuis, la liste s'est beaucoup allongée (Vogler and Kalisz 2001; Barrett 2003; Goodwillie *et al.* 2005). La distribution des taux d'autofécondation est continue et légèrement bimodale (**Figure 5**).

Au fil du siècle dernier, plusieurs arguments ont été invoqués pour expliquer la distribution des systèmes de reproduction et leur évolution. Historiquement, deux approches ont coexisté, qui ont fini par se rejoindre. La première s'intéressait aux systèmes de reproduction d'un point de vue génétique. Elle a engendré un grand nombre de modèles théoriques sur l'évolution du taux d'autofécondation (Lloyd 1979; Lande and Schemske 1985;

Holsinger 1988; Jain 1976; Uyenoyama and Waller 1991a; b; c ; Charlesworth 2006). En parallèle, une approche plus naturaliste étudiait comment les contraintes écologiques des populations des plantes influencent la reproduction, avec un intérêt particulier pour l'écologie de la pollinisation. En effet, l'immobilité des plantes est une contrainte majeure[3] puisqu'elle les rend dépendantes d'un vecteur pour le transfert du pollen. Les modèles plus récents incorporent souvent des contraintes génétiques, écologiques et fonctionnelles (Lloyd 1992; Robertson 1992; Porcher and Lande 2005; Johnston *et al.* 2009).

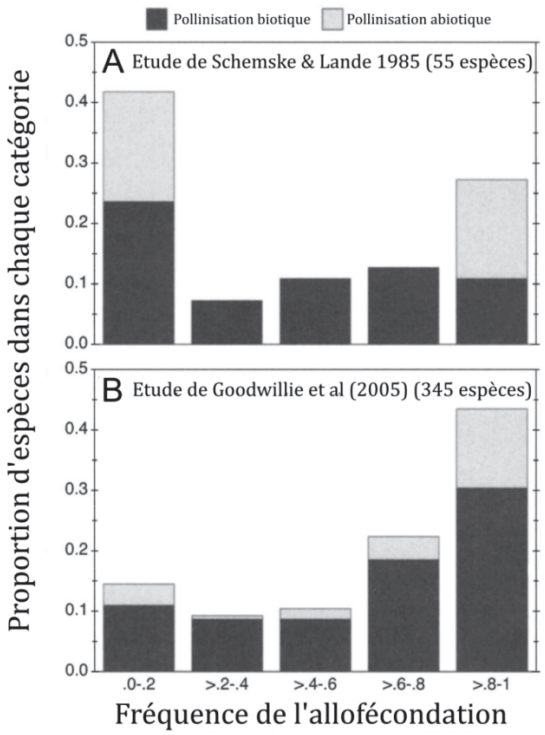

Figure 5. Distribution des taux d'allofécondation estimés chez les plantes à fleurs. En gris clair : plantes pollinisées par des vecteurs non biotiques (eau, vent). En gris foncé : plantes pollinisées par des vecteurs vivants (par exemple : insectes, oiseaux). A) Distribution publiée dans l'étude de Schemske et Lande (1985). B) Distribution complétée à 345 espèces. Si plusieurs estimations étaient présentes pour une espèce, le taux d'allofécondation a été moyenné sur les années puis sur les populations. Figure adaptée de Goodwillie *et al.* (2005).

[3] Et la réalisation soudaine de tout ce que cela impliquait, pendant mon premier cours de biologie végétale de Licence, est probablement au cœur de mon intérêt pour les plantes.

Facteurs génétiques

Les plantes hermaphrodites transmettent leur patrimoine génétique à travers leurs ovules, mais également à travers leur pollen. Une plante allogame transmet une copie de son génome via la fécondation d'un gamète femelle dans un ovule et une copie de son génome via chaque grain de pollen. Dans une population allofécondante, un mutant qui effectue de l'autofécondation tout en exportant la grande majorité de son pollen transmet ses gènes via ses ovules, où les gamètes sont fécondés grâce à son propre pollen, et en fertilisant d'autres plantes (soit une augmentation de 50% par rapport aux plantes allofécondantes (Fisher 1941; Charlesworth and Charlesworth 1979). C'est ce qui a été appelé « l'avantage de transmission » de l'autofécondation.

Face à cet avantage, qui devrait naturellement conduire à la fixation de l'autofécondation, les modèles ont tout d'abord opposé la dépression de consanguinité, la réduction relative de valeur sélective des individus issus de l'autofécondation par rapport aux individus issus de l'allofécondation (Lande and Schemske 1985). La dépression de consanguinité devait sélectionner des systèmes allogames si supérieure à 0.5 et sélectionner des systèmes autogames si inférieure à 0.5. En l'état, ce modèle n'expliquait pas la large fraction de taux d'autofécondation intermédiaires observée par les mêmes auteurs (Schemske and Lande 1985). De nombreux modèles ont ensuite complexifié la modélisation de la dépression de consanguinité (résumés dans Charlesworth 2006), modélisé son évolution et son interaction avec l'environnement (Cheptou and Mathias 2001) ou la dynamique des populations (Cheptou and Dieckmann 2002). Un certain nombre de ces modèles prédisent l'évolution de systèmes de reproduction mixte à l'équilibre.

Plusieurs mécanismes peuvent générer de la dépression de consanguinité (**Figure 6**). L'hypothèse dite de la dominance est basée sur l'existence de mutations récessives délétères dans le génome, qui ségrégent dans la population et qui sont plus exprimées lors de l'autofécondation que lors de l'allofécondation. Cette hypothèse est pour le moment considérée comme responsable d'une large partie de la dépression de consanguinité (Charlesworth and Willis 2009). L'hypothèse de la superdominance est basée sur les locus où les hétérozygotes ont une meilleure valeur sélective que les homozygotes. Parfois, ce mécanisme peut être confondu avec le précédent si deux locus très proches (et donc recombinant peu) portent chacun une mutation délétère récessive sur chacune des copies du chromosome. Dans cette configuration, les hétérozygotes ont une valeur sélective plus importante car à chacun des deux locus, la

mutation dominante cache la mutation délétère (**Figure 6**). On parle alors de pseudo-superdominance. Plus difficile encore à quantifier est la manière dont de multiples mutations affectent le génome : est-ce que les effets des mutations sont indépendants et se multiplient entre eux ? Enfin, l'étude de Vergeer *et al.* (2012) suggère que des facteurs épigénétiques peuvent également être impliqués dans l'expression de la dépression de consanguinité.

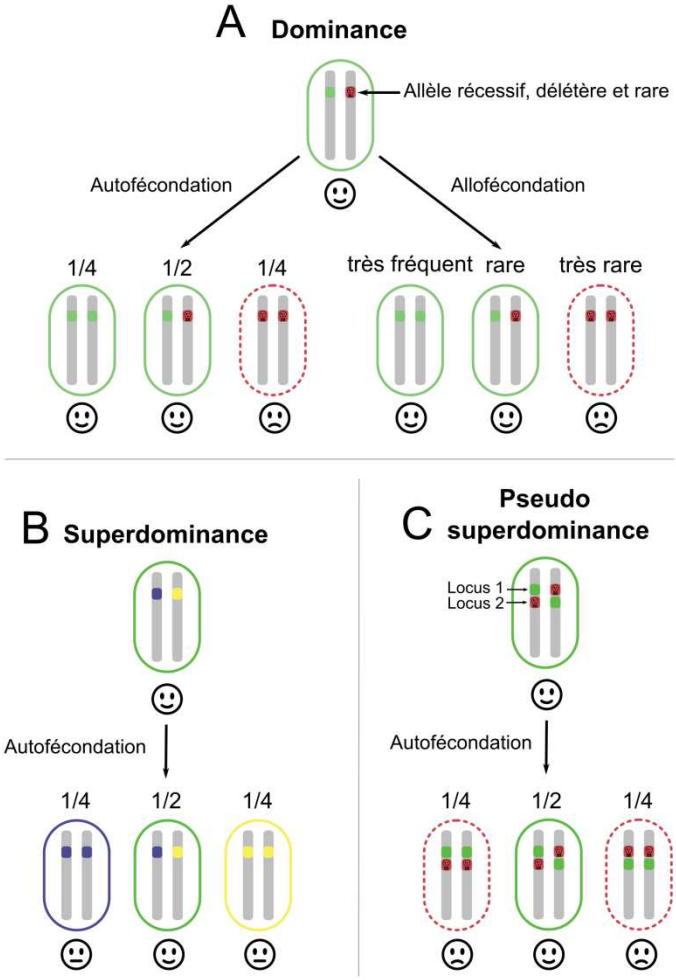

Figure 6. Mécanismes principaux générant de la dépression de consanguinité. A) Mécanisme de dominance impliquant un allèle délétère récessif rare (rouge) et un allèle neutre ou bénéfique dominant commun dans la population (vert). B) Mécanisme de superdominance selon lequel l'hétérozygote (jaune-bleu) a une meilleure valeur sélective que les homozygotes (bleu-bleu et jaune-jaune). C) Mécanisme de pseudo-superdominance qui est en fait un mécanisme de dominance, mais avec deux locus impliqués très proches l'un de l'autre et recombinant donc peu. Si des mutations délétères récessives (rouges) ne sont pas sur le même chromosome, alors le résultat final ressemble à de la superdominance.

Au vu de la variété des mécanismes produisant de la dépression de consanguinité, et dans la mesure où ils dépendent pour la plupart de l'histoire de purge de la population, il n'est pas surprenant qu'il existe une large variation de la dépression de consanguinité entre espèces, populations et même parfois traits (Husband and Schemske 1996; Keller and Waller 2002; Crnokrak and Barrett 2002; Winn *et al.* 2011; Angeloni *et al.* 2011). En outre, il arrive qu'elle ne s'exprime pas de la même manière dans tous les environnements (Byers and Waller 1999; Crnokrak and Roff 1999; Keller and Waller 2002; Armbruster and Reed 2005; Fox and Reed 2011). Le stress semble augmenter l'intensité de la dépression de consanguinité, mais pas toujours (Armbruster and Reed 2005; Fox and Reed 2011), ce qui a conduit certains auteurs à proposer que l'histoire de sélection plutôt que le stress en tant que tel explique cette variation (Agrawal and Whitlock 2010; Long *et al.* 2013).

Contraintes environnementales

Outre la dépression de consanguinité et l'avantage de transmission, un certain nombre de facteurs environnementaux peuvent influencer le système de reproduction. La plus large variation de la distribution des taux d'autofécondation dans le cas de la pollinisation biotique (**Figure 5**) suggère que les pollinisateurs, quels qu'ils soient, ajoutent de la complexité à la reproduction. En effet, sauf si les plantes ont la capacité de s'autoféconder de manière autonome, elles sont dépendantes de vecteurs pour transporter les grains de pollen vers les organes femelles. Cette dépendance soumet les traits susceptibles de favoriser ce transfert de pollen à une forte sélection. L'autofécondation autonome est une manière de contourner le problème et correspond à l'avantage dit de « l'assurance reproductive », car en cas d'absence de pollinisateurs, une plante possédant cette capacité peut se reproduire là où ses congénères allogames ne le peuvent pas. Dans la mesure où le transfert de pollen est souvent une étape limitante en terme de qualité et de quantité (Ashman *et al.* 2004; Knight *et al.* 2005; Aizen and Harder 2007), cet avantage peut être crucial.

Néanmoins, tout n'est pas si simple. Si la quantité de pollen produit est limitée, s'autoféconder réduit mécaniquement le potentiel exporté pour l'allofécondation ; c'est ce que l'on appelle le décompte de pollen (« pollen discounting »). Plus il est fort, plus l'avantage de transmission est réduit. De surcroît, cette diminution est accentuée s'il y a de la dépression de consanguinité dans la population. Non seulement on remplace une allofécondation par une autofécondation, mais cette dernière produit des descendants avec une plus faible valeur sélective.

De manière similaire, le nombre d'ovules étant souvent limité, la fécondation avec du pollen autogame revient, si tous les ovules de la plante sont fécondés, à réduire le nombre d'ovules fécondés avec du pollen allogame (décompte en ovules ou « seed discounting »). Si les modèles considèrent en général que le nombre total d'ovules est fixe, ce qui crée du décompte en ovules, cela ne veut pas dire que la relation entre nombre d'ovules allofécondés et autofécondés soit nécessairement négative (Johnston 1998).

Les modèles d'évolution des systèmes de reproduction qui prennent en compte l'effet du « pollen discounting » prédisent que ces effets peuvent contrebalancer certains avantages de l'autofécondation (Holsinger 1991; Lloyd 1992; Johnston 1998). En particulier, la relation entre le nombre d'ovules allo- et autofécondés, et la relation entre le nombre d'ovules autofécondés et la quantité de pollen exporté peuvent prendre des formes très variables, qui dépendent notamment du comportement des pollinisateurs et de la structure des fleurs. Un modèle décrivant plusieurs de ces relations, correspondant à des scénarios biologiques distincts, prédit l'évolution de taux d'autofécondation allant de zéro à un, en passant par des taux intermédiaires stables, et ce y compris avec de fortes valeurs de dépression de consanguinité (Johnston *et al.* 2009).

Système de reproduction et dispersion

Les différents avantages et désavantages de l'autofécondation font que l'on prédit son évolution dans certaines conditions, et le maintien de l'allofécondation dans d'autres. Un cas particulier qui a stimulé de nombreuses recherches est l'évolution de la capacité à s'autoféconder lors de la colonisation. Baker (1955) a prédit que les individus colonisant de nouveaux habitats à grande distance de leur population d'origine pourront plus facilement fonder une population s'ils ont la capacité de s'autoféconder ou de se reproduire asexuellement. Stebbins, dans son article de (1957), a insisté sur la généralité de ce phénomène et a surnommé ce filtre démographique « Baker's law ». Les discussions récentes sur la définition la plus utile de ce principe et ses cadres d'applications ont généré des échanges scientifiques stimulants sur l'évolution, la plasticité du taux d'autofécondation, et son interaction avec des processus démographiques et évolutifs (Cheptou 2012; Pannell *et al.* 2015).

Lorsqu'il disperse très loin de sa population d'origine, un individu a toutes les chances d'arriver dans un endroit où il est moins adapté, isolé de ses congénères et potentiellement sans pollinisateurs efficaces. Dans cette situation, il est intuitif que les individus *capables* de

s'autoféconder ou de se reproduire végétativement ont un avantage par rapport à des individus complètement allofécondants. De plus, si l'autofécondation peut faciliter l'adaptation au moins dans un premier temps, pour différentes raisons décrites dans la première partie de cette introduction, l'autofécondation pourrait être encore plus avantageuse dans ce contexte. Si la dépression de consanguinité n'est pas trop forte, on peut donc s'attendre à ce que les dispersions à longue distance soient associées à une transition vers la capacité de s'autoféconder, si l'espèce n'avait pas déjà cette capacité. Cette capacité à l'autofécondation peut être acquise génétiquement ou plastiquement et se décliner sous la forme de nombreux mécanismes : transition d'un système auto-incompatible vers un système auto-compatible (Pannell *et al.* 2015 pour revue), perte partielle de l'hétérostylie, modification de la structure florale, augmentation du nombre de fleurs hermaphrodites ou cléistogames (Levin 2010, et 2012 pour revue).

Le principe de Baker a également été appliqué dans le contexte des métapopulations, où la dispersion se fait souvent à moins longue distance et où des individus dispersants ne sont pas nécessairement complètement isolés. Cela a généré des prédictions distinctes sur l'évolution de l'autofécondation que le principe original. Dans ce contexte, l'autofécondation donne un plus faible avantage, surtout lorsque l'on incorpore des effets négatifs tels que la dépression de consanguinité. Si le succès de la colonisation dépend de la densité en plantes dans l'environnement colonisé et si le nombre de migrants est faible, les modèles prédisent des résultats cohérents avec le principe de Baker (Pannell and Barrett 1998; Dornier *et al.* 2008) et les données (Cheptou 2012; Pannell *et al.* 2015 pour revue). Néanmoins, d'autres modèles impliquant de l'hétérogénéité temporelle et spatiale de la pollinisation et de la dépression de consanguinité prédisent l'évolution d'une association entre allofécondation et forte capacité à la dispersion d'une part, et entre autofécondation et faible capacité de dispersion d'autre part (Cheptou and Massol 2009; Massol and Cheptou 2011). En effet, dans ces modèles, le système de reproduction et la capacité à disperser évoluent de manière à ce que les individus payent le coût de la consanguinité ou le coût de la dispersion (probabilité d'arriver dans une parcelle de mauvaise qualité), mais pas les deux à la fois.

Système de reproduction et adaptation locale

Ces modèles sur la dispersion entre environnements (de pollinisation) différents nous ramènent à l'étude originelle qui a stimulé les travaux sur le taux d'autofécondation et les plantes pseudo-métallicoles. Antonovics (1968) prédisait l'évolution de l'autofécondation dans

une population subissant une forte pression de sélection et recevant du pollen portant des allèles délétères en provenance de populations voisines, car l'autofécondation limitait l'incorporation des allèles délétères et favorisait donc l'évolution de l'adaptation locale. Un modèle plus récent s'est intéressé à la question de l'évolution de l'autofécondation et de l'adaptation locale en incluant la dépression de consanguinité et la dépression d'allofécondation, qui peut être générée lors du croisement de deux génotypes adaptés à des conditions différentes (Epinat and Lenormand 2009). L'évolution de l'autofécondation n'est prédite que quand le taux de migration est faible, la sélection forte, et le taux de recombinaison efficace faible. Ce modèle, quoique plus complexe que les précédents, n'inclut cependant pas de nombreux facteurs tels que l'avantage de l'assurance reproductive associé à l'autofécondation. Il illustre le fait que malgré de nombreuses études sur les systèmes de reproduction, nous ne comprenons pas encore bien la relation entre adaptation locale, taux d'autofécondation et dépression de consanguinité.

Ma thèse se situe dans ce contexte, à l'interface de l'étude de l'adaptation aux sols toxiques et de l'évolution des systèmes de reproduction. Je me suis intéressée à l'effet de fortes pressions de sélection hétérogène (la concentration en éléments métalliques à l'état de traces dans le sol) sur le système de reproduction d'une plante, en étudiant comment le taux d'autofécondation varie entre populations adaptées à des conditions très différentes, et comment l'adaptation à la toxicité affecte la dépression de consanguinité. J'ai pour cela utilisé la plante modèle *Noccaea caerulescens* que je vais vous présenter.

III. Portrait-robot de *Noccaea caerulescens*

Noccaea caerulescens J. Presl & C. Presl F.K. Mey est une petite Brassicaceae qui est capable de tolérer et d'hyperaccumuler des éléments métalliques traces [4], tels que le zinc, le cadmium ou le nickel. On la trouve dans toute l'Europe avec une distribution fragmentée, de l'Espagne à la Scandinavie, de l'Angleterre à la République Tchèque. Certaines régions ont été beaucoup étudiées, telles que les Cévennes (Escarré *et al.* 2000; Dubois *et al.* 2003; Jiménez-Ambriz *et al.* 2007), la Belgique et le Luxembourg (Frérot *et al.* 2003; Dechamps *et al.* 2007, 2008, 2011), le

[4] La notion de métaux lourds, quoi que bien enracinée dans la littérature sur les plantes pseudo-métallicoles, n'est pas une définition scientifiquement précise. Par tradition, j'utiliserai parfois ce terme dans ce document, comme référant au zinc, cadmium, nickel et autres métaux toxiques à fortes concentrations.

Jura Suisse (Besnard *et al.* 2009), mais des populations de toute l'Europe ont été ponctuellement étudiées (Koch *et al.* 1998; Gonneau 2014). En France, *N. caerulescens* est surtout trouvée dans les massifs montagneux (Alpes, Massif Central, Jura, Pyrénées) et pousse dans des biotopes aux conditions extrêmement variées, que ce soit en termes de pH du sol, d'altitude, de ressources minérales, ou de climat (Gonneau 2014). Une caractérisation fine de ces paramètres a conduit à la définition de quatre groupes édaphiques : calaminaire ou métallicole, vivant dans d'anciennes mines ; vivant sur sol naturellement riche en serpentine ; non métallicole, vivant sur sol non minier acide ; et vivant sur sol non minier neutrophile (Gonneau 2014). Dans la mesure où elle peut vivre sur des sols à fortes concentrations en éléments traces et sur des sols « normaux », *N. caerulescens* est considérée comme une pseudo-métallophyte.

Noccaea caerulescens fleurit de février ou mars à juin. Dans le nord de l'Europe, elle tend à être annuelle à bisanuelle dans les populations métallicoles, et bisanuelle à pérenne dans les populations non métallicoles (Dechamps *et al.* 2011). Elle est surtout annuelle et bisanuelle dans le sud de la France. Les graines du printemps et de l'été germent à l'automne après les pluies. Les plantent passent l'hiver à l'état de rosettes. Environ 30% des rosettes survivent et fleurissent au printemps suivant et 11% survivent mais ne fleurissent pas au printemps suivant, et sont donc potentiellement bisannuelles (Dubois 2005). Lorsqu'elle fleurit, elle peut produire une à plusieurs dizaines d'inflorescences avec des fleurs blanches ou légèrement rosées (**Figure 7**). Elle produit des silliques en forme de cœur. Les graines n'ont pas de structures de dispersion et tombent sur le sol. En revanche, des inflorescences sèches sont parfois retrouvées à plusieurs mètres des plantes, ce qui suggère qu'une fois sèches, elles peuvent se casser et être dispersées par le vent (Rileys 1956 et observations personnelles).

Une systématique du genre compliquée

Noccaea caerulescens a divergé des autres lignées du genre *Noccaea* il y a environ 1 million d'années, pendant le Pléistocène (Koch and German 2013). La systématique du genre *Noccaea* et la caractérisation de cette espèce en particulier sont extrêmement compliquées. Initialement, sur la base de critères morphologiques, *N. caerulescens* était incluse dans le genre *Thlaspi* L.. Ce genre regroupait beaucoup d'espèces au sein des Brassicaceae, mais était mal résolu, ce qui a conduit certains auteurs à le qualifier de « dumping site » (Al-Shehbaz 2014). Néanmoins, sur la base de critères de morphologie des graines et de marqueurs enzymatiques, le genre *Thlaspi* L. a été inclus dans le genre *Noccaea*, et *Thlaspi caerulescens* est devenue *Noccaea caerulescens*

(détails dans Koch and German 2013; et Al-Shehbaz 2014). Il est intéressant de noter que l'accumulation d'éléments traces est un trait extrêmement répandu dans le genre *Noccaea* : presque toutes les plantes accumulent le zinc, environ la moitié accumule le nickel et quelques-unes accumulent le plomb (Koch and German 2013).

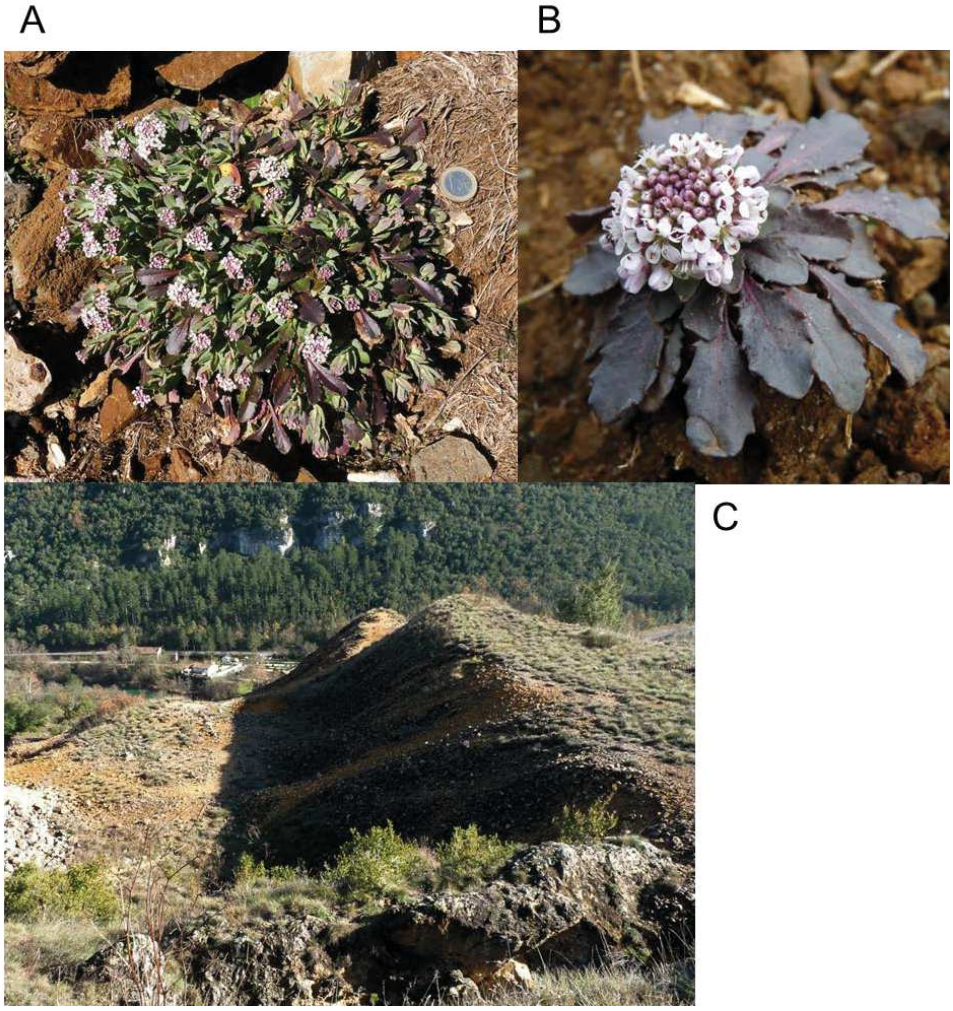

Figure 7. *Noccaea caerulescens* et l'un de ses habitats. A) et B) Deux individus de *N. caerulescens* dans la population de Saint Bresson. C) Photo d'une partie de la mine des Avinières, avec plusieurs des milieux communément trouvés dans les mines de la région : pentes peu végétalisées et talus plus herbeux. Note : on ne voit pas ici de zones plus boisées, que l'on peut également trouver dans les populations métallicoles. Photos : M. Mousset.

Noccaea caerulescens a été désignée par plusieurs noms illégitimes dans la littérature : *Thlaspi alpestre* L. (Rileys 1956 par exemple), *Thlaspi sylvestre* Jord., ou *Thlaspi virens* Jord.. *Thlaspi alpestre* en particulier est à considérer avec précaution car cette dénomination pouvait également désigner le taxon actuellement nommé *Noccaea brachypetala* Jord. F. K. Mey (Koch *et*

al. 1998; Koch and German 2013). La confusion entre *N. caerulescens* et *N. brachypetala* n'est pas complètement résolue : il existe des noms qui, dans certaines zones géographiques sont utilisés comme synonymes de *N. caerulescens* et de *N. brachypetala* (Koch and German 2013). Pourtant, les deux taxons sont distinguables grâce à un ensemble de traits morphologiques y compris dans les endroit où ils coexistent (Martos *et al.* 2016).

Enfin, *N. caerulescens* a été séparée en plusieurs sous-espèces, dont le nombre va de deux à sept en fonction des auteurs. Le traitement systématique le plus connu définissait deux sous-espèces, *ssp calaminare* et *ssp caerulescens*, qui représentaient les écotypes vivant sur sols pollués, et sur sols normaux. Néanmoins, Koch *et al.* (1998) ont montré avec des marqueurs enzymatiques que cette distinction n'est pas pertinente. Plusieurs études utilisant des marqueurs neutres confirment ce résultat et suggèrent que l'écotype métallicole a évolué plusieurs fois de manière indépendante (Dubois *et al.* 2003; Besnard *et al.* 2009; Gonneau 2014).

Histoire récente de l'espèce

Gonneau (2014) a utilisé la structure génétique à des marqueurs nucléaires et chloroplastiques pour inférer l'histoire des populations d'Europe de l'Ouest. Il montre qu'il existe deux groupes phylogéographiques différents, à l'est et à l'ouest de l'aire étudiée. La divergence génétique à des marqueurs chloroplastiques suggère que différentes populations dérivent de plusieurs refuges glaciaires établis lors du dernier maximum glaciaire (-23000 à -180000 ans), probablement en Italie et dans la péninsule ibérique ou les Pyrénées. La diversité génétique allant en décroissant du sud vers le nord est cohérente avec un scénario de recolonisation de l'Europe à partir de refuges au sud. L'auteur discute de la possibilité que les populations des Cévennes aient dérivé d'un refuge proche de l'Italie, alors que les stations du reste du Massif Central auraient dérivé d'un refuge ibérique ou pyrénéen. Les données des microsatellites suggèrent l'existence de trois groupes divergents : un au sud du Massif Central (incluant les populations des Cévennes étudiées dans cette thèse), un regroupant des populations du nord-ouest du Massif Central, des Pyrénées et des Alpes, et un troisième regroupant des populations du Jura et des Vosges. La distinction entre stations du sud du Massif Central et les autres est donc cohérente entre les deux types de marqueurs. L'analyse ABC, qui utilise des méthodes bayésiennes pour inférer l'histoire démographique des différentes populations indique que les populations du sud du Massif Central ont divergé en premier et que les deux autres unités ont divergé plus récemment, ce qui est également cohérent avec

l'hypothèse de deux re-colonisations indépendantes. Il est à noter que dans ces analyses, c'est la géographie et non pas le type de sol qui structure la diversité génétique, ce qui confirme que la distinction des deux sous-espèces n'est pas soutenue génétiquement, d'où le terme actuellement préféré d' « écotype ».

À une échelle plus régionale, Besnard *et al.* (2009) ont également trouvé plusieurs groupes dans le Jura Suisse et les Alpes dont la distribution est similaire à d'autres espèces de cette aire géographique. Cela a conduit les auteurs à suggérer que ces groupes sont issus de différents refuges glaciaires ou de différentes voies de recolonisation à partir d'un même refuge. Dans leur étude, la diversité neutre n'était pas expliquée par la concentration en métal. Par contre, la différenciation entres paires de populations pour des gènes potentiellement impliqués dans la tolérance ou l'accumulation des métaux était corrélée aux différences de concentration en métaux des sols. Cela suggère un plus faible flux de gènes entre populations métallicoles et non métallicoles à ces locus.

Caryotype de *Noccaea caerulescens*

Noccaea caerulescens est une plante diploïde avec 2n = 14 chromosomes (Rileys 1956; Koch *et al.* 1998). La taille de son génome est à peu près 267 Mb (Mandáková *et al.* 2015). Le caryotype de *N. caerulescens* a subi un certain nombre d'inversions et remaniements par rapport au caryotype ancestral (Mandáková *et al.* 2015). Un certain nombre de ces remaniements sont partagés avec deux espèces proches accumulatrices d'éléments traces (quoiqu'à de plus faibles concentrations), mais des remaniements importants sont spécifiques à *N. caerulescens*. Ces remaniements sont partagés par toutes les populations testées, venant de toute l'aire de répartition, et indépendamment du type de sol. Ces inversions auraient conduit à des différences d'expression de gènes et à la création de clusters de gènes impliqués dans l'homéostasie des métaux. Si ces réarrangements ont joué un rôle dans l'évolution de la tolérance et de l'hyperaccumulation, on peut émettre l'hypothèse que l'absence de variation intra-spécifique du caryotype chez *N. caerulescens* pourrait en partie expliquer la tolérance constitutive de cette espèce aux métaux lourds ; les variations entre écotypes (et même populations) seraient alors dues à des différences d'expression plus « locales », telles que des différences de séquences dans les promoteurs des gènes ou des duplications (Ó Lochlainn *et al.* 2011; Craciun *et al.* 2012).

Un système de reproduction mixte

Noccaea caerulescens est hermaphrodite, auto-compatible et protogyne : le pistil croît hors du bouton floral et est mature avant que les anthères ne s'ouvrent (Rileys 1956 et observations personnelles) (**Figure 8**). Cette caractéristique est couramment interprétée comme favorisant l'allogamie. Sur la base du coefficient de consanguinité (F_{IS}), *N. caerulescens* a un système de reproduction mixte dans une grande partie de son aire de répartition : dans le Sud de la France (Dubois *et al.* 2003; Jiménez-Ambriz *et al.* 2007), en Belgique et dans le Luxembourg (Dubois *et al.* 2003), en Suisse (Besnard *et al.* 2009) et dans de nombreuses populations étudiées ponctuellement (Koch *et al.* 1998; Gonneau 2014). Dans les populations du Sud de la France, les populations non métallicoles ont des F_{IS} plus hauts et des ratios pollen/ovule plus faibles que les populations métallicoles, ce qui suggère un système plus consanguin. Ces résultats sont basés sur un faible nombre de marqueurs moléculaires (marqueurs enzymatiques et microsatellites), ce qui les rend imprécis. De plus, la méthode d'estimation du taux d'autofécondation par les F_{is} est sujette à un certain nombre de biais. S'il est indubitable que cette espèce a un système de reproduction mixte, les connaissances sur les taux d'autofécondation, leur variation entre populations et entre écotypes sont imprécises.

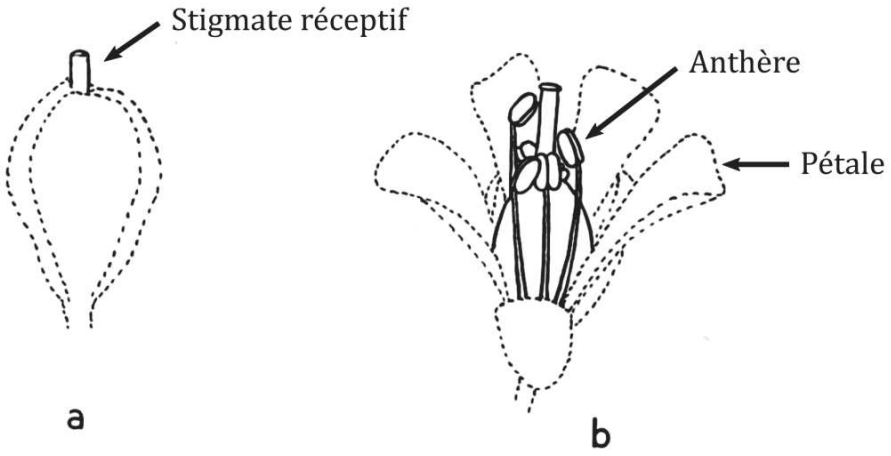

Figure 8. Bouton floral et ouverture de la fleur de *Noccaea caerulescens*. A) Bouton floral, avec le stigmate réceptif dépassant des pétales. B) Le bouton floral s'ouvre, le stigmate est encore réceptif et les anthères vont s'ouvrir sous peu. Figure adaptée de Rileys (1956).

Bases physiologiques de la tolérance et de l'hyperaccumulation

Cette thèse n'ayant pas pour objet la tolérance physiologique, mais plutôt l'effet des éléments traces sur la valeur sélective des plantes, je ne vais que brièvement expliquer les grands mécanismes de la tolérance et de l'hyperaccumulation (pour revue Assunção, Schat, *et al.* 2003; Verbruggen *et al.* 2009; Hanikenne and Nouet 2011). D'un point de vue physiologique, la tolérance aux éléments traces et leur accumulation [5] sont dépendantes de plusieurs mécanismes : entrée des ions métalliques dans les racines, import dans les cellules, stockage dans les cellules racinaires, translocation via les vaisseaux du xylème vers les parties aériennes, entrée dans les cellules des parties aériennes, chélation ou séquestration dans les vacuoles. Les molécules impliquées dans ces mécanismes peuvent être communes, ou varier pour les différents ions métalliques.

Une large variété de techniques utilisées

Il existe de nombreux moyens d'étudier les mécanismes physiologiques de la tolérance et de l'hyperaccumulation. Parmi ceux mis en œuvre chez *N. caerulescens*, on peut citer :

- des analyses des concentrations en métaux dans différentes parties de la plante (Gonneau *et al.* 2014), de cinétique de l'entrée des ions dans les racines, de suivi du flux des ions métalliques, spectroscopie aux rayons X (voir Milner and Kochian 2008 pour plus d'exemples)

- des croisements intra spécifiques avec étude de la ségrégation de la tolérance et de l'hyperaccumulation (par exemple, Frérot *et al.* 2003, 2005).

- des analyses de QTL (« quantitative trait loci analysis »), utilisant des croisements entre plantes de différentes populations qui varient dans leur capacité à tolérer et accumuler différents métaux (par exemple, les accessions de « Ganges » et « La Calamine », Deniau (2006), ou « Lellingen » et « La Calamine », Assunçao (2003)). Les analyses QTL, de par leur puissance limitée, permettent en général de localiser quelques gènes candidats à fort effet.

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[5] L'hyperaccumulation repose sur le dépassement de concentrations seuil dans les tissus aériens de la plante. Lesdites concentrations seuil dépendent des éléments traces considérés, sont décidées arbitrairement et sont parfois sujettes à discussion. De manière générale, une plante hyperaccumulatrice est capable d'accumuler de très grandes quantités d'un élément trace dans ses tissus foliaires, avec une translocation très efficace des racines aux feuilles et en général un rapport de concentrations des feuilles sur sol supérieur à un. Les détails de cette discussion n'étant pas pertinents pour la compréhension de cette thèse, je ne m'apesantirai pas plus sur le sujet.

- des études fonctionnelles de gènes candidats (Craciun *et al.* 2012; Iqbal *et al.* 2013) : clonage dans des levures ou chez *Arabidopsis thaliana* (L.) Heynh, étude et manipulation de l'expression des gènes dans diverses conditions etc.

- des analyses du transcriptome. A l'opposé, mais complémentaires des analyses fonctionnelles, les analyses comparant l'expression du génome entre deux espèces ou deux accessions donnent des informations générales sur les gènes exprimés dans chacune d'elles. Ces techniques apportent énormément d'informations sur les processus cellulaires différant entre individus d'accessions ou d'espèces différentes. Hammond *et al.* (2006) ont comparé l'expression des gènes de *Noccaea caerulescens* et *Thlaspi arvense*. Rigola *et al.* (2006) ont comparé l'expression de *A. thaliana* et *N. caerulescens* en utilisant une bibliothèque d'EST. Halimaa *et al.* (2014) ont étudié l'expression des gènes dans les racines de différentes accessions de *N. caerulescens* qui varient dans leurs capacités à hyperaccumuler le zinc, le cadmium et le nickel. Ils ont montré que le facteur qui permet le mieux de distinguer ces accessions est l'expression des gènes impliqués dans la gestion des métaux. Ces études ont permis de comparer l'expression de nombreux gènes connus pour être potentiellement impliqués dans la tolérance et l'accumulation des métaux, mais aussi de suggérer de nouveaux gènes candidats prometteurs.

De la solution du sol à la solution des vacuoles, périple d'un atome de métal

La tolérance et l'hyperaccumulation dépendent de nombreuses familles de molécules. Des métaux différents peuvent interagir avec des voix cellulaires différentes, mais certaines d'entre elles ont une affinité forte pour plusieurs métaux. Malgré des similitudes entre espèces (*N. caerulescens* et *A. halleri* par exemple) indiquant que les grandes fonctions sont conservées, il existe des différences y compris entre accessions de *N. caerulescens* (Iqbal *et al.* 2013, par exemple), et les détails des différents systèmes ne sont pas encore maîtrisés.

Noccaea caerulescens se caractérise par une faible accumulation des métaux dans les racines et une très importante translocation vers les parties aériennes (**Figure 9**). Les différents ions métalliques rentrent dans le système racinaire via différentes familles de transporteurs. Ils peuvent ensuite être stockés dans la vacuole des cellules racinaires grâce à des transporteurs transmembranaires de différentes familles. Une fois dans la vacuole, les ions peuvent former des complexes avec le malate ou d'autres acides organiques. Ils peuvent également être chélatés dans le cytoplasme, avec de l'histidine par exemple (notamment pour le nickel), ou avec des metallothionéines, du glutathion et des phytochélatines (pour le cadmium). Les nicotianamines

sont également impliquées dans la chélation du cadmium et du zinc. Chez *N. caerulescens*, toutefois, la majorité des ions métalliques sont activement pompés dans les vaisseaux du xylème, qui les transportent jusqu'aux parties aériennes de la plante. Les ions métalliques ou les complexes entre des ions et d'autres molécules sortent des cellules grâce à des transporteurs transmembranaires. Dans les parties aériennes, ces mêmes molécules, plus des transporteurs de la famille des ZIP sont responsables du déchargement des ions métalliques depuis le xylème vers les cellules. Chez *N. caerulescens,* l'accumulation et la détoxification se font surtout dans les cellules épidermiques, soit par séquestration dans la vacuole, soit par chélation dans la cellule à un certain nombre de ligands (**Figure 9**).

Tolérance aux éléments traces

Il faut tout d'abord noter que le type de test (croissance hydroponique ou sur sol contaminé manuellement ou importé des mines), les concentrations et mélanges de métaux, et les méthodes d'extraction des métaux peuvent influencer le résultat des tests de tolérance et d'accumulation (Escarré *et al.* 2013; Gonneau *et al.* 2014). Néanmoins, de grandes lignes ressortent.

Les populations de *N. caerulescens* varient dans leur capacité à accumuler et tolérer différents métaux. La tolérance à de fortes concentrations de zinc est retrouvée dans toutes les populations de *N. caerulescens* (Baker *et al.* 1994; Meerts and Van Isacker 1997). Les populations métallicoles ont néanmoins une plus grande tolérance que les populations issues de sites non métallicoles (Meerts and Van Isacker 1997; Escarré *et al.* 2000; Assuncao *et al.* 2003; Frérot *et al.* 2005). La variation de tolérance entre familles à l'intérieur des populations semble plus forte chez les populations non métallicoles (Meerts and Van Isacker 1997). Ceci serait cohérent avec une forte sélection pour la tolérance dans les populations métallicoles, qui diminuerait la variation aux locus responsables de la tolérance. La tolérance au cadmium est moins répandue, et les populations venant de sites à serpentine semblent être plus sensibles que les autres à cet élément (Roosens *et al.* 2003; Assuncao *et al.* 2003). Les populations métallicoles de *N. caerulescens* ont également une tolérance au plomb (Baker *et al.* 1994; Meerts and Van Isacker 1997) et au nickel (Baker *et al.* 1994).

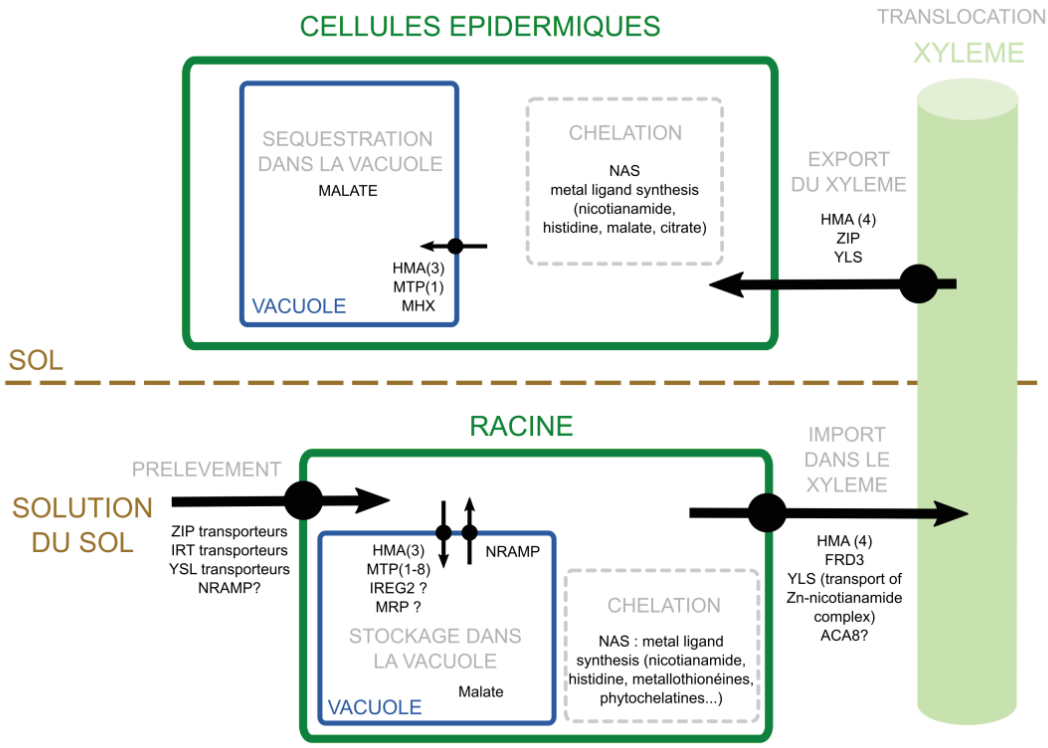

Figure 9. Mécanismes de l'homéostasie des éléments traces et principales molécules et familles de molécules impliquées. Schéma inspiré de Verbruggen *et al.* (2009).

Des transplantations en jardin commun et en populations naturelles ont été effectuées pour étudier l'adaptation des plantes des écotypes métallicoles et non métallicoles à la pollution du sol, avec des populations du sud de la France et du nord de l'Europe.

Dechamps *et al.* (2007) ont utilisé deux populations de chaque écotype provenant du nord de l'Europe et trois niveaux de toxicité artificielle du sol (0 $mg.kg^{-1}$, 1000 $mg.kg^{-1}$, 8000 $mg.kg^{-1}$ de zinc). Les populations métallicoles étaient peu affectées par la toxicité. Leur cycle de vie différait en fonction du niveau de toxicité, mais au bout de deux ans, la production de graines était équivalente dans tous les traitements, ce qui démontrait que ces plantes utilisaient différentes stratégies pour maintenir l'homéostasie. Ce résultat suggère que les populations métallicoles sont exposées à différentes concentrations en populations naturelles et que le coût associé à la tolérance est faible (dans les conditions expérimentales en tout cas). A l'opposé, les populations non métallicoles souffraient en général de la toxicité. La survie des plantes et les traits liés à la reproduction étaient globalement affectés par la toxicité, mais la croissance était moins affectée.

Jiménez-Ambriz *et al.* (2007) ont effectué une expérience très similaire mais avec des populations du sud de la France et seulement deux niveaux de toxicité (0 $mg.kg^{-1}$, 1000 $mg.kg^{-1}$ de zinc). À part pour le nombre d'inflorescences, les populations métallicoles souffraient peu de la toxicité, ce qui est cohérent avec les résultats précédents. Chez les populations non métallicoles, quasiment tous les traits étaient négativement affectés par la toxicité, ce qui confirme également les résultats de Dechamps *et al.* (2007). En outre, il n'y avait pas de différence de réponse à la toxicité entre populations d'un même écotype. Il existait, pour de nombreux traits, de la variation entre familles, mais il n'y avait quasiment pas d'interaction entre famille et toxicité. Les auteurs ont également effectué des mesures en populations naturelles ; généralement, les seules différences morphologiques entre populations métallicoles et non métallicoles portaient sur la taille des graines et sur le nombre d'inflorescences.

Dechamps *et al.* (2008) ont transplanté des plantes d'écotype métallicole sur sol non minier en populations naturelles et des plantes de l'écotype non métallicole sur des sites miniers. Sur sol métallifère, les plantes métallicoles avaient toujours une meilleure performance que les plantes non métallicoles, ce qui confirme les résultats obtenus en conditions contrôlées et met en évidence l'adaptation locale des populations métallicoles à leur environnement. En environnement non métallicole, les plantes métallicoles survivaient aussi bien que les non métallicoles. Une interaction entre la population et le site de test était clairement visible, partiellement provoquée par des attaques d'herbivores différentes entre sites, affectant différemment différentes populations, et en particulier les populations métallicoles.

Hyperaccumulation d'éléments traces

Noccaea caerulescens semble hyperaccumuler le zinc dans toutes les populations, qu'elles proviennent de sols métallifères ou non (par exemple, Reeves *et al.* 2001; Assuncao *et al.* 2003; Molitor *et al.* 2005; Gonneau *et al.* 2014). Néanmoins, les populations non métallicoles accumulent plus de zinc que les populations métallicoles (Meerts and Van Isacker 1997; Escarré *et al.* 2000, 2013; Frérot *et al.* 2003; Roosens *et al.* 2003; Assuncao *et al.* 2003; Gonneau *et al.* 2014). La capacité à accumuler le zinc peut varier entre populations de *N. caerulescens* (Pollard and Baker 1996; Escarré *et al.* 2000; Lombi *et al.* 2000) et entre familles à l'intérieur des populations (Pollard and Baker 1996; Meerts and Van Isacker 1997; Escarré *et al.* 2000; Molitor *et al.* 2005). Gonneau *et al.* (2014) ont cependant trouvé peu de variation dans les populations

métallicoles qui tendaient à répondre de la même manière à la toxicité. *Noccaea caerulescens* accumule également le cadmium dans un certain nombre des populations métallicoles et non métallicoles en France et au Luxembourg (Escarré *et al.* 2000; Lombi *et al.* 2000; Molitor *et al.* 2005). Il semble toutefois qu'à l'inverse du zinc, certaines populations métallicoles accumulent de plus larges concentrations que les populations non métallicoles (Escarré *et al.* 2000; Roosens *et al.* 2003; Gonneau *et al.* 2014) mais pas toutes (Escarré *et al.* 2013). Les populations vivant sur sol serpentinique sont capables d'accumuler le nickel (Reeves *et al.* 2001; Assuncao *et al.* 2003; Escarré *et al.* 2013). En condition contrôlées, des populations non métallicoles accumulent davantage ce métal que les populations métallicoles (Gonneau *et al.* 2014). Certaines populations sont capables d'accumuler le plomb (Baker *et al.* 1994; Meerts and Van Isacker 1997; Gonneau 2014) et, comme pour le zinc, les populations non métallicoles en accumulent davantage que les populations métallicoles (Meerts and Van Isacker 1997).

Plan de la thèse

Le but proximal de cette thèse était de caractériser différents aspects du système de reproduction de *Noccaea caerulescens* et d'étudier comment ce dernier varie en fonction de l'histoire d'adaptation aux conditions minières. Ultimement, je souhaitais mieux comprendre un des facteurs qui a pu contraindre comme favoriser l'adaptation des populations métallicoles à leurs conditions de vie particulières.

Chapitre 1

J'ai tout d'abord étudié de manière fine le système de reproduction de *N. caerulescens* dans dix populations des Cévennes. Cette étude avait deux grands buts. Le premier était de caractériser précisément les taux d'autofécondation en utilisant des méthodes plus adéquates que celles utilisés précédemment, car ces données sont la base de la discussion de toute ma thèse. Le second but était de tester si le patron d'adaptation à la toxicité du sol expliquait d'une manière ou d'une autre la variation du système de reproduction. Pour cela, j'ai caractérisé les taux d'autofécondation de chaque population et les flux de gènes entre les populations pendant deux saisons reproductives. J'ai comparé les taux d'autofécondation des populations ayant des histoires sélectives différentes : population vivant dans d'anciennes mines et populations vivant sur des sols calcaires typiques de la région. Cette étude a fait l'objet d'une publication dans *Annals of Botany*.

Chapitre 2

Le but de ce chapitre était de mieux comprendre la variation du taux d'autofécondation observée entre écotypes, compte tenu du fait que l'hypothèse de la nécessité d'un isolement reproducteur des populations métallicoles n'est pas fondée. J'ai testé un facteur qui était depuis longtemps suspecté d'interférer avec la reproduction : la densité de plantes en fleur. Outre son effet sur la pollinisation d'autres espèces, la densité est globalement plus forte dans les populations métallicoles que dans les populations non métallicoles de *N. caerulescens*. Pour tester l'effet de ce facteur, j'ai cherché à réduire la variation environnementale et donc étudié l'effet de la densité à l'intérieur de deux populations métallicoles. J'ai utilisé deux types de méthodes pour estimer le taux d'autofécondation, à deux échelles légèrement différentes.

Chapitre 3

Le premier but de ce chapitre était de caractériser la dépression de consanguinité chez *N. caerulescens*, car c'est un paramètre important du système de reproduction. Le second but était de tester des attendus théoriques concernant l'interaction entre la dépression de consanguinité et l'environnement où elle est testée. J'ai mené une expérience en conditions contrôlées pour découvrir si la dépression de consanguinité augmente dans un environnement stressant, si elle dépend de l'écotype, et s'il y a une interaction entre l'écotype (qui représente l'histoire d'adaptation), le stress appliqué et la dépression de consanguinité.

Annexe

En annexe, on pourra trouver une publication décrivant les marqueurs microsatellites développés chez *N. caerulescens* à l'ISEM et au LEEP à Lille, marqueurs dont une partie a été utilisée au cours des trois chapitres de cette thèse.

Bibliographie

Agrawal A, Whitlock M. 2010. Environmental duress and epistasis: how does stress affect the strength of selection on new mutations? *Trends in Ecology & Evolution* **25**: 450–458.

Aguilée R, Shaw F, Rousset F, Shaw R, Ronce O. 2013. How does pollen versus seed dispersal affect niche evolution? *Evolution; International Journal of Organic Evolution* **67**: 792–805.

Aizen M, Harder L. 2007. Expanding the limits of the pollen-limitation concept: effects of pollen quantity and quality. *Ecology* **88**: 271–281.

Al-Shehbaz I. 2014. A synopsis of the genus *Noccaea* (coluteocarpeae, Brassicaceae). *Harvard Papers in Botany* **19**: 25–51.

Angeloni F, Ouborg N, Leimu R. 2011. Meta-analysis on the association of population size and life history with inbreeding depression in plants. *Biological Conservation* **144**: 35–43.

Antonovics J. 1968. Evolution in closely adjacent plant populations V. Evolution of self-fertility. *Heredity* **23**: 219–238.

Antonovics J. 2006. Evolution in closely adjacent plant populations X: long-term persistence of prereproductive isolation at a mine boundary. *Heredity* **97**: 33–37.

Armbruster P, Reed D. 2005. Inbreeding depression in benign and stressful environments. *Heredity* **95**: 235–242.

Ashman T-L, Knight T, Steets J, Amarasekare P, Burd M, Campbell D, Dudash M, Johnston M, Mazer S, Mitchell R, Morgan M, Wilson W. 2004. Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology* **85**: 2408–2421.

Assunção A, Bookum T, Nelissen H, Vooijs R, Schat H, Ernst W. 2003. A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **159**: 383–390.

Assuncao A, Bookum W, Nelissen H, Vooijs R, Schat H, Ernst W. 2003. Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159**: 411–419.

Assunção A, Schat H, Aarts M. 2003. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159**: 351–360.

Baker H. 1955. Self-compatibility and establishment after "long-distance" dispersal. *Evolution* **9**: 347–349.

Baker A, Reeves R, Hajar A. 1994. Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. & C. Presl (Brassicaceae). *New Phytologist* **127**: 61–68.

Barrett S. 2003. Mating strategies in flowering plants: the outcrossing–selfing paradigm and beyond. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **358**.

Bataillon T, Kirkpatrick M. 2000. Inbreeding depression due to mildly deleterious mutations in finite populations: size does matter. *Genetics Research* **75**: 75–81.

Besnard G, Basic N, Christin P-A, Savova-Bianchi D, Galland N. 2009. *Thlaspi caerulescens* (Brassicaceae) population genetics in western Switzerland: is the genetic structure affected by natural variation of soil heavy metal concentrations? *New Phytologist* **181**: 974–984.

Burgarella C, Gayral P, Ballenghien M, Bernard A, David P, Jarne P, Correa A, Hurtrez-Boussès S, Escobar J, Galtier N, Glémin S. 2015. Molecular evolution of freshwater snails with contrasting mating systems. *Molecular Biology and Evolution* **32**: 2403–2416.

Byers D, Waller D. **1999**. Do plant populations purge their genetic load? Effects of population size and mating history on inbreeding depression. *Annual Review of Ecology and Systematics* **30**: 479–513.

Caisse M, Antonovics J. **1978**. Evolution in closely adjacent plant populations IX. Evolution of reproductive isolation in clinal popuations. *Heredity* **40**: 371–384.

Charlesworth D. **2006**. Evolution of plant breeding systems. *Current Biology* **16**: 726–735.

Charlesworth D, Charlesworth B. **1979**. The evolutionary genetics of sexual systems in flowering plants. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **205**: 513–530.

Charlesworth D, Willis J. **2009**. The genetics of inbreeding depression. *Nature Reviews Genetics* **10**: 783–796.

Cheptou P-O. **2012**. Clarifying Baker's Law. *Annals of Botany* **109**: 633–641.

Cheptou P-O, Dieckmann U. **2002**. The evolution of self-fertilization in density-regulated populations. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **269**: 1177 –1186.

Cheptou P-O, Massol F. **2009**. Pollination fluctuations drive evolutionary syndromes linking dispersal and mating system. *The American Naturalist* **174**: 46–55.

Cheptou P-O, Mathias A. **2001**. Can varying inbreeding depression select for intermediary selfing rates? *American Naturalist* **157**: 361–373.

Cheverud J, Vaughn T, Pletscher S, King-Ellison K, Bailiff J, Adams E, Erickson C, Bonislawski A. **1999**. Epistasis and the evolution of additive genetic variance in populations that pass through a bottleneck. *Evolution* **53**: 1009–1018.

Craciun A, Meyer C-L, Chen J, Roosens N, Groodt R, Hilson P, Verbruggen N. **2012**. Variation in HMA4 gene copy number and expression among *Noccaea caerulescens* populations presenting different levels of Cd tolerance and accumulation. *Journal of Experimental Botany* **63**: 4179–4189.

Crnokrak P, Barrett S. **2002**. Perspective: purging the genetic load: a review of the experimental evidence. *Evolution* **56**: 2347–2358.

Crnokrak P, Roff R. **1999**. Inbreeding depression in the wild. *Heredity* **83**: 260–270.

Dechamps C, Elvinger N, Meerts P, Lefebvre C, Escarré J, Colling G, Noret N. **2011**. Life history traits of the pseudometallophyte *Thlaspi caerulescens* in natural populations from Northern Europe. *Plant Biology* **13**: 125–135.

Dechamps C, Lefèbvre C, Noret N, Meerts P. **2007**. Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. *New Phytologist* **173**: 191–198.

Dechamps C, Noret N, Mozek R, Escarré J, Lefèbvre C, Gruber W, Meerts P. **2008**. Cost of adaptation to a metalliferous environment for *Thlaspi caerulescens*: a field reciprocal transplantation approach. *New Phytologist* **177**: 167–177.

Deniau A, Pieper B, Bookum W, Lindhout P, Aarts M, Schat H. **2006**. QTL analysis of cadmium and zinc accumulation in the heavy metal hyperaccumulator *Thlaspi caerulescens*. *Theoretical and Applied Genetics* **113**: 907–920.

Dornier A, Munoz F, Cheptou P-O. **2008**. Allee effect and self-fertilization in hermaphrodites: reproductive assurance in a structured metapopulation. *Evolution* **62**: 2558–2569.

Dubois S. 2005. *Etude d'un réseau de populations métallicoles et non-métallicoles de* Thlaspi caerulescens *(Brassicaceae). Structure génétique, démographie et pressions de sélection.* Université Montpellier II Sciences et Techniques du Languedoc, Montpellier, France.

Dubois S, Cheptou P-O, Petit C, Meerts P, Poncelet M, Vekemans X, Lefèbvre C, Escarré J. **2003**. Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* **157**: 633–641.

Epinat G, Lenormand T. **2009**. The evolution of assortative mating and selfing with in- and outbreeding depression. *Evolution* **63**: 2047–2060.

Escarré J, Lefèbvre C, Frérot H, Mahieu S, Noret N. **2013**. Metal concentration and metal mass of metallicolous, non metallicolous and serpentine *Noccaea caerulescens* populations, cultivated in different growth media. *Plant and Soil* **370**: 197–221.

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière Y, Delay B. **2000**. Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. *New Phytologist* **145**: 429–437.

Fisher R. **1941**. Average excess and average effect of a gene substitution. *Annals of Human Genetics* **11**: 53–63.

Fox C, Reed D. **2011**. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution* **65**: 246–258.

Fox C, Scheibly K, Reed D. **2008**. Experimental evolution of the genetic load and its implications for the genetic basis of inbreeding depression. *Evolution* **62**: 2236–2249.

Frérot H, Lefèbvre C, Petit C, Collin C, Dos Santos A, Escarré J. **2005**. Zinc tolerance and hyperaccumulation in F1 and F2 offspring from intra and interecotype crosses of *Thlaspi caerulescens*. *New Phytologist* **165**: 111–119.

Frérot H, Petit C, Lefèbvre C, Gruber W, Collin C, Escarré J. **2003**. Zinc and cadmium accumulation in controlled crosses between metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* (Brassicaceae). *New Phytologist* **157**: 643–648.

Glémin S, Muyle A. **2014**. Mating systems and selection efficacy: a test using chloroplastic sequence data in Angiosperms. *Journal of Evolutionary Biology* **27**: 1386–1399.

Glémin S, Ronfort J. **2013**. Adaptation and maladaptation in selfing and outcrossing species: new mutations versus standing variation. *Evolution* **67**: 225–240.

Goldberg E, Kohn J, Lande R, Robertson K, Smith S, Igić B. **2010**. Species selection maintains self-incompatibility. *Science* **330**: 493–495.

Gonneau C. 2014. *Distribution, écologie et évolution de l'hyperaccumulation des éléments en traces par Noccaea caerulescens.* Université de Lorraine, Nancy, France

Gonneau C, Genevois N, Frérot H, Sirguey C, Sterckeman T. **2014**. Variation of trace metal accumulation, major nutrient uptake and growth parameters and their correlations in 22 populations of *Noccaea caerulescens*. *Plant and Soil* **384**: 271–287.

Goodwillie C, Kalisz S, Eckert C. **2005**. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution, and Systematics* **36**: 47–79.

Halimaa P, Lin Y-F, Ahonen V, Blande D, Clemens S, Gyenesei A, Häikiö E, Kärenlampi S, Laiho A, Aarts M, Pursiheimo J-P, Schat H, Schmidt H, Tuomainen M, Tervahauta A. **2014**. Gene expression differences between *Noccaea caerulescens* ecotypes help to identify candidate genes for metal phytoremediation. *Environmental Science & Technology* **48**: 3344–3353.

Hammond J, Bowen H, White P, Mills V, Pyke K, Baker A, Whiting S, May S, Broadley M. **2006**. A comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* shoot transcriptomes. *New Phytologist* **170**: 239–260.

Hanikenne M, Nouet C. **2011**. Metal hyperaccumulation and hypertolerance: a model for plant evolutionary genomics. *Current Opinion in Plant Biology* **14**: 252–259.

Hartfield M. **2016**. Evolutionary genetic consequences of facultative sex and outcrossing. *Journal of Evolutionary Biology* **29**: 5–22.

Hartfield M, Glémin S. **2014**. Hitchhiking of deleterious alleles and the cost of adaptation in partially selfing species. *Genetics* **196**: 281–293.

Hereford J. **2010**. Does selfing or outcrossing promote local adaptation? *American Journal of Botany* **97**: 298–302.

Holsinger K. **1988**. Inbreeding depression doesn't matter: the genetic basis of mating-system evolution. *Evolution* **42**: 1235–1244.

Holsinger K. **1991**. Mass-action models of plant mating systems: the evolutionary stability of mixed mating systems. *The American Naturalist* **138**: 606–622.

Husband B, Schemske D. **1996**. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* **50**: 54–70.

Igic B, Busch J. **2013**. Is self-fertilization an evolutionary dead end? *New Phytologist* **198**: 386–397.

Iqbal M, Nawaz I, Hassan Z, Hakvoort H, Bliek M, Aarts M, Schat H. **2013**. Expression of HMA4 cDNAs of the zinc hyperaccumulator *Noccaea caerulescens* from endogenous NcHMA4 promoters does not complement the zinc-deficiency phenotype of the *Arabidopsis thaliana* hma2hma4 double mutant. *Frontiers in Plant Science* **4**.

Jain S. **1976**. The evolution of inbreeding in plants. *Annual Review of Ecology and Systematics* **7**: 469–495.

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O. **2007**. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**: 199–215.

Johnston M. **1998**. Evolution of intermediate selfing rates in plants: pollination ecology versus deleterious mutations. *Genetica* **102**: 267–278.

Johnston M, Porcher E, Cheptou P-O, Eckert C, Elle E, Geber M, Kalisz S, Kelly J, Moeller D, Vallejo-Marín M, Winn A. **2009**. Correlations among fertility components can maintain mixed mating in plants. *The American Naturalist* **173**: 1–11.

Keller L, Waller D. **2002**. Inbreeding effects in wild populations. *Trends in Ecology & Evolution* **17**: 230–241.

Kimura M, Maruyama T, Crow J. **1963**. The mutation load in small populations. *Genetics* **48**: 1303–1312.

Knight T, Steets J, Vamosi J, Mazer S, Burd M, Campbell D, Dudash M, Johnston M, Mitchell R, Ashman T-L. **2005**. Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology, Evolution, and Systematics* **36**: 467–497.

Koch M, German D. **2013**. Taxonomy and systematics are key to biological information: *Arabidopsis*, *Eutrema* (Thellungiella), *Noccaea* and *Schrenkiella* (Brassicaceae) as examples. *Frontiers in Plant Science* **4**.

Koch M, Mummenhoff K, Hurka H. **1998**. Systematics and evolutionary history of heavy metal tolerant *Thlaspi caerulescens* in Western Europe: evidence from genetic studies based on isozyme analysis. *Biochemical Systematics and Ecology* **26**: 823–838.

Lande R. **1977**. On comparing coefficients of variation. *Systematic Biology* **26**: 214–217.

Lande R, Porcher E. **2015**. Maintenance of quantitative genetic variance under partial self-fertilization, with implications for evolution of selfing. *Genetics* **200**: 891–906.

Lande R, Schemske D. **1985**. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* **39**: 24–40.

Leimu R, Fischer M. **2008**. A meta-analysis of local adaptation in plants. *PLoS One* **3**: e4010.

Lenormand T. **2002**. Gene flow and the limits to natural selection. *Trends in Ecology & Evolution* **17**: 183–189.

Levin D. 2010. Environment-enhanced self-fertilization: implications for niche shifts in adjacent populations. *Journal of Ecology* **98**: 1276–1283.

Levin D. 2012. Mating system shifts on the trailing edge. *Annals of Botany* **109**: 613–620.

Li Q-J, Xu Z-F, Kress J, Xia Y-M, Zhang L, Deng X-B, Gao J-Y, Bai Z-L. 2001. Pollination: flexible style that encourages outcrossing. *Nature* **410**: 432–432.

Lloyd D. 1979. Some reproductive factors affecting the selection of self-fertilization in plants. *The American Naturalist* **113**: 67–79.

Lloyd D. 1992. Self- and cross-fertilization in plants. II. The selection of self- fertilization. *International Journal of Plant Sciences* **153**: 370–380.

Lohr J, Haag C. 2015. Genetic load, inbreeding depression, and hybrid vigor covary with population size: an empirical evaluation of theoretical predictions. *Evolution* **69**: 3109–3122.

Lombi E, Zhao F, Dunham S, McGrath S. 2000. Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. *New Phytologist* **145**: 11–20.

Long T, Rowe L, Agrawal A. 2013. The effects of selective history and environmental heterogeneity on inbreeding depression in experimental populations of *Drosophila melanogaster*. *The American naturalist* **181**: 532–544.

Lopez S, Rousset F, Shaw F, Shaw R, Ronce O. 2008. Migration load in plants: role of pollen and seed dispersal in heterogeneous landscapes. *Journal of Evolutionary Biology* **21**: 294–309.

Mandáková T, Singh V, Krämer U, Lysak M. 2015. Genome structure of the heavy metal hyperaccumulator *Noccaea caerulescens* and its stability on metalliferous and nonmetalliferous soils. *Plant Physiology* **169**: 674–689.

Martos S, Gallego B, Sáez L, López-Alvarado J, Cabot C, Poschenrieder C. 2016. Characterization of Zinc and Cadmium hyperaccumulation in three *Noccaea* (Brassicaceae) populations from non-metalliferous sites in the Eastern Pyrenees. *Frontiers in Plant Science* **7**.

Massol F, Cheptou P-O. 2011. Evolutionary syndromes linking dispersal and mating system: the effect of autocorrelation in population conditions. *Evolution* **65**: 591–598.

McNeilly T, Antonovics J. 1968. Evolution in closely adjacent plant populations IV. Barriers to gene flow. *Heredity* **23**: 205–218.

Meerts P, Van Isacker N. 1997. Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133**: 221–231.

Milner M, Kochian L. 2008. Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Annals of Botany* **102**: 3–13.

Molitor M, Dechamps C, Gruber W, Meerts P. 2005. *Thlaspi caerulescens* on nonmetalliferous soil in Luxembourg: ecological niche and genetic variation in mineral element composition. *New Phytologist* **165**: 503–512.

Nordborg M. 2000. Linkage disequilibrium, gene trees and selfing: an ancestral recombination graph with partial self-fertilization. *Genetics* **154**: 923–929.

Ó Lochlainn S, Bowen H, Fray R, Hammond J, King G, White P, Graham N, Broadley M. 2011. Tandem quadruplication of HMA4 in the Zinc (Zn) and Cadmium (Cd) hyperaccumulator *Noccaea caerulescens*. *PLoS ONE* **6**: e17814.

Pannell J, Auld J, Brandvain Y, Burd M, Busch J, Cheptou P-O, Conner J, Goldberg E, Grant A-G, Grossenbacher D, Hovick SM, Igic B, Kalisz S, Petanidou T, Randle AM, de Casas RR, Pauw A, Vamosi JC, Winn AA. 2015. The scope of Baker's law. *New Phytologist* **208**: 656–667.

Pannell J, Barrett S. 1998. Baker's law revisited: reproductive assurance in a metapopulation. *Evolution* **52**: 657–668.

Peterson M, Kay K. 2015. Mating system plasticity promotes persistence and adaptation of colonizing populations of hermaphroditic angiosperms. *The American Naturalist* **185**: 28–43.

Pollard A, Baker A. 1996. Quantitative genetics of zinc hyperaccumulation in *Thlaspi caerulescens*. *New Phytologist* **132**: 113–118.

Porcher E, Lande R. 2005. The evolution of self-fertilization and inbreeding depression under pollen discounting and pollen limitation. *Journal of Evolutionary Biology* **18**: 497–508.

Reeves R, Schwartz C, Morel J-L, Edmondson J. 2001. Distribution and metal-accumulating behavior of *Thlaspi caerulescens* and associated metallophytes in France. *International Journal of Phytoremediation* **3**: 145–172.

Rigola D, Fiers M, Vurro E, Aarts M. 2006. The heavy metal hyperaccumulator *Thlaspi caerulescens* expresses many species-specific genes, as identified by comparative expressed sequence tag analysis. *New Phytologist* **170**: 753–766.

Rileys R. 1956. The influence of the breeding system on the genecology of *Thlaspi Alpestre* L. *New Phytologist* **55**: 319–330.

Robertson A. 1992. The relationship between floral display size, pollen carryover and geitonogamy in *Myosotis colensoi* (Kirk) Macbride (Boraginaceae). *Biological Journal of the Linnean Society* **46**: 333–349.

Roosens N, Verbruggen N, Meerts P, Ximénez-Embrùn P, Smith J. 2003. Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of *Thlaspi caerulescens* from western Europe. *Plant, Cell & Environment* **26**: 1657–1672.

Schemske D, Lande R. 1985. The evolution of self-fertilization and inbreeding depression in plants. II. empirical observations. *Evolution* **39**: 41–52.

Stebbins G. 1957. Self-fertilization and population variability in the higher plants. *The American Naturalist* **91**: 337–354.

Takebayashi N, Morrell P. 2001. Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. *American Journal of Botany* **88**: 1143–1150.

Uyenoyama M, Waller D. 1991a. Coevolution of self-fertilization and inbreeding depression II. Symmetric overdominance in viability. *Theoretical Population Biology* **40**: 47–77.

Uyenoyama M, Waller D. 1991b. Coevolution of self-fertilization and inbreeding depression I. Mutation-selection balance at one and two loci. *Theoretical Population Biology* **40**: 14–46.

Uyenoyama M, Waller D. 1991c. Coevolution of self-fertilization and inbreeding depression. III. Homozygous lethal mutations at multiple loci. *Theoretical Population Biology* **40**: 173–210.

Verbruggen N, Hermans C, Schat H. 2009. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist* **181**: 759–776.

Vergeer P, Wagemaker N, Ouborg N. 2012. Evidence for an epigenetic role in inbreeding depression. *Biology Letters* **8**: 798–801.

Vogler D, Kalisz S. 2001. Sex among the flowers: the distribution of plant mating *systems*. *Evolution* **55**: 202–204.

Willis J. 1999. The role of genes of large effect on inbreeding depression in *Mimulus guttatus*. *Evolution* **53**: 1678–1691.

Winn A, Elle E, Kalisz S, Cheptou P-O, Eckert C, Goodwillie C, Johnston M, Moeller D, Ree R, Sargent R, Vallejo-Marín M. 2011. Analysis of inbreeding depression in mixed-mating plants provides evidence for selective interference and stable mixed mating. *Evolution* **65**: 3339–3359.

CHAPITRE 1

LOWER SELFING RATES IN METALLICOLOUS POPULATIONS THAN IN NON-METALLICOLOUS POPULATIONS OF THE PSEUDOMETALLOPHYTE *NOCCAEA CAERULESCENS* (BRASSICACEAE) IN SOUTHERN FRANCE

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Lower selfing rates in metallicolous populations than in non-metallicolous populations of the pseudometallophyte *Noccaea caerulescens* (Brassicaceae) in Southern France

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• **Background and Aims** The pseudometallophyte *Noccaea caerulescens* is an excellent model to study evolutionary processes, as it grows both on normal and on heavy-metal-rich, toxic soils. The evolution and demography of populations are critically impacted by mating system and, yet, information about the *N. caerulescens* mating system is limited.
• **Methods** Mean selfing rates were assessed using microsatellite loci and a robust estimation method (RMES) in five metallicolous and five non-metallicolous populations of *N. caerulescens* in Southern France, and this measure was replicated for two successive reproductive seasons. As a part of the study, the patterns of gene flow among populations were analysed. The mating system was then characterized at a fine spatial scale in three populations using the MLTR method on progeny arrays.
• **Key Results** The results confirm that *N. caerulescens* has a mixed mating system, with selfing rates ranging from 0·2 to 0·5. Selfing rates did not vary much among populations within ecotypes, but were lower in the metallicolous than in the non-metallicolous ecotype, in both seasons. Effective population size was also lower in non-metallicolous populations. Biparental inbreeding was null to moderate. Differentiation among populations was generally high, but neither ecotype nor isolation by distance explained it.
• **Conclusions** The consequences of higher selfing rates on adaptation are expected to be weak to moderate in non-metallicolous populations and they are expected to suffer less from inbreeding depression, compared to metallicolous populations.

Key words: Mating system, metallicolous, microsatellite markers, MLTR, *Noccaea caerulescens* (formerly *Thlaspi caerulescens*), pseudometallophyte, RMES, selfing, self-fertilization.

INTRODUCTION

Organisms with the ability to grow on extremely toxic soils, such as mine wastes or mine smelters, have triggered a lot of interest recently (McNeilly and Antonovics, 1968; Antonovics *et al*., 1971; Bradshaw, 1984; Macnair, 1987; Pauwels *et al*., 2008). *Noccaea caerulescens* is one such organism, and is now a model species for unravelling the genetic and physiological mechanisms of metal tolerance and hyperaccumulation (Assunção *et al*., 2003*b*; Hanikenne and Nouet, 2011, and references therein), with potential applications in phytostabilization or phytoextraction (Chaney *et al*., 1997; but see Ernst, 2005). *Noccaea caerulescens* is a pseudometallophyte herb, as it can grow on both metal-rich soils and other types of soils. It is constitutively tolerant to soils containing high concentrations of trace elements such as cadmium (Cd), zinc (Zn) and lead (Pb) (e.g. Meerts and Van Isacker, 1997; Escarré *et al*., 2000) and is also an hyperaccumulator, because it can accumulate extremely large quantities of nickel (Ni), Zn and Cd in its tissues (Assunção *et al*., 2003*a*; Verbruggen *et al*., 2009, and references therein). It is related to *Arabidopsis thaliana*, which conveniently makes some genetic resource transfers possible (Rigola *et al*., 2006). *Noccaea caerulescens* is also an excellent model to investigate eco-evolutionary processes such as the adaptation to stressful environments or the evolution of local adaptation (Dubois *et al*., 2003; Dechamps *et al*., 2007; Jiménez-Ambriz *et al*., 2007; Noret *et al*., 2007; Besnard *et al*., 2009; Fones *et al*., 2013).

Some populations of *N. caerulescens* have higher levels of tolerance to trace elements than others (Meerts and Van Isacker, 1997; Escarré *et al*., 2000; Assunção *et al*., 2003*a*), leading to the description of different ecotypes based on edaphic conditions: in particular, a metallicolous (or calamine) ecotype that grows on metalliferous soils such as former mining sites, more tolerant but accumulating less Zn, and a non-metallicolous ecotype that grows on non-metalliferous soils, less tolerant but accumulating more Zn (Meerts and Van Isacker, 1997; Escarré *et al*., 2000; Assunção *et al*., 2003*a*). Previous studies showed genetic differentiation between ecotypes at some candidate loci involved in metal metabolism (Besnard *et al*., 2009), as well as local adaptation of metallicolous populations, based on both reciprocal transplants in natural populations (Dechamps *et al*.,

2008) and common garden experiments (Meerts and Van Isacker, 1997; Dechamps *et al*., 2007; Jiménez-Ambriz *et al*., 2007). However, these ecotypes could not be distinguished using neutral markers (Koch *et al*., 1998; Dubois *et al*., 2003; Jiménez-Ambriz *et al*., 2007), suggesting either independent recurrent adaptation to different edaphic conditions or enough gene flow between ecotypes to homogenize genomes except around genes involved in local adaptation.

The mating system, especially the selfing rate, is a key factor to consider in this context. Indeed, it affects both the ecology and evolution of organisms, and in particular their adaptation to extreme conditions in heterogeneous environments (Levin, 2010), such as metalliferous and non-metalliferous soils on which the self-compatible species *N. caerulescens* grows. For example, autonomous selfing provides reproductive insurance when pollinators or mates are scarce, which could help in colonizing new habitats (Baker, 1955; Levin, 2010, Pannell, 2015; but see Cheptou, 2012). This positive demographic effect may be counterbalanced by inbreeding depression, i.e. the lower fitness of selfed compared with outcrossed progeny, which may increase the demographic vulnerability of small and inbred populations. Note, however, that if inbreeding depression affects mostly the early stages of the life cycle in populations where the number of juveniles greatly exceeds the carrying capacity, it may have very little impact on the prospects of population survival.

Selfing affects all evolutionary forces by modifying patterns of gene flow, decreasing recombination efficacy, increasing genetic drift and exposing genetic variation to selection. This may have both positive and negative effects on population survival and adaptation. On the one hand, recessive mutations in inbred individuals can be purged through selection when deleterious, and be fixed when beneficial (Caballero and Hill, 1992). In the latter case, this would speed up adaptation to new environments (Glémin and Ronfort, 2013). On the other hand, selfing reduces effective population size (Nordborg, 2000) and effective recombination (Holden 1979, Hartfield and Glémin, 2014), and consequently may lead to a loss of standing genetic diversity, and compromise the adaptive capacities of highly selfing species in the long term (Stebbins, 1957; Wright *et al*., 2013; Lande and Porcher, 2015). Finally, the mating system may constrain local adaptation in heterogeneous environments by affecting gene flow across habitats with divergent selection (Lopez *et al*., 2008), such as contaminated soils next to non-contaminated soils. In the classic study on the joint evolution of mating system and local adaptation, Antonovics (1968) found that *Agrostis tenuis* and *Anthoxanthum odoratum* plants growing in a mine had higher autonomous selfing rates than did plants growing on a nearby non-contaminated pasture, consistent with his theoretical prediction of an advantage to selfing in such heterogeneous habitats. However, further theoretical work showed that the relationships between local adaptation and selfing rates could be complicated by the joint evolution of inbreeding depression in heterogeneous environments (Epinat and Lenormand, 2009; Ronce *et al*., 2009). In particular, Epinat and Lenormand (2009) predicted that the evolution of increased selfing in heterogeneous habitats may be impeded by the evolution of higher inbreeding depression at the local adaptation locus. In addition, after the initial results of Antonovics (1968), replicated by Cuguen *et al*. (1989) and Lefèbvre (1970) (but see Lefèbvre, 1973, 1976; Dulya and Mikryukov, 2015), the accumulated empirical evidence did not provide overall support to the idea of a general and strong association between selfing and plant local adaptation (for two meta-analyses, see Leimu and Fischer, 2008; Hereford, 2010).

Despite its key role in the ecology and adaptation to heterogeneous habitats, the mating system of *N. caerulescens* has not been characterized in detail. The species is self-compatible, and, based on F_{IS} values, has a mixed mating system (Koch *et al*., 1998; Dubois *et al*., 2003; Jiménez-Ambriz *et al*., 2007; Besnard *et al*., 2009). Dubois *et al*. (2003) found that, in contrast to the finding of Antonovics (1968), non-metallicolous populations had higher inbreeding than metallicolous populations. This result was, however, derived from estimations of F_{IS} based on four enzymatic markers with limited polymorphism, and on the measurement of pollen–ovule ratios. Estimates of selfing rates based on fixation indexes suffer from several methodological biases (e.g. in the presence of null alleles), and are sensitive to population characteristics such as sub-structuration (Jarne and David, 2008), biparental inbreeding in particular (Jarne and David, 2008; Wang *et al*., 2012). Selfing rates in plants with a mixed mating system are known to vary from one season to another (e.g. Cheliak *et al*., 1985; Barrett *et al*., 1993; Eckert *et al*., 2009; Coates *et al*., 2013), due to environment variation affecting both pollinator and plant communities. Traits potentially affecting the mating system such as the height of flowering stalks vary significantly across years in natural populations of *N. caerulescens* (Jiménez-Ambriz *et al*., 2007). Estimating mating system parameters in more than one reproductive season would thus allow a more robust evaluation of the variation of mating system between populations and of the stability of these patterns through time.

Here we present a detailed analysis of the mating system in metallicolous and non-metallicolous populations of *N. caerulescens*, focusing on a set of geographically close populations to minimize potential causes of variation in the mating system. Ten populations from the Causses and Cévennes region in Southern France were investigated using 14 microsatellite markers. The ten populations were used for two consecutive reproductive seasons, and one population was used for four seasons. We described patterns of gene flow among populations, as reflected by their genetic structure, and estimated their effective size. Selfing rates within each population were estimated using a method robust to scoring artefacts, and based on correlations in homozygosity across loci (RMES; David *et al*., 2007). To gain more insight into the relative role of selfing and other forms of inbreeding, we further analysed a distinct data set, corresponding to progeny arrays of genotypes in three of the ten populations, using the MLTR method (Ritland, 2002).

MATERIALS AND METHODS

Species presentation

Noccaea caerulescens (J. Presl & C. Presl F.K. Mey) is a pseudometallophyte from the *Brassicaceae* family. It is annual to perennial, flowers in spring and grows one to several dozen inflorescences with small white to light purple flowers. Its main pollinators in the studied area have not yet been extensively described, but observations in several populations for several

years reveal that Hymenoptera (domesticated and wild bees, bumble-bees), small Diptera and Lepidoptera visit *N. caerulescens* flowers (I. Decombeix, A. Mignot, C. Petit, L. Prat, ISEM, Montpellier, pers. comm.). These pollinators broadly correspond to previous observations of hover flies, flies, Lepidoptera and bees visiting flowers in Germany and Great Britain (Knuth, 1908; Rileys, 1956). Most individuals from populations in Southern France exhibit an annual life cycle (Dubois, 2005): seeds produced during a given spring and summer will germinate the following autumn after the first abundant rains and will spend the winter as rosettes; about 30 % of the rosettes will survive and flower the following spring and about 11 % will survive and stay in the non-reproductive state the following spring (Dubois, 2005). In the studied area, situated north of Montpellier, *N. caerulescens* grows on relatively open substrate. Metallicolous populations occur on nutrient-poor, schistous or dolomitic soils, while non-metallicolous populations grow on limestone soils.

Methods to estimate selfing rates

Different approaches are available to study mating systems, using different types of data and different assumptions (Jarne and David, 2008). We used two of them to better characterize the mating system of *N. caerulescens*. The RMES software (David *et al*., 2007), which is based on a population structure approach, infers the mean population selfing rate using grown individuals sampled directly in the populations. Its estimation integrates selfing rates across several generations. The MLTR software (Ritland, 2002), which is based on a progeny array approach, uses family structured data to infer population mating system parameters from seeds collected in the populations. In contrast to the former approach, its estimated selfing rate represents a snapshot of the mating structure in the previous generation, and is usually less affected by early acting inbreeding depression because progeny are typically sampled as seedlings.

Sampling for the population structure approach

We chose five populations with high heavy metal concentrations (hereafter called metallicolous populations or MET) and five populations on calcareous soil (non-metallicolous populations or NONMET) in the Causses and Cévennes, the southern part of Massif Central and north of Montpellier (France). All sites (Fig. 1; Table 1) were close to Ganges and separated by $<$1–38 km. We sampled leaves on plants in May–June 2013, and in February–April 2014. These plants were at the rosette or flowering stage and were assumed to represent mostly the offspring of the 2012 and 2013 reproductive seasons, respectively. Plants were sampled in approximately the same areas in the populations for the two years. For all sites, leaves were collected on 40–50 plants along a random walk, on individuals separated by $>$1 m whenever possible to limit sampling of related individuals; they were subsequently oven dried (40 °C during 48–72 h), and kept in silica gel. In the Saint Bresson (SB) population, we sampled approx. 50 plants each year twice. As the sample size affects the estimation of genetic diversity, we provide separate estimates for each sample (their size is similar to that of the other populations), but we pool the two samples to estimate selfing rates with more precision. For this population, we also sampled leaves during spring 2011 and 2012 and were thus able to obtain selfing rate estimates for four consecutive reproductive seasons.

Sampling for the progeny array approach

Thirteen to twenty seeds from several inflorescences of the same plant were collected at maturity (June–July) on 20 mothers per population in Saint Hippolyte (HI; MET) and Baraquette (BQ; NONMET) in 2007, and in SB (MET) in 2011. Seeds from each family were sown in the year of harvest, on burnt clay (HI and BQ) or vermiculite (SB) in randomized containers in a temperate glasshouse at the experimental garden of LabEx CeMEB (Plateforme des Terrains d'Expériences du LabEx CeMEB). Seedlings were collected at the four-leaf stage, oven dried and genotyped. The mother genotype was available only for SB; it was thus inferred for the other populations.

Microsatellite genotyping

Neutral diversity was assessed using 15 microsatellite loci organized in two multiplexes (NcM1 and NcM2) and following the protocol described in Mousset *et al*. (2015). Two microsatellite markers, 6g4 and 6e4 (Jiménez-Ambriz *et al*., 2007), were added to NcM1 and NcM2, respectively, following the same protocol (6g4, fluorochrome PET, final concentration 0·8 μm; 6e4, fluorochrome 6FAM, final concentration 0·4 μm). DNA was extracted using a classic cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990), amplified using PCR and genotyped using capillary electrophoresis on an ABI PRISM 3130 xl Genetic Analyzer (SB 2010, 2011 and progeny array 2011) and an ABI PRISM 3500 xl Genetic Analyzer (all other populations). Raw data were analysed using GeneMapper® version 5.0 (Applied Biosystems). The use of a different Genetic Analyzer led to different migration conditions, and thus different bins. As a consequence, allelic frequencies from SB 2010 and 2011, and progeny arrays from 2011 samples were not compared with other populations genotyped later. Nevertheless, the RMES method relies only on the presence or absence of heterozygotes within populations to compute mean selfing rates. We were thus able to compare the 2010 and 2011 estimates of mean selfing rates with the 2012 and 2013 estimates using the population structure approach.

In the 2011 SB population, null alleles were obvious for some markers in some families; Ncpm31 and Tc-up2 were removed from the analysis, and thus the analysis was performed on 13 loci. Due to amplification issues, 6g4 was barely readable in the genotypes in the ten populations of 2012 and 2013 and thus were removed from their analyses (which was thus performed on 14 loci).

Genetic diversity

We used GENETIX (version 4.05, Belkhir *et al*., 2004) to compute allelic frequencies, number of alleles, inbreeding

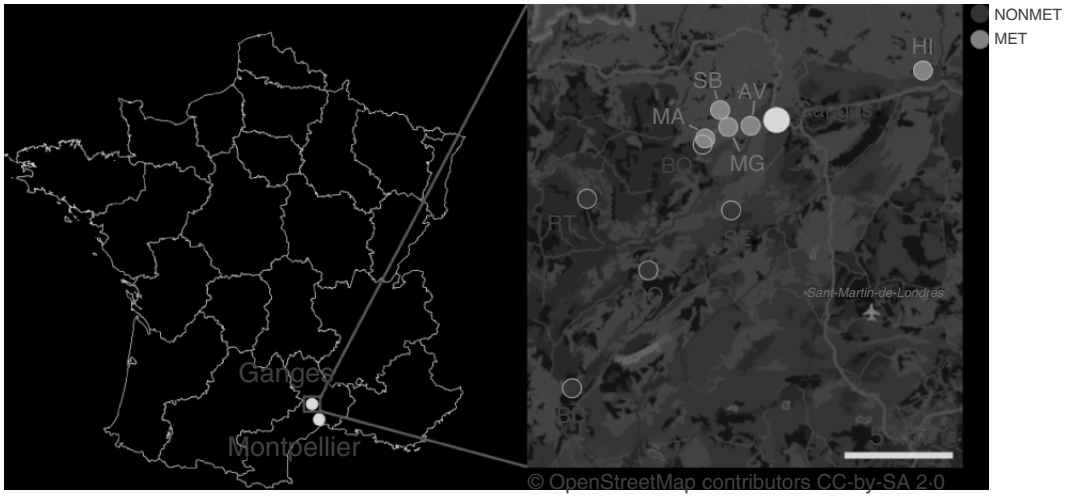

FIG. 1. Geographical location of the ten natural populations of *Noccaea caerulescens* sampled in the South of France in 2012 and 2013. Non-metallicolous populations are represented in light grey, and metallicolous populations in dark grey. Background maps: © OpenStreetMaps Contributors. AV, Avinières; BD, Saint Baudille; BQ, Baraquette; CO, Coulet; HI, Saint Hippolyte; MA, Malines; MG, Moyen-Âge; RT, Navacelles Route; SB, Saint Bresson; SE, Séranne.

TABLE 1. *Name, location and sample size of the sampled natural populations of* Noccaea caerulescens

Ecotype	Population	Sample size for the season					Latitude (N)	Longitude (E)	Locality
		2007	2010	2011	2012	2013			
MET	AV[‡,§]				50	49	43·932138	3·662831	Saint-Laurent-le-Minier
	HI	20*15			49	44	43·971409	3·833329	Saint-Hippolyte-du-Fort
	MA[‡,§]				42	48	43·922939	3·617699	Saint-Laurent-le-Minier
	MG[†,‡,§]				52	52	43·931157	3·640581	Saint-Laurent-le-Minier
	SB[‡]		43	44 20*20	87	105	43·943303	3·632505	Pommiers
NONMET	BD				48	47	43·744648	3·486100	Montpeyroux
	BQ[¶]	20*15			45	53	43·918536	3·615228	Saint-Laurent-le-Minier
	CO				51	52	43·829053	3·561568	Saint-Maurice-Navacelles
	RT[¶]				56	44	43·880013	3·500879	Saint-Maurice-Navacelles
	SE[¶]				49	48	43·871755	3·643483	Gorniès

Years represent the reproductive season studied. Samples correspond to leaves, except for the progeny array approach for which seeds have been collected. For these samples, numbers separated by an asterisk represent the number of families and the number of offspring genotyped per family. Concentrations of trace elements can be found in the publications listed below, when available.

MET, metallicolous; NONMET, non-metallicolous. AV, Avinières; BD, Saint Baudille; BQ, Baraquette; CO, Coulet; HI, Saint Hippolyte; MA, Malines; MG, Moyen-Âge; RT, Navacelles Route; SB, Saint Bresson; SE, Séranne.

[†]Also known as Petra Alba in Escarré *et al*. (2011).

[‡]Dubois *et al*. (2003).

[§]Escarré *et al*. (2011).

[¶]Dubois (2005).

coefficient F_{IS} [using the Weir and Cockerham (1984) method with 1000 bootstrap 95 % confidence intervals (CIs)] and the amount of linkage disequilibrium between loci, and Genepop (Rousset, 2008) to test for linkage equilibrium among loci. Combining the information of these two software programs, no pair of loci had a statistically significant correlation coefficient higher than 50 % in more than two populations; therefore, we kept all loci in the following analyses. We used the permutation test in FSTAT (Goudet, 2005) to compare the observed and expected heterozygosity, F_{IS} and F_{ST}, of the metallicolous and non-metallicolous ecotypes (5000 permutations of populations between ecotypes).

Analysis of gene flow among populations

Partitioning of genetic variance. We used Genepop to calculate pairwise F_{ST} values between pairs of populations (for each year) and the HIERFSTAT R package (Goudet, 2005; R Core Team, 2014) to partition genetic variance among ecotypes, populations and plants (95 % CIs were based on 1000 bootstraps).

Isolation by distance. We tested for the presence of isolation by distance among populations using the R package 'fields' (Nychka *et al*., 2014) to calculate the geographic distance matrix among populations based on their GPS coordinates. We then tested for the presence of isolation by distance each year

with Mantel tests in the 'adegenet' R package (Jombart and Ahmed, 2011), within each ecotype and on the whole data set. Additionally, following the guidelines provided by Rousset (1997), we regressed $F_{ST}/(1 - F_{ST})$ on the geographic distance.

Principal component analysis. To better describe the structure of genetic diversity, we calculated a centred, non-scaled principal component analysis (PCA) on allele frequencies in 2012 and 2013 using the R packages 'adegenet' and 'ade4' (Dray and Dufour, 2007; Jombart and Ahmed, 2011).

Mating system analysis

Population structure approach (RMES). We estimated mean population selfing rates with the RMES software (Robust Multilocus Estimate of Selfing, version 2009; David *et al.*, 2007). RMES uses the distribution of heterozygosity across all loci to infer selfing rates, and therefore provides estimates that are robust to several common problems of microsatellite data, such as the presence of null alleles or partial dominance. RMES maximizes likelihood (precision used: 0·0001) to estimate selfing rates (along with its 95 % CI) and computes the likelihood profile (i.e. the likelihood of all possible selfing rates at regularly spaced intervals). We used both pieces of information in two different approaches to compare selfing rates between populations, ecotypes and years of sampling.

Likelihood ratio tests (LRTs) can been used to test for differences in selfing rates between populations (a single selfing rate, *s*, for all populations vs. a different *s* for each population; David *et al.*, 2007). Our problem was different as we wanted to test for differences in the distributions of *s* (and especially for differences in the mean of the distributions) between metallicolous and non-metallicolous populations, the 'ecotype' being equivalent to a fixed factor and the 'population within ecotype' being a source of random variation, similarly to a mixed model analysis of variance (ANOVA). To implement this approach we assumed that in each ecotype, population selfing rates (*s*) followed a logit-normal distribution and looked for values of the mean and standard deviation of logit(*s*) that maximize the likelihood over all data. We also maximized the likelihood under specific constraints, e.g. assuming that the two ecotypes had the same standard deviation and/or the same mean, or that they were equal to pre-defined values (e.g. 0). Models with and without a relevant constraint were then compared using standard LRTs. To fit these models, we used a Mathematica 9 routine (Wolfram Research, Inc., 2012) that computes the relevant likelihoods by combining population-specific likelihood profiles given by RMES and uses a simple iterative procedure to maximize them. Analyses were performed separately for the years 2012 and 2013. We found that the among-population standard deviation did not differ between ecotypes, and from zero in either ecotype. Therefore, we tested separately for the 2012 and 2013 data whether mean *s* were significantly different between ecotypes by performing LRT on models with a single or two means for the two ecotypes, assuming s.d. = 0 within each ecotype.

To investigate temporal variation in mating system across years, we tested separately for the two ecotypes if they had the same selfing rate in the two sampled reproductive seasons. Using the same framework as before, we used LRT to compare, for each ecotype separately, a model allowing a different *s* per season with a model estimating a single overall *s*. Finally, we similarly tested for variation by year in selfing rates in SB for the four seasons.

Progeny array approach. We used MLTR (version 3.3; Ritland, 2002) on the progeny structured data sets to estimate mating system parameters using maximum likelihood estimations in HI, BQ and SB. We used the Newton–Raphson algorithm to obtain single locus and multilocus estimates of selfing rate (s_s and s_m), correlations of selfing between families r_t (variance in selfing rates among mother plants.), correlations of paternity among siblings r_p (the inverse of the estimated number of fathers for the progeny of a single mother) and paternal inbreeding coefficient, *F*. We used 1000 bootstraps with whole families resampling to estimate 95 % CIs for these estimates. Comparing single locus- and multilocus-based selfing rates enables us to estimate the contribution of biparental inbreeding in the population, i.e. mating among related plants. Estimates of mating system parameters when assuming equal allele frequencies in the pollen cloud and ovule pool, and when relaxing this assumption, were similar. As the CIs were larger in the latter case, we used the most parsimonious model.

Effective population size

We took advantage of the two sampling years to estimate variance effective size in each population (ten populations sampled for two consecutive reproductive seasons). We used the MLNe software (Wang and Whitlock 2003) to estimate effective size, with the maximum likelihood approach, assuming no immigration in populations. This method is based on the fact that, as effective size decreases, allele frequencies vary more widely through time due to drift effects.

RESULTS

Within-population genetic diversity

The mean number of alleles per locus was 5·4 ± 1 in metallicolous populations and 6·2 ± 0·7 in non-metallicolous populations (Table 2). Population allelic frequencies within each population were similar across the two sampled years. Pairwise F_{ST} within populations between the two sampling years was therefore small (<0·06). Mean genetic diversity was higher in non-metallicolous populations than in metallicolous populations, although the difference was not significant (MET 2012, 0·54 ± 0·10 and MET 2013, 0·55 ± 0·11; NONMET 2012, 0·63 ± 0·04 and NONMET 2013, 0·63 ± 0·04; permutation test, $P_{2012} = 0{\cdot}13$, $P_{2013} = 0{\cdot}16$; Table 2). Mean observed heterozygosity was similar in metallicolous and non-metallicolous populations (MET 2012, 0·43 ± 0·08 and MET 2013, 0·46 ± 0·10; NONMET 2012, 0·42 ± 0·07; NONMET 2013, 0·41 ± 0·06; permutation test, $P_{2012} = 0{\cdot}89$, $P_{2013} = 0{\cdot}55$; Table 2). F_{IS} was lower in metallicolous populations than in non-metallicolous populations (MET 2012, 0·22 ± 0·03 and MET 2013, 0·19 ± 0·04; NONMET 2012, 0·35 ± 0·11 and NONMET 2013, 0·37 ± 0·07; $P_{2012} = 0{\cdot}04$; $P_{2013} = 0{\cdot}007$, Table 2). The mean inbreeding coefficient F_{IS} was different from zero in all populations for the two years (except in the two SB sub-samples in 2012),

TABLE 2. *Sample size and genetic diversities in all ten populations of* Noccaea caerulescens *in 2012 and 2013*

Ecotype	Population	Year	Sample size	Mean allelic richness	H_{obs}	H_{exp}	F_{IS}
MET	AV	2012	50	5·7	0·49	0·65	**0·25**
		2013	49	6·5	0·55	0·65	**0·17**
	HI	2012	49	5·4	0·40	0·51	**0·24**
		2013	44	5·5	0·42	0·51	**0·20**
	MA	2012	42	6·1	0·55	0·66	**0·19**
		2013	48	6·9	0·58	0·69	**0·18**
	MG	2012	52	4·4	0·37	0·46	**0·20**
		2013	52	3·5	0·38	0·44	**0·15**
	SB	2012	(SB1) 44	5·1	0·44	0·48	0·1
			(SB2) 43	4·1	0·42	0·43	0·04
		2013	(SB3) 58	5·1	0·44	0·51	**0·15**
			(SB4) 47	5·1	0·42	0·46	**0·09**
NONMET	BD	2012	48	6·1	0·31	0·62	**0·51**
		2013	47	5·4	0·43	0·61	**0·31**
	BQ	2012	45	6·1	0·44	0·67	**0·36**
		2013	53	6·0	0·37	0·63	**0·43**
	CO	2012	51	5·7	0·41	0·59	**0·33**
		2013	52	5·8	0·33	0·57	**0·45**
	RT	2012	56	6·3	0·49	0·61	**0·20**
		2013	44	6·3	0·47	0·69	**0·33**
	SE	2012	49	7·6	0·45	0·69	**0·36**
		2013	48	7·1	0·46	0·66	**0·31**

F_{IS} values in bold are statistically different from zero.

H_{obs}, observed heterozygosity; H_{exp}, expected heterozygosity; F_{IS}, inbreeding coefficient. MET, metallicolous; NONMET, non-metallicolous. AV, Avinières; BD, Saint Baudille; BQ, Baraquette; CO, Coulet; HI, Saint Hippolyte; MA, Malines; MG, Moyen-Âge; RT, Navacelles Route; SB, Saint Bresson; SE, Séranne.

SB1, SB2, SB3 and SB4 correspond to different samplings in the SB population in the two seasons.

indicating a general deficit in heterozygotes. This confirms the mixed mating system of *N. caerulescens* and suggests that metallicolous populations may have a less inbred mating system than do non-metallicolous populations.

Among-population genetic structure

Population differentiation was relatively high in general given the short geographical distances between them, but ecotype structuration did not account for it. Pairwise F_{ST} ranged from 0·07 to 0·42 in 2012 and 2013 (Supplementary Data Table S1). Mean pairwise F_{ST} was 0·29 (s.d. = 0·1) among metallicolous populations in both 2012 and 2013, and 0·14 (s.d. = 0·02) and 0·16 (s.d. = 0·03) among non-metallicolous populations in 2012 and 2013, respectively. The difference in F_{ST} was significant in 2012 ($P = 0·02$) but not in 2013 ($P = 0·11$). Hierarchical fixation indexes, as reported in Table 3 for both years, indicated differentiation among populations within ecotypes but very little structuration by ecotypes. The high within-population fixation index is consistent with the high F_{IS} measured in all populations.

Isolation by distance could not be detected in the data set. Partial Mantel tests for each ecotype and for each year revealed no pattern of isolation by distance (2012 MET, $P = 0·19$; NONMET, $P = 0·07$; all populations $P = 0·28$; 2013 MET, $P = 0·22$; NONMET, $P = 0·21$; all populations $P = 0·28$). Similarly, the slopes of the regression of $F_{ST}/(1 - F_{ST})$ on the logarithm of distance were not significantly different from zero, within metallicolous populations, within non-metallicolous populations or among all populations (Supplementary Data Fig. S1), thus confirming the absence of isolation by distance, at least at the scale of the few kilometres separating our populations.

TABLE 3. *Hierarchical fixation indexes between ecotypes, populations and individuals of* Noccaea caerulescens *in 2012 and 2013*

Source of variation	Year	Fixation index (CI)
Among ecotypes	2012	0·04 (0·01–0·06)
	2013	0·04 (0·01–0·07)
Among populations within ecotypes	2012	0·25 (0·2–0·29)
	2013	0·24 (0·20–0·28)
Among individuals among populations	2012	0·28 (0·26–0·3)
	2013	0·28 (0·25–0·32)

CI, confidence intervals (1000 bootstraps); *F*, hierarchical fixation index.

The PCA did not discriminate ecotypes (Supplementary Data Fig. S2); instead, the principal components each separated one of the metallicolous populations from all other populations, suggesting greater genetic originality of the metallicolous populations. The first four components explained only 27·4 % of the inertia in 2012 and 23·2 % in 2013. None of these axes discriminated all metallicolous populations from all non-metallicolous populations. Most genetic variation was thus not explained by ecotype differentiation, and each metallicolous population tended to be differentiated from all other populations.

Effective population size

Estimated effective population size varied quite a lot between populations and was, on average, smaller in non-metallicolous populations than in metallicolous populations (Table 4). In one population, the effective population size was at the maximum the software can compute. Several upper

Table 4. *Variance in effective size in* Noccaea caerulescens *estimated by MLNe using the maximum likelihood approach, with 95 % confidence intervals*

Ecotype	Population	N*e*
MET	AV	755 (89–10 000)
	HI	209 (58–10 000)
	MA	10 000 (105–10 000)
	MG	107 (38–10 000)
	SB	118 (64–445)
NONMET	BD	43 (26–100)
	BQ	49 (30–117)
	CO	63 (38–157)
	RT	23 (18–32)
	SE	50 (32–103)

MET, metallicolous; NONMET, non-metallicolous. AV, Avinières; BD, Saint Baudille; BQ, Baraquette; CO, Coulet; HI, Saint Hippolyte; MA, Malines; MG, Moyen-Âge; RT, Navacelles Route; SB, Saint Bresson; SE, Séranne.

values of CIs reach the upper limit of estimation, all in metallicolous populations. Taken together, these results suggest moderate to large variance effective size in metallicolous populations (>100) and small to moderate variance effective size for non-metallicolous populations, generally <100 and with size as small as 23.

Mating system

Populations of *N. caerulescens* in the studied area exhibited a clear mixed mating system: selfing rates estimated by RMES ranged between 0·2 and 0·5, depending on populations and years (Fig. 2), and the mean selfing rate of populations was $0{\cdot}25 \pm 0{\cdot}05$ in metallicolous populations and $0{\cdot}39 \pm 0{\cdot}09$ in non-metallicolous populations. Population selfing rates estimated by RMES were consistently lower than those generated via F_{IS} (Supplementary Data Fig. S3), and ecotype selfing rates were approx. 25 % smaller in RMES compared with F_{IS} based estimations, for both ecotypes.

We found clear differences between ecotypes. The LRTs showed that, in both 2012 and 2013, logit-normal distributions of selfing rates had different means for the two ecotypes, but equal standard deviations (Table 5). This standard deviation converged towards zero (due to numerical precision, estimated standard deviations cannot be smaller than 0·002) so we simplified the models by assuming s.d. = 0 for both ecotypes (see the Materials and Methods). Using RMES both in 2012 and in 2013, we found significant differences in selfing rates between the two ecotypes (Tables 6 and 7), as a model estimating two different selfing rates in the two ecotypes clearly outperformed a constrained model with equal selfing rates across ecotypes.

There was no evidence of temporal variation. Within each ecotype, we found no consistent significant difference in selfing rates across the two years in populations of the same ecotype (Tables 6 and 7). Similarly, no temporal variation was detected during four consecutive years in SB (Table 6; Fig. 3).

Analysis of the progeny structured data set (Fig. 4) indicated that the paternal inbreeding coefficient F ranged between 0·09 (95 % CI 0–0·21) and 0·17 (95 % CI 0·05–0·35), suggesting moderate inbreeding of fathers. Multilocus selfing rate estimation ranged from 0·18 (95 % CI 0·12–0·23) to 0·49 (95 % CI 0·37–0·60). These estimates are within the range of values observed in RMES. From the difference between multilocus and single locus estimates of selfing, we determined that biparental inbreeding varied between 0·03 (95 % CI 0–0·07) in SB and 0·19 (95 % CI 0·12–0·25) in BQ. The correlation of paternity ranged between 0·032 (95 % CI 0·01–0·051) in SB and 0·35 (95 % CI 0·18–0·47) in BQ, which correspond to 31 and three different fathers per mother plant, respectively. The correlation of selfing among individuals of the same family ranged from 0·05 (95 % CI 0–0·11) to 0·27 (95 % CI 0·1–0·43) in the three populations, indicating small to moderate variance in selfing rates among mother plants.

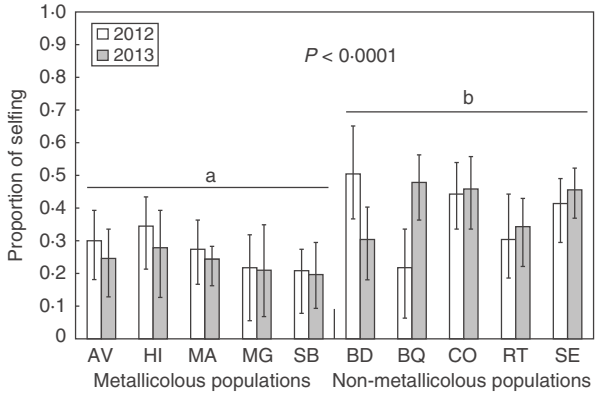

FIG. 2. Mean selfing rate in the ten natural populations of *Noccaea caerulescens* sampled in the South of France in 2012 and 2013. Estimates were obtained using the maximum likelihood method in RMES. Error bars represent 95 % bootstrap confidence intervals. Selfing rates represented by different letters are statistically significantly different. Analysis was performed on 14 loci.

TABLE 5. *Description of the models fitted on selfing rates in* Noccaea caerulescens *using the full information of the likelihood profiles, and result of model comparisons based on log likelihood tests*

Year	Mean(s)	s.d.	Log likelihood	Difference in deviance	d.f.	*P*-value
2012	$\mu_{MET\text{-}2012}$, $\mu_{NONMET\text{-}2012}$	$\sigma_{MET\text{-}2012}$, $\sigma_{NONMET\text{-}2012}$	−4·90			
	$\mu_{\mathbf{MET\text{-}2012}}$, $\mu_{\mathbf{NONMET\text{-}2012}}$	$\boldsymbol{\sigma_{2012}}$	−4·97	0·14	1	0·71
	μ_{2012}	σ_{2012}	−7·31	4·68	1	**0·03**
2013	$\mu_{MET\text{-}2013}$, $\mu_{NONMET\text{-}2013}$	$\sigma_{MET\text{-}2013}$, $\sigma_{NONMET\text{-}2013}$	−2·86			
	$\mu_{\mathbf{MET\text{-}2013}}$, $\mu_{\mathbf{NONMET\text{-}2013}}$	$\boldsymbol{\sigma_{2013}}$	−2·86	0·00	1	1
	μ_{2013}	σ_{2013}	−8·70	11·67	1	**0·001**

MET, metallicolous; NONMET, non-metallicolous; μ, mean of the logit-normal distribution; σ, standard deviation of the logit-normal distribution of selfing rates among populations.

The best models are in bold.

DISCUSSION

In this study, we characterized the variation in the mating system of *N. caerulescens* in both space and time, in the region of Montpellier, Southern France, by estimating gene flow both between and within populations.

TABLE 6. *Description of the models fitted on selfing rates in* Noccaea caerulescens *and results of model comparisons*

Populations	Year	Estimated selfing rate(s)	Log likelihood	Delta deviance	d.f.	*P*-value
All pop	2012	$\underline{s_{MET\text{-}2012}}$, $\underline{s_{NONMET\text{-}2012}}$	−3366·1	6·5	1	**0·01**
	2012	s_{2012}	−3369·3			
All pop	2013	$\underline{s_{MET\text{-}2013}}$, $\underline{s_{NONMET\text{-}2013}}$	−3739·8	14·3	1	**0·0002**
	2013	s_{2013}	−3746·9			
MET	2012–2013	$s_{MET\text{-}2012}$, $s_{MET\text{-}2013}$	−3912·8	0·1	1	0·75
	2012–2013	$\underline{s_{MET}}$	−3912·9			
NONMET	2012–2013	$s_{NONMET\text{-}2012}$, $s_{NONMET\text{-}2013}$	−3193·0	0·2	1	0·65
	2012–2013	$\underline{s_{NONMET}}$	−3193·2			
All pop	2012–2013	$\underline{s_{MET}}$, $\underline{s_{NONMET}}$	−7106·1	21·7	1	**<0·0001**
	2012–2013	s	−7116·9			
SB	2010–2011–2012–2013	$s_{SB\text{-}2010}$, $s_{SB\text{-}2011}$, $s_{SB\text{-}2012}$, $s_{SB\text{-}2013}$	−2014·8	2·4	3	0·5
	2010–2011–2012–2013	s_{SB}	−2016·0			

The models were fitted using maximum log likelihood computed in RMES, and compared using log likelihood ratio tests.

MET, metallicolous; NONMET, non-metallicolous; pop, population; s represents the constrained or unconstrained selfing rate estimated for one population or a group of populations.

The best models are in bold, and the value of underlined estimated selfing rates are provided in Table 7.

TABLE 7. *Mean selfing rates within ecotype of* Noccaea caerulescens *for each year and 95 % likelihood confidence intervals*

Ecotype	Year of estimation	Selfing rate (CI)
MET	2012	0·26 (0·20–0·31)
	2013	0·24 (0·19–0·27)
	Two years constrained equal	0·25 (0·21–0·28)
NONMET	2012	0·38 (0·33–0·43)
	2013	0·41 (0·37–0·45)
	Two years constrained equal	0·40 (0·3–0·43)

MET, metallicolous; NONMET, non-metallicolous.

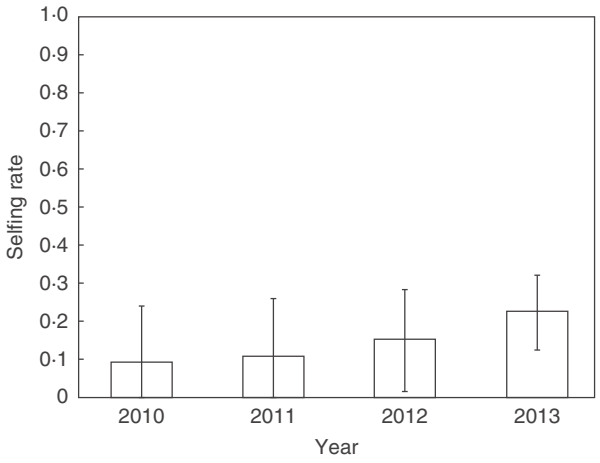

FIG. 3. Mean selfing rate in the SB population of *Noccaea caerulescens*, for four consecutive seasons. Estimates were obtained using the maximum likelihood method in RMES. Error bars represent 95 % likelihood confidence intervals. Analysis was performed on 14 loci.

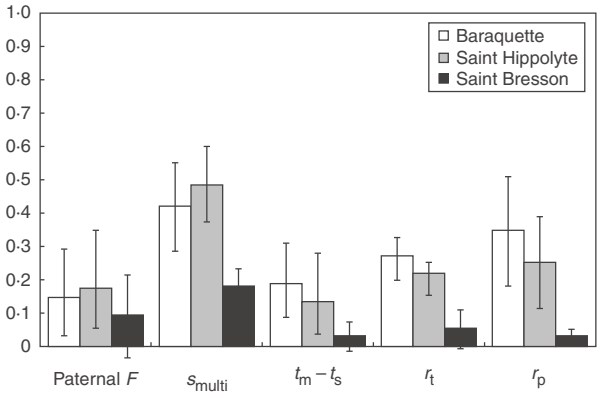

FIG. 4. Mating system parameters obtained with MLTR in the BQ (2007), HI (2007) and SB (2011) populations of *Noccaea caerulescens*. Error bars represent the 95 % confidence interval obtained after 1000 bootstraps. s_{multi}, multilocus estimate of the selfing rate; $t_m - t_s$, estimation of biparental inbreeding; r_t, correlation of selfing among families; r_p, correlation of paternity. Analysis was performed on 15 loci for BQ and HI, and 13 loci for SB.

Gene flow between populations

We found that populations of *N. caerulescens* in Southern France are in general relatively strongly differentiated despite distances of a few kilometres between them, and that metallicolous populations tend to be more differentiated than non-metallicolous populations. The magnitude of genetic differentiation among populations, which is quite typical of plants with a mixed mating system (Loveless and Hamrick, 1984; Hamrick and Godt, 1996), and the lack of isolation by distance point to little gene flow among populations. Such a result was previously found in the same region (Dubois *et al*., 2003; Jiménez-Ambriz *et al*., 2007) and at a larger scale (Gonneau, 2014; although isolation by distance was occasionally detected in his data set) in this species, and in some other pseudometallophyte species (Mengoni *et al*., 2000; Deng *et al*., 2007; Słomka *et al*., 2011). Isolation by distance and lower F_{ST} values were, however, detected in the self-incompatible *Arabidopsis halleri* at a larger scale (Meyer *et al*., 2009; Pauwels *et al*., 2012). Neutral differentiation between ecotype is very small here, suggesting that the ecotype structure does not have much effect on gene flow among populations. This lack or small effect of ecotype on the structure of neutral genetic variation is relatively common in pseudometallophytes (Mengoni *et al*., 2000; Deng *et al*., 2007; Bizoux *et al*., 2008; Słomka *et al*., 2011); geographical grouping sometimes

explains neutral genetic variation better than ecotypic characteristics (Baumbach and Hellwig, 2007; Pauwels *et al*., 2012). Given that gene flow seems to occur at smaller distances than between polluted and non-polluted sites, we do not expect local adaptation in the metallicolous ecotype here to select for higher selfing rates than in the non-metallicolous ecotype or other types of zygotic barriers, such as described in Antonovics (1968) or Antonovics (2006). Instead, our results suggest that both metallicolous and non-metallicolous populations are distributed as isolated islands in the landscape.

The higher differentiation of metallicolous populations remains to be explained, given their higher effective size and lower inbreeding (see discussion below). The strongly selective environment in contaminated sites, or their ecological isolation, may further restrict gene flow into those populations.

Gene movement within populations

Methodological improvements. We confirmed that *N. caerulescens* has a mixed mating system using a recent method that is robust to several biases affecting previously used F_{IS}-derived estimates of mean selfing rate (Dubois *et al.,* 2003). F_{IS}-derived estimates rely on assumptions such as inbreeding equilibrium, the absence of population sub-division or biparental inbreeding, and are sensitive to genotyping errors, small population sizes and small sample sizes (Jarne and David, 2008; Wang *et al*., 2012). The method implemented in RMES also assumes inbreeding equilibrium and absence of population sub-division, but population sub-division should bias RMES estimates only if one sub-population is consistently less heterozygous at all loci than the others. We have no reasons to think that it should be the case within our populations. In addition, RMES is robust to the presence of null alleles (David *et al*., 2007; Jarne and David, 2008) as well as to small sample sizes (Wang *et al*., 2012), and simulations show that the bias generated by biparental inbreeding is small (Wang *et al*., 2012). We consequently expect our estimates to be more accurate than are estimates derived from F_{IS}. Ecotype selfing rate were approx. 25 % smaller in RMES compared with F_{IS}-based estimations, suggesting that the latter estimations may be upwardly biased by biparental inbreeding, which we could detect using progeny arrays in some populations, and by allelic dropouts, which have been detected in some populations for markers in our data set (Mousset *et al*., 2015).

Multilocus individual heterozygosity is affected by selfing in previous generations (Enjalbert and David, 2000), which could lower our power to detect temporal variation. We found, however, that outcrossing rates are relatively high (>50 %), meaning that estimates of selfing rates are mostly determined by the proportion of individuals that were produced by self-fertilization in the immediately preceding generation, with relatively little influence of earlier generations (Enjalbert and David, 2000). Therefore, we believe that the selfing rate did not vary much in time, as otherwise it would have been detected.

Despite the expectation that selfing rates may vary across reproductive seasons in species with a mixed mating system, due in particular to climatic constraints, and despite observations that they sometimes do (e.g. Cheliak *et al*., 1985; Barrett *et al*., 1993; Eckert *et al*., 2009; Coates *et al*., 2013), such variation is not a general rule (Barrett *et al*., 1993; Eckert *et al*., 2009). For instance, Eckert *et al*. (2009) found no temporal variation of the mating system in about half of the 30 studies that they reviewed. The stability of the mating system in the presently studied set of populations, however, remains to be tested for a greater number of years and populations.

Mating system in N. caerulescens. Mean selfing rates of the ten *N. caerulescens* populations estimated in RMES and MLTR ranged from 0·18 to 0·51, and biparental inbreeding (approximated by the amount of apparent selfing based on the comparison of single locus and multilocus estimates) ranged from zero to 0·19. Selfing rates were similar through years and among populations of the same ecotype, but the metallicolous ecotype had a lower selfing rate than did the non-metallicolous ecotype (MET, 0·25; NONMET, 0·4), as previously found by Dubois *et al*. (2003), despite their less robust methodology. Besnard *et al*. (2009) measured F_{IS} for 17 populations of *N. caerulescens* in the Jura Mountains (Switzerland): they also found a mixed mating system (selfing from 0 to 0·90) but did not report any correlation between F_{IS} and soil metal concentration. Contrasting selfing rates in metallicolous and non-metallicolous populations with contrasted soil toxicity in other parts of the species range would be necessary to evaluate the generality of the pattern that we have documented regionally.

Differences in selfing rates between ecotypes could stem from differences in plant traits affecting pollinator behaviour and self-fertilization mechanisms such as autonomous selfing, or from purely external factors such as differences in pollinator and plant communities in polluted and non-polluted sites.

Variation in mating system between ecotypes could be due to variation in inbreeding depression. Inbreeding depression is a major factor driving mating system evolution (Lande and Schemske, 1985; Goodwillie *et al*., 2005; Charlesworth, 2006), and it varies strongly with environmental conditions (Armbruster and Reed, 2005; Fox and Reed, 2011). Selfing rates were measured on adults collected in the field. We would underestimate selfing rates if inbreeding depression were acting on traits affecting survival before sampling (such as seed survival, germination or juvenile survival) and thus eliminating inbred individuals. Different levels of inbreeding depression in metallicolous and non-metallicolous populations could thus, in part, explain the differences in estimated selfing rates between the two ecotypes. If we assume no inbreeding depression in the non-metallicolous ecotype and the same initial selfing rate of 0·4 for both ecotypes, inbreeding depression for survival in the metallicolous ecotype would have to reach 50 % to explain the difference in estimated selfing rates between the ecotypes when sampled at the adult stage. Preliminary estimates of inbreeding depression in *N. caerulescens* suggest that inbreeding depression for survival and its variation between ecotypes is not sufficient to explain entirely such differences in estimated selfing rates (M. Mousset, unpubl. res.). This strengthens our observation that the two ecotypes do differ in their mating system. If inbreeding depression were higher in metallicolous populations, this could, however, have selected for plant traits resulting in lower selfing rates in these populations.

Variation in plant traits may explain differences in mating system between ecotypes. Traits directly modifying the mating system include floral morphology, floral display (Elzinga *et al*., 2007; Goodwillie *et al*., 2010; Devaux *et al*., 2014) or

phenology, which all can increase pollinator attraction or, alternatively, enable autonomous selfing. Dubois *et al*., (2003) showed that metallicolous populations from the Causses and Cévennes region had a higher ratio of pollen to ovules than non-metallicolous populations, and concluded that this observation is consistent with a more allogamous mating system. Jiménez-Ambriz *et al*. (2007) observed a marginally higher number of flowering stalks in the field for a metallicolous population of *N. caerulescens* from the same region as in our study. It could increase attraction to pollinators, thus increasing outcrossing if more pollinators reach the patch and forage by switching between plants. A larger floral display was, however, sometimes found conversely to increase geitonogamous selfing, i.e. selfing among flowers of the same plant (de Jong *et al*., 1993; Harder and Barrett, 1995; Galloway *et al*., 2002; Lau *et al*., 2008; Devaux *et al*., 2014), so the role of this trait in explaining differences in mating system among ecotypes remains to be tested.

Differences in pollination service in the two types of sites could lead to differences in selfing rates. Pollinators determine the outcrossed fraction of seeds, but may also be responsible for an unknown fraction of selfing, through facilitated selfing (pollinator-mediated within-flower selfing) and geitonogamous selfing (pollinator-mediated between-flower selfing). Differences in diversity, abundance, identity, efficiency or behaviour of pollinators can thus affect self-fertilization rates directly. Metal concentrations in the soil or hyperaccumulating plants may directly affect pollinators through unintentional ingestion of trace elements, or indirectly modify pollinator community through modification of plant community. The diversity, demography and abundance of solitary wild bees were found to decrease along two gradients of heavy metal pollutions (Moroń *et al*., 2012, 2014). These results do not mean we should always expect higher selfing rates in metallicolous populations due to reduced pollination service (Dulya and Mikryukov, 2015). Indeed, some pollinator species are expected to adapt to the edaphic conditions, and the effects on mating system would depend on species identity, abundance and behaviour toward the considered plant species (Meindl and Ashman, 2015). Further studies of plant and pollinator cohorts, as well as their phenologies are needed to characterize pollination accurately in the two ecotypes of *N. caerulescens* and their effects of mating system.

Pollinator behaviour further depends on the spatial distribution of flowering species and thus on factors including density, population fragmentation or marginality. Dubois *et al*. (2003) noted that non-metallicolous populations of *N. caerulescens* seem to have lower densities than metallicolous populations, which could partly explain differences in selfing rates observed between ecotypes. A test of the effect of density on mating system at the fine spatial scale is currently performed in several metallicolous populations of *N. caerulescens* of Southern France.

Consequences of mating system differences for adaptation

Does the moderate difference in selfing rates observed in metallicolous (25 %) and non-metallicolous (40 %) ecotypes impose different constraints on adaptation and demography of the two ecotypes? Selfing causes non-random gamete sampling and hitchhiking effects, which decreases the effective population size. Hitchhiking effects are however weak for intermediate selfing rates as estimated in both ecotypes (Hartfield and Glémin, 2014). All else being equal, non-metallicolous populations would then suffer from a moderate decrease in effective size of about 10 % compared with metallicolous populations due to their more inbred mating system (Charlesworth, 1993, cited in Glémin and Ronfort, 2013). This moderate decrease of effective size should have little effect on the probability of adaptation proceeding from standing variation in the two ecotypes. Our estimates of effective sizes from temporal variance in gene frequency suggest that non-metallicolous populations indeed have a reduced effective size compared with metallicolous populations. Even though the latter estimates are not very precise, the difference in effective size seems to be larger than that expected only from the difference in selfing rates, suggesting that other factors reduce effective population sizes in non-metallicolous populations, such as demography, patchiness, or variance in reproductive success. In particular, estimates of effective size in non-metallicolous populations may have been affected by the stronger demographic stochasticity in these populations.

The higher inbreeding of non-metallicolous populations may still facilitate adaptation through the fixation of *de novo* mutations due to the increased expression of recessive rare mutations. For instance, metallicolous populations would thus have a 22 % lower probability of fixation of *de novo* beneficial recessive mutations than non-metallicolous populations (for a mutation with selection coefficient $s = 0{\cdot}01$ and dominance coefficient $h = 0{\cdot}1$, Glémin and Ronfort, 2013). Purging of recessive deleterious mutations in more inbred populations should result in a lower equilibrium frequency of recessive deleterious mutations in non-metallicolous populations (Gillespie, 1998, p. 96), yielding lower inbreeding depression in these populations (e.g. a decrease of 30 % of inbreeding depression at a single locus for $h = 0{\cdot}1$; Gillespie, 1998, p.96, cited in Glémin, 2003). However, both data (Winn *et al*., 2011) and theoretical predictions based on multilocus models (e.g. Lande and Porcher,2015; Roze, 2015) suggest that the relationship between inbreeding depression and selfing rate is weak for intermediate selfing rates. The smaller effective population size in non-metallicolous populations may also result in fixation of weakly deleterious recessive mutations, increasing the drift load but further depressing inbreeding depression (Glémin, 2003). In conclusion, all else being equal, non-metallicolous populations may show less inbreeding depression than metallicolous populations, while the consequences of higher selfing rates in non-metallicolous populations on their adaptation are expected to be weak to moderate, depending on the genetic basis for adaptation (new mutations vs. standing variation).

Conclusions

Our study confirms the mixed mating system of *N. caerulescens* and provides for the first time precise estimates of selfing rates through two different methods in a set of nearby populations in Southern France. We have discussed several alternative hypotheses that may explain the proximate and ultimate causes

of ecotypic differences in mating system. These hypotheses are now being tested by measures of inbreeding depression, pollinator communities and behaviour, and plant traits in metallicolous and non-metallicolous ecotypes. Testing whether metallicolous and non-metallicolous ecotypes differ in mating system in other regions of the large geographical range of *N. caerulescens* would allow assessment of the generality of the present pattern and better elucidation its causes.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: regression of the pairwise $F_{ST}/(1 - F_{ST})$ in both years on the logarithm of the geographic distances between populations of *Noccaea caerulescens*. Figure S2: projection of individuals in the principal components plane of a principal components analysis in *Noccaea caerulescens*. Figure S3: correlation between selfing rates of *Noccaea caerulescens* derived from F_{IS} and estimated with the RMES method. Table S1: pairwise F_{ST} between populations of *Noccaea caerulescens*.

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LITTERATURE CITED

Antonovics J. 1968. Evolution in closely adjacent plant populations V. Evolution of self-fertility. *Heredity* **23**: 219–238.

Antonovics J. 2006. Evolution in closely adjacent plant populations X: long-term persistence of prereproductive isolation at a mine boundary. *Heredity* **97**: 33–37.

Antonovics J, Bradshaw AD, Turner RG. 1971. Heavy metal tolerance in plants. *Advances in Ecological Research* **7**: 1–85.

Armbruster P, Reed DH. 2005. Inbreeding depression in benign and stressful environments. *Heredity* **95**: 235–242.

Assunção AGL, Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO. 2003*a*. Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159**: 411–419.

Assunção AGL, Schat H, Aarts MGM. 2003*b*. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159**: 351–360.

Baker HG. 1955. Self-compatibility and establishment after 'long-distance' dispersal. *Evolution* **9**: 347–349.

Barrett SC, Husband BC, Cole WW. 1993. Variation in outcrossing rates in *Eichhornia paniculata*: temporal changes in populations of contrasting style morph structure. *Plant Species Biology* **8**: 141–148.

Baumbach H, Hellwig FH. 2007. Genetic differentiation of metallicolous and non-metallicolous *Armeria maritima* (Mill.) Willd. taxa (Plumbaginaceae) in Central Europe. *Plant Systematics and Evolution* **269**: 245–258.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Catch F. 2004. *GENETIX 4.0. 5.2., Software under Windows™ for the genetics of the populations*. University of Montpellier, Montpellier, France.

Besnard G, Basic N, Christin P-A, Savova-Bianchi D, Galland N. 2009. *Thlaspi caerulescens* (Brassicaceae) population genetics in western Switzerland: is the genetic structure affected by natural variation of soil heavy metal concentrations? *New Phytologist* **181**: 974–984.

Bizoux J-P, Daïnou K, Raspé O, Lutts S, Mahy G. 2008. Fitness and genetic variation of *Viola calaminaria*, an endemic metallophyte: implications of population structure and history. *Plant Biology* **10**: 684–693.

Bradshaw AD. 1984. Adaptation of plants to soils containing toxic metals – a test for conceit. *Origins and Development of Adaptation* **102**: 4–19.

Caballero A, Hill WG. 1992. Effects of partial inbreeding on fixation rates and variation of mutant genes. *Genetics* **131**: 493–507.

Chaney RL, Malik M, Li YM, *et al.* 1997. Phytoremediation of soil metals. *Current Opinion in Biotechnology* **8**: 279–284.

Charlesworth B. 1993. Evolutionary mechanisms of senescence. *Genetica* **91**: 11–19.

Charlesworth D. 2006. Evolution of plant breeding systems. *Current Biology* **16**: 726–735.

Cheliak WM, Dancik BP, Morgan K, Yeh FCH, Strobeck C. 1985. Temporal variation of the mating system in a natural population of Jack pine. *Genetics* **109**: 569–584.

Cheptou P-O. 2012. Clarifying Baker's Law. *Annals of Botany* **109**: 633–641.

Coates DJ, Williams MR, Madden S. 2013. Temporal and spatial mating-system variation in fragmented populations of *Banksia cuneata*, a rare bird-pollinated long-lived plant. *Australian Journal of Botany* **61**: 235–242.

Cuguen J, Acheroy M, Loutfi AL, Petit D, Vernet P. 1989. Breeding system differentiation in *Arrhenatherum elatius* populations: evolution toward selfing? *Evolutionary Trends in Plants* **3**: 17–24.

David P, Pujol B, Viard F, Castela V, Goudet J. 2007. Reliable selfing rate estimates from imperfect population genetic data. *Molecular Ecology* **16**: 2474–2487.

Dechamps C, Lefèbvre C, Noret N, Meerts P. 2007. Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. *New Phytologist* **173**: 191–198.

Dechamps C, Noret N, Mozek R, *et al.* 2008. Cost of adaptation to a metalliferous environment for *Thlaspi caerulescens*: a field reciprocal transplantation approach. *New Phytologist* **177**: 167–177.

Deng J, Liao B, Ye M, Deng D, Lan C, Shu W. 2007. The effects of heavy metal pollution on genetic diversity in zinc/cadmium hyperaccumulator *Sedum alfredii* populations. *Plant and Soil* **297**: 83–92.

Devaux C, Lepers C, Porcher E. 2014. Constraints imposed by pollinator behaviour on the ecology and evolution of plant mating systems. *Journal of Evolutionary Biology* **27**: 1413–1430.

Doyle JJ, Doyle JL. 1990. DNA extraction from Arabidopsis. *Focus* **12**: 13–15.

Dray S, Dufour A-B. 2007. The ade4 package: implementing the duality diagram for ecologists. *Journal of Statistical Software* **22**: 1–20.

Dubois S. 2005. *Étude d'un réseau de populations métallicoles et non-métallicoles de Thlaspi caerulescens (Brassicaceae). Structure génétique, démographie et pressions de sélection*. PhD thesis, Université Montpellier II Sciences et Techniques du Langedoc, Montpellier, France.

Dubois S, Cheptou P-O, Petit C, *et al.* 2003. Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* **157**: 633–641.

Dulya OV, Mikryukov VS. 2015. Genetic variation and selfing rate in *Lychnis flos-cuculi* along an industrial pollution gradient. *New Phytologist*. doi:10.1111/nph.13636.

Eckert CG, Ozimec B, Herlihy CR, Griffin CA, Routley MB. 2009. Floral morphology mediates temporal variation in the mating system of a self-compatible plant. *Ecology* **90**: 1540–1548.

Elzinga JA, Atlan A, Biere A, Gigord L, Weis AE, Bernasconi G. 2007. Time after time: flowering phenology and biotic interactions. *Trends in Ecology and Evolution* **22**: 432–439.

Enjalbert J, David JL. 2000. Inferring recent outcrossing rates using multilocus individual heterozygosity: application to evolving wheat populations. *Genetics* **156**: 1973–1982.

Epinat G, Lenormand T. 2009. The evolution of assortative mating and selfing with in- and outbreeding depression. *Evolution* **63**: 2047–2060.

Ernst W. 2005. Phytoextraction of mine wastes – options and impossibilities. *Chemie der Erde – Geochemistry* **65** Supplement 1: 29–42.

Escarré J, Lefèbvre C, Gruber W, *et al.* 2000. Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. *New Phytologist* **145**: 429–437.

Escarré J, Lefèbvre C, Raboyeau S, *et al.* 2011. Heavy metal concentration survey in soils and plants of the Les Malines mining district (Southern France): implications for soil restoration. *Water, Air, and Soil Pollution* **216**: 485–504.

Fones HN, Eyles CJ, Bennett MH, Smith JAC, Preston GM. 2013. Uncoupling of reactive oxygen species accumulation and defence signalling in the metal hyperaccumulator plant *Noccaea caerulescens*. *New Phytologist* **199**: 916–924.

Fox CW, Reed DH. 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution* **65**: 246–258.

Galloway LF, Cirigliano T, Gremski K. 2002. The contribution of display size and dichogamy to potential geitonogamy in *Campanula americana*. *International Journal of Plant Sciences* **163**: 133–139.

Gillespie JH. 1998. *Population genetics: a concise guide*. Baltimore, MD: The John Hopkins University Press.

Glémin S. 2003. How are deleterious mutations purged? Drift versus nonrandom mating. *Evolution* **57**: 2678–2687.

Glémin S, Ronfort J. 2013. Adaptation and maladaptation in selfing and outcrossing species: new mutations versus standing variation. *Evolution* **67**: 225–240.

Gonneau C. 2014. *Distribution, écologie et évolution de l'hyperaccumulation des éléments en traces par Noccaea caerulescens*. PhD thesis, Université de Lorraine, Nancy, France.

Goodwillie C, Kalisz S, Eckert CG. 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution, and Systematics* **36**: 47–79.

Goodwillie C, Sargent RD, Eckert CG, *et al.* 2010. Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. *New Phytologist* **185**: 311–321.

Goudet J. 2005. hierfstat, a package for r to compute and test hierarchical F-statistics. *Molecular Ecology Notes* **5**: 184–186.

Hamrick JL, Godt MJW. 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society B: Biological Sciences* **351**: 1291–1298.

Hanikenne M, Nouet C. 2011. Metal hyperaccumulation and hypertolerance: a model for plant evolutionary genomics. *Current Opinion in Plant Biology* **14**: 252–259.

Harder LD, Barrett SCH. 1995. Mating cost of large floral displays in hermaphrodite plants. *Nature* **373**: 512–515.

Hartfield M, Glémin S. 2014. Hitchhiking of deleterious alleles and the cost of adaptation in partially selfing species. *Genetics* **196**: 281–293.

Hereford J. 2010. Does selfing or outcrossing promote local adaptation? *American Journal of Botany* **97**: 298–302.

Holden LR. 1979. New properties of the two-locus partial selfing model with selection. *Genetics* **93**: 217–236.

Jarne P, David P. 2008. Quantifying inbreeding in natural populations of hermaphroditic organisms. *Heredity* **100**: 431–439.

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O. 2007. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**: 199–215.

Jombart T, Ahmed I. 2011. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics* **27**: 3070–3071.

de Jong TJ, Waser NM, Klinkhamer PGL. 1993. Geitonogamy: the neglected side of selfing. *Trends in Ecology and Evolution* **8**: 321–325.

Knuth P. 1908. *Handbook of flower pollination*, **Vol. II**. Oxford: Clarendon Press.

Koch M, Mummenhoff K, Hurka H. 1998. Systematics and evolutionary history of heavy metal tolerant *Thlaspi caerulescens* in Western Europe: evidence from genetic studies based on isozyme analysis. *Biochemical Systematics and Ecology* **26**: 823–838.

Lande R, Porcher E. 2015. Maintenance of quantitative genetic variance under partial self-fertilization, with implications for evolution of selfing. *Genetics* **200**: 891–906.

Lande R, Schemske DW. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* **39**: 24–40.

Lau JA, Miller RE, Rausher MD. 2008. Selection through male function favors smaller floral display size in the common morning glory *Ipomoea purpurea* (Convolvulaceae). *American Naturalist* **172**: 63–74.

Lefèbvre C. 1970. Self-fertility in maritime and Zinc mine populations of *Armeria maritima* (Mill.) Willd. *Evolution* **24**: 571–577.

Lefèbvre C. 1973. Outbreeding and inbreeding in a zinc–lead mine population of *Armeria maritima*. *Nature* **243**: 96–97.

Lefèbvre C. 1976. Breeding system and population structure of *Armeria maritima* (Mill.) Willd. on a zinc–lead mine. *New Phytologist* **77**: 187–192.

Leimu R, Fischer M. 2008. A meta-analysis of local adaptation in plants. *PLoS One* **3**: e4010. doi: 10.1371/journal.pone.0004010.

Levin DA. 2010. Environment-enhanced self-fertilization: implications for niche shifts in adjacent populations. *Journal of Ecology* **98**: 1276–1283.

Lopez S, Rousset F, Shaw FH, Shaw RG, Ronce O. 2008. Migration load in plants: role of pollen and seed dispersal in heterogeneous landscapes. *Journal of Evolutionary Biology* **21**: 294–309.

Loveless MD, Hamrick JL. 1984. Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics* **15**: 65–95.

Macnair MR. 1987. Heavy metal tolerance in plants: a model evolutionary system. *Trends in Ecology and Evolution* **2**: 354–359.

McNeilly T, Antonovics J. 1968. Evolution in closely adjacent plant populations IV. Barriers to gene flow. *Heredity* **23**: 205–218.

Meerts P, Van Isacker N. 1997. Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133**: 221–231.

Meindl GA, Ashman T-L. 2015. Effects of floral metal accumulation on floral visitor communities: introducing the elemental filter hypothesis. *American Journal of Botany* **102**: 379–389.

Mengoni A, Gonnelli C, Galardi F, Gabbrielli R, Bazzicalupo M. 2000. Genetic diversity and heavy metal tolerance in populations of *Silene paradoxa* L. (Caryophyllaceae): a random amplified polymorphic DNA analysis. *Molecular Ecology* **9**: 1319–1324.

Meyer C-L, Vitalis R, Saumitou-Laprade P, Castric V. 2009. Genomic pattern of adaptive divergence in *Arabidopsis halleri*, a model species for tolerance to heavy metal. *Molecular Ecology* **18**: 2050–2062.

Moroń D, Grześ IM, Skórka P, *et al.* 2012. Abundance and diversity of wild bees along gradients of heavy metal pollution. *Journal of Applied Ecology* **49**: 118–125.

Moroń D, Szentgyörgyi H, Skórka P, Potts SG, Woyciechowski M. 2014. Survival, reproduction and population growth of the bee pollinator, *Osmia rufa* (Hymenoptera: Megachilidae), along gradients of heavy metal pollution. *Insect Conservation and Diversity* **7**: 113–121.

Mousset M, Flaven E, Justy F, *et al.* 2015. Characterization and multiplexing of 21 microsatellite markers for the herb *Noccaea caerulescens* (Brassicaceae). *Applications in Plant Sciences* **3**: 1500052.

Nordborg M. 2000. Linkage disequilibrium, gene trees and selfing: an ancestral recombination graph with partial self-fertilization. *Genetics* **154**: 923–929.

Noret N, Meerts P, Vanhaelen M, Santos AD, Escarré J. 2007. Do metal-rich plants deter herbivores? A field test of the defence hypothesis. *Oecologia* **152**: 92–100.

Nychka D, Furrer R, Sain S. 2014. Tools for spatial data. *R package version*, **6**(11).

Pannell JR. 2015. Evolution of the mating system in colonizing plants. *Molecular Ecology* **24**: 2018–2037.

Pauwels M, Vekemans X, Godé C, Frérot H, Castric V, Saumitou-Laprade P. 2012. Nuclear and chloroplast DNA phylogeography reveals vicariance among European populations of the model species for the study of metal tolerance, *Arabidopsis halleri* (Brassicaceae). *New Phytologist* **193**: 916–928.

Pauwels M, Willems G, Roosens N, Frérot H, Saumitou-Laprade P. 2008. Merging methods in molecular and ecological genetics to study the adaptation of plants to anthropogenic metal-polluted sites: implications for phytoremediation. *Molecular Ecology* **17**: 108–119.

R Core Team. 2014. *R: a language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rigola D, Fiers M, Vurro E, Aarts MGM. 2006. The heavy metal hyperaccumulator *Thlaspi caerulescens* expresses many species-specific genes, as identified by comparative expressed sequence tag analysis. *New Phytologist* **170**: 753–766.

Rileys R. 1956. The influence of the breeding system on the genecology of *Thlaspi alpestre* L. *New Phytologist* **55**: 319–330.

Ritland K. 2002. Extensions of models for the estimation of mating systems using n independent loci. *Heredity* **88**: 221–228.

Ronce O, Shaw FH, Rousset F, Shaw RG. 2009. Is inbreeding depression lower in maladapted populations? A quantitative genetics model. *Evolution* **63**: 1807–1819.

Rousset F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* **145**: 1219–1228.

Rousset F. 2008. genepop'007: a complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* **8**: 103–106.

Roze D. 2015. Effects of interference between selected loci on the mutation load, inbreeding depression, and heterosis. *Genetics* **201**: 745–757.

Słomka A, Sutkowska A, Szczepaniak M, Malec P, Mitka J, Kuta E. 2011. Increased genetic diversity of *Viola tricolor* L. (Violaceae) in metal-polluted environments. *Chemosphere* **83**: 435–442.

Stebbins GL. 1957. Self-fertilization and population variability in the higher plants. *American Naturalist* **91**: 337–354.

Verbruggen N, Hermans C, Schat H. 2009. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist* **181**: 759–776.

Wang J, Whitlock MC. 2003. Estimating effective population size and migration rates from genetic samples over space and time. *Genetics* **163**: 429–446.

Wang J, El-Kassaby YA, Ritland K. 2012. Estimating selfing rates from reconstructed pedigrees using multilocus genotype data. *Molecular Ecology* **21**: 100–116.

Weir BS, Cockerham CC. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.

Winn AA, Elle E, Kalisz S, *et al.* 2011. Analysis of inbreeding depression in mixed-mating plants provides evidence for selective interference and stable mixed mating. *Evolution* **65**: 3339–3359.

Wolfram Research, Inc. 2012. *Mathematica*. Champaign, IL, USA.

Wright SI, Kalisz S, Slotte T. 2013. Evolutionary consequences of self-fertilization in plants. *Proceedings of the Royal Society B: Biological Sciences* **280**: 20130133.

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CHAPITRE 2

EFFECT OF DENSITY ON THE SELFING RATE OF *NOCCAEA CAERULESCENS* IN METALLICOLOUS POPULATIONS OF SOUTH OF FRANCE

Abstract

Mating system can strongly affect levels of adaptation within populations. Here we explore how this variation affects adaptation to heterogeneous selection in the pseudometallophyte *Noccaea caerulescens*, which can grow on both toxic mine wastes and normal soils. It has higher self-fertilisation rates in non-metallicolous populations, which have lower densities of flowering plants than metallicolous populations. We tested whether variation of density alone could explain variation in selfing rates by analysing the variation of mating system between plants with different local densities of flowering neighbours, within two mines, using two methods to estimate selfing rates. We found no effect of density on the selfing rates of *Noccaea caerulescens* in either population. These results suggest that density, at least on this local scale, has little effect on mating system in this species.

Introduction

Mating systems in general and self-fertilization in particular have complex effects on adaptation to new environments because selfing can affect demographic dynamics after colonization as well as the evolutionary forces of selection, drift and gene flow (Levin 2010; Glémin and Ronfort 2013; Peterson and Kay 2015). For instance, some plants have colonized and adapted to highly toxic metal-contaminated soils, such as mine wastes. The Brassicaceae *Noccaea caerulescens* is one of them, with populations living on regular, uncontaminated soils, and populations having colonized highly contaminated former mines (Meerts and Van Isacker 1997; Escarré *et al.* 2000; Assunção, Bookum, *et al.* 2003). *Noccaea caerulescens* has a mixed mating system (Koch *et al.* 1998; Dubois *et al.* 2003; Jiménez-Ambriz *et al.* 2007; Besnard *et al.* 2009; Mousset *et al.* 2016). In a seminal paper, Antonovics (1968) predicted that plants living within a mine should self-fertilize more than their congeners outside the mine to prevent the import of non-adapted genes. This prediction was confirmed by empirical estimates of selfing rates (Antonovics 1968; Lefèbvre 1970; Cuguen *et al.* 1989). Yet, several recent studies of mating system variation in metal-tolerant plants have found the reverse pattern (Dubois *et al.* 2003; Dulya and Mikryukov 2015; Mousset *et al.* 2016). This suggests that mating systems are also constrained by variation in pollination environment, as along pollution gradients. Such constraints, were often ignored by early genetic models on the evolution of mating system. Because of relaxed competition with other species, conspecific density can be high in polluted sites (Dulya and Mikryukov 2015) and it was hypothesized that it may facilitate outcrossing in former mines, compared to unpolluted sites (Koch *et al.* 1998; Dubois *et al.* 2003). We here test the idea that higher density of neighbouring conspecifics at a local scale is associated with higher outcrossing in *N. caerulescens* populations in the South of France.

Higher densities can increase the rate at which pollinators visit plants (Klinkhamer and de Jong 1990; Kunin 1993, 1997; Grindeland *et al.* 2005; Feldman 2008, but see Bosch and Waser 1999, 2001; Mustajärvi *et al.* 2001; Dauber *et al.* 2010). Higher attractiveness, in turn, often leads to higher seed set in plants occurring in dense patches (Kunin 1993, 1997; Bosch and Waser 1999, 2001; Feldman 2008), although this can depend on species (Bosch and Waser 2001) or population (Dauber *et al.* 2010). Besides increasing plant attractiveness to pollinators, density modifies foraging behaviour in other important ways. Several authors have shown that at high densities, pollinators fly more *between* plants than *within* plants (Karron *et al.* 1995), visit fewer flowers on a single plant (Klinkhamer and de Jong 1990; Bosch and Waser 1999;

Mustajärvi *et al.* 2001; Grindeland *et al.* 2005) and switch more between plants (Mustajärvi *et al.* 2001). This type of behaviour can explain that Murawski and Hamrick (1991) found more pollen donors in high-density patches than in low density patches. Because of these behaviours, plants growing in high-density patches or populations should outcross more than isolated plants. Indeed, higher densities are often associated with higher outcrossing rates (Farris and Mitton 1984; Vaquero *et al.* 1989; Murawski and Hamrick 1991, 1992; van Treuren *et al.* 1993, 1994; Karron *et al.* 1995; Franceschinelli and Bawa 2000; Duncan *et al.* 2004). There are some exceptions, where higher density was associated with higher selfing rates, which were sometimes attributed to biparental inbreeding wrongly assigned to selfing (Ellstrand *et al.* 1978; Watkins and Levin 1990). The effect of density on pollinators visitation rates, seed set and outcrossing rate may thus vary, depending on the studied pollinators, the plant species, the scale of the experiment, or even the year for a given population (Kunin 1993, 1997; Bosch and Waser 2001; Feldman 2008; Dauber *et al.* 2010). Nevertheless, density remains a prime factor likely to affect mating system variation in natural populations.

A confounding factor in studies of how plant density affects mating systems in the floral display, the number of open flowers on a plant. This also modifies pollinator behaviour and by consequence, mating system. Larger display sizes can increase plant visitation rates (Grindeland *et al.* 2005), and the number of flowers pollinated on the same plant (Hessing 1988; Robertson 1992; de Jong *et al.* 1993; Robertson and MacNair 1995; Kunin 1997; Ohashi and Yahara 1998; Grindeland *et al.* 2005). Floral display size can also affect the proportion of within-plant movements compared to among-plants movements (Karron *et al.* 2004), affecting rated of between-flowers selfing (geitonogamous selfing). Geitonogamy is common in hermaphroditic entomophilous plants and has been shown to increase with floral display size (de Jong *et al.* 1993; Harder and Barrett 1995; Galloway *et al.* 2002; Karron *et al.* 2004, 2009; Lau *et al.* 2008; Goodwillie *et al.* 2010).

In *Noccaea caerulescens*, we sought to test how plant density and display size influences mating system in ways that might drive differences in mating system observed between polluted and non-polluted sites. To separate the effect of plant density from other sources of environmental variation that might affect pollinators' communities and behaviours, we tested the effect of density on selfing rates within-populations, in two different metallicolous populations where the local density of flowering plants varied more than within non-metallicolous populations. In the first population, we studied the effect of the distance to the closest neighbours (i.e. of local density) on individual selfing rates, controlling for the number of

inflorescences of the focal plant. The density of plants in that population was very high during that reproductive season. We thus also studied a second population showing more spatial variation in plant density to test the relationship between plant density and selfing rates. In this second population, we tested the effect of density on selfing rates at a slightly higher spatial scale. Density did not influence selfing rates in either of these two populations. In the first population (where it could be tested), we also observed no increase in selfing rates in response to the number of floral stalks.

Material & Methods

Species

Noccaea caerulescens J. Presl & C. Presl F.K. Mey grows from Mediterranean regions to Czechoslovakia and Scandinavia (Rileys 1956; Ingrouille and Smirnoff 1986; Koch *et al.* 1998). It flowers in spring and has a mostly annual life cycle in the South of France (Dubois 2005). It grows one to several dozen inflorescences, with white to purplish small flowers. Its main pollinators in the South of France region, where we conducted our study have not been extensively described, but we observed visitations of Hymenoptera (domesticated and wild bees, bumblebees), small Diptera and Lepidoptera (I. Decombeix, A. Mignot, C. Petit, L. Prat, ISEM, Montpellier, pers. comm.). These pollinators broadly correspond to those previously observed in Germany and Great Britain (Knuth 1908; Rileys 1956).

Studied sites

The Saint Bresson site (49.943231N 3.632352E) is a metalliferous site in Southern France, located in the Cevennes mountains, north of Montpellier. It is close to Ganges and Saint-Laurent-le-Minier, where several *N. caerulescens* accessions come from (e.g. Lombi et al., 2000; Assunção et al., 2003; Halimaa et al., 2014). The high concentrations of Lead, Zinc and Cadmium found in the soil (Dubois *et al.* 2003) derive from past mining activities (“Mine de Mas Lacombe”, in Pommiers), which stopped in 1925 (Dubois 2005). The Saint Bresson site is heterogeneous, with a large portion of the population growing on bare, exposed soil with very low vegetation richness and cover, and another part growing on a slightly more shaded area, with higher vegetation cover (Dubois 2005). The soil is a schistous substrate and its pH ranges from 6.7 under the trees to 7.2 on open, bare areas (Dubois 2005). The population is at an altitude of 450 m and has a sunny orientation.

The Saint Hippolyte population (43.971409N; 3.833329E) is located on a former mining site ("Mine de la Boissière", closed in 1955) nearby Saint-Hippolyte-du-Fort, and 16 km from the SB population. It seemed to have a larger variation of density within the population than the SB population. The population is at an altitude of 248 m and has a dolomitic soil.

Methods used to estimate self-fertilization rates

We use two methods to estimate selfing rates in each population and relate these estimated rates to the pollination environment. These methods are based on two different types of sampling. The MLTR software (Ritland 2002) uses family structured data to infer population or individual mating system parameters. The estimated selfing rate represents a snapshot of the selfing rate in the previous generation. This method was used in the Saint Bresson population (SB), to obtain individual selfing rates for different focal plants, using the information provided by both the genotype of the focal plant (the mother) and the genotype of twenty seedlings. In contrast, the RMES software (David *et al.* 2007) uses the distribution of heterozygosity across all loci to infer selfing rates in any user-defined group of individuals (e.g. a population or a sub-population). This method integrates effects of selfing on heterozygosity over several generations and is robust to several problems of microsatellite data, such as the presence of null alleles or partial dominance. This method was used in the Saint Hippolyte population (HI) to estimate mean selfing rates among different quadrats to test whether selfing rates depend on conspecific density within the quadrats.

Sampling

Saint Bresson population – Progeny Arrays Approach

We selected twenty "focal" plants on two transects crossing the open part of the population, with at least one meter between them and marked them using coloured wire. We measured the distance to the ten closest flowering *N. caerulescens* neighbours (an inverse measure of local density of conspecifics). On the focal plants and on the neighbours, we recorded the number of inflorescences as a proxy of floral display. We used the distance to the tenth most remote neighbour to calculate a mean number of inflorescences per m^2 in the immediate proximity of the focal plant. Hereafter, we will call the focal plant and its ten closest neighbours a "patch", for simplification.

At the beginning of April 2011, one leaf of each focal plant was collected and desiccated in paper bags filled with silica gel. In May 2011, the inflorescences from the focal plants harbouring ripe fruits were collected. In January 2012, seeds from each focal plant were sown together in rectangular containers filled with vermiculite. Pots were randomized in a temperate glasshouse that belongs to the Plateforme des Terrains d'Expériences du LabEx CeMEB (Montpellier) and watered when needed. In February and March 2012, twenty seedlings per focal plant (the "progeny array") were collected at the two- or four-leaves stage, dried 24h at 60°C and kept using silica gel until genotyping.

Saint Hippolyte population – Population Structure Approach

In April 2014, we first randomly picked coordinates in the population to obtain an estimate of how the density of *N. caerulescens* varies in the population. At these points, we measured the number of *N. caerulescens* in a circle of 75 cm diameter. This yielded 134 density "points" across the population.

Secondly, we placed quadrats in high and low density areas in several sectors of the mine (hereafter called "zones") that had been previously defined (**Figure 1**). The size of the quadrat was adjusted to the density of the area so that we could sample seeds on fifteen to twenty individuals. We estimated the area of the quadrat and used it to estimate the density of *Noccaea caerulescens* as the number of flowering individuals per m^2 for each quadrat (**Table 1**). This generated two groups of quadrats, with respectively mean density of 12.1 plants.m^{-2} (sd = 4.6) and 118.2 plants.m^{-2} (sd = 69.8). We sampled different zones of the mine to obtain estimates of selfing rates in the whole population. This spatial level will, however, only be used in the AMOVA analysis (see below).

However, this sampling did not allow us to include isolated individuals so we collected seeds from isolated plants in the zones and measured the local density within a one-meter radius circle around these plants (**Figure 1**). Five isolated quadrats were added, with a mean density of 0.8 (sd = 0.4), accounting for 14 additional individuals.

Table 1. Area and quadrat densities of the HI population.

Zone	Quadrat	Area (m^2)	Density of individuals ($./m^2$)	Density of inflorescences ($./m^2$)	Number of genotyped individuals	Included in the AMOVA
1	Q2	2.56	9.8	17.6	16	Yes
2	Q3	1.24	12.9	26.6	18	Yes
4	Q1	2.08	15.4	38.0	18	No
5	Q2	1.49	5.4	12.7	18	Yes
5	Q3	2.07	16.9	35.7	17	Yes
1	Q9	0.79	29.3	100.6	19	Yes
1	Q10	0.79	178.3	238.1	19	Yes
2	Q9	0.79	180.8	301.8	20	Yes
3	Q9	0.79	63.7	168.1	11	No
5	Q5	0.79	182.1	527.1	15	Yes
5	Q8	0.79	75.1	163.0	19	Yes

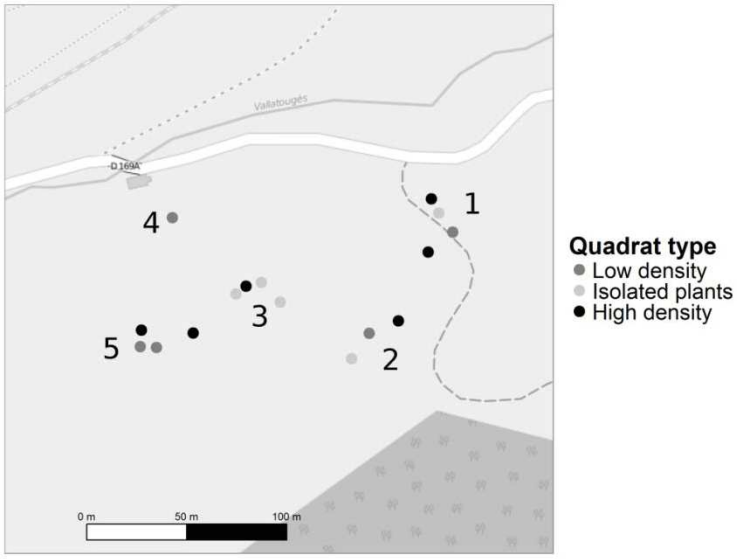

Figure 1. Map of the sampling scheme in the Saint Hippolyte population. Grey: low density quadrats. Light grey: isolated individuals. Dark: high-density quadrats. Numbers represent the "zone" of the mine were the quadrats were set.

In October 2015, two seeds per plant were sown in individual pots filled with a sterilized mix (1/3 regular soil, 1/3 potting substrate, 1/3 sand), randomized in the same glasshouse as in the previous experiment, and watered when needed. On the 20th and 21th of December 2015, all seedlings were collected, dried 72h at 40°C and conserved using silica-gel until genotyping. This resulted in 87 plants in the low-density group (101 including the isolated plants) and 103 plants in the high-density group.

Microsatellite genotyping

Neutral diversity was assessed in Saint Bresson (20 mothers with 20 seedlings each) and in Saint Hippolyte (204 plants from two densities groups) using 15 microsatellite loci organized in two multiplexes (NcM1 and NcM2) and following the protocol described in Mousset *et al.* (2016). Two microsatellites markers, 6g4 and 6e4 (Jiménez-Ambriz *et al.* 2007) were added to NcM1 and NcM2 respectively, following the same protocol (6g4: fluorochrom PET, final concentration 0.8 µM; 6e4: fluorochrom 6FAM, final concentration: 0.4 µM). DNA was extracted using a classic CTAB protocol (Doyle 1990), amplified using PCR, and genotyped using capillary electrophoresis on ABI PRISM 3130 xl Genetic Analyzer (SB) and ABI PRISM 3500 xl Genetic Analyzer (HI). Raw data were analysed using GeneMapper® version 5.0 (Applied Biosystems). In the SB population, null alleles were obvious for some markers in some families; Ncpm31 and Tc-up2 were removed from the analysis, thus performed on 13 loci. These data were produced through the technical facilities of the LabEx "Centre Méditerranéen de l'Environnement et de la Biodiversité", through the GENSEQ platform.

Statistical analysis

Estimation of selfing rates in SB

We used MLTR (version 3.3; Ritland, 2002) to estimate the self-fertilization rate of each focal plant in the SB population, using both progeny and focal plant genotypes, and using the method of moments, most adapted given our progeny array sizes. We asked several questions:

1) Do higher selfing rates of the focal plants correlate with longer mean distance to the ten closest neighbours (hereafter "distance to neighbours", an inverse function of density)? We expect this relation if pollinators switch more among plants of a same patch when the local density is high and/or if pollinators stay longer on isolated plants, increasing facilitated or geitonogamous selfing rates on these plants. We controlled for the number of inflorescences of the focal plant, as more inflorescences (i.e. a larger floral display) are expected to increase (geitonogamous) selfing rates.
2) Similarly, we tested whether the mean number of inflorescences per m^2, which is another proxy for density, had an effect on selfing rates, controlling for floral display of the focal plant.

3) Does biparental inbreeding decrease with increasing distance to flowering neighbours? We would expect this if limited dispersion of the seeds leads to related individual clustering together and if pollinators switch more between close (related) plants.

We studied the relation between selfing rate, mean distance to neighbours (respectively mean number of inflorescences.m^{-2}) and the number of inflorescence of the focal plant with a linear model. Selfing rate was the response variable, and mean distance to neighbours (respectively mean number of inflorescences.m^{-2}), number of inflorescences of the focal individual and their interactions were the explanatory variables. We used non-parametric Spearman correlation test to study the relationship between biparental inbreeding and distance to neighbours, and the number of flowering stalks in the focal and in the patch. All analysis were performed in R (R Core Team, 2014).

Hierarchical structure analysis in HI

We checked the RMES assumption of no genetic sub-structuration of the sample by investigating the structure of genetic variation between zones and quadrats with an AMOVA. We performed this analysis on zones one, two and five, where we had more than one quadrat, with more than 15 sequenced individuals (**Table 1**). We used the HIERFSTAT R package (R Core Team, 2014; Goudet, 2005) to partition genetic variance among areas, quadrats within zones, and plants within quadrats (95% confidence intervals were based on 1000 bootstraps).

Estimation of selfing rates in HI

We estimated selfing rates in the HI dataset with RMES (David *et al.* 2007). RMES maximizes likelihood (precision: 0.0001) to estimate selfing rates (along with the 95% confidence intervals), and this enables us to use likelihood-ratio tests (LRT) to test for differences in selfing rates between different groups (a single selfing rate in all groups versus a different selfing rate in each group).

We first estimated selfing rates at the smallest scale that we had: quadrats. For each density level, we first estimated selfing rate for each quadrat. We then tested with a LRT test whether a model with different selfing rates for each quadrat was better than a model with a constrained selfing rate for all quadrats belonging to the same class of density. We then compared the two levels of densities by summing the log-likelihood of the two constrained models (one selfing rate for all quadrats within each density level), and comparing this sum to the log-likelihood of a model assuming a constrained selfing rate for all quadrats of both densities.

Because selfing rates seemed more variable in one density level than in the other, we decided to perform a slightly more complex analysis, that compares models with either varying mean and variance of selfing rates. A similar approach was used in Mousset *et al.* (2016). This analysis assumes that in each density level, quadrat selfing rates follow a logit-normal distribution with mean *s* and standard deviation *sd*. It estimates the *s* and *sd* that maximize the likelihood over all data. It is also possible to maximize likelihood under different constraints (for example, assuming that the distributions in both low and high density have different means or standard deviation). Models with and without a constraint are then compared using standard likelihood-ratio tests. This analysis was performed using a Mathematica 9 routine (Wolfram Research, Inc, 2012). Details can be found in Mousset *et al.* (2016).

Secondly, because our analysis of genetic structure showed that most of within population structure was explained by the zone, we pooled the individuals of all quadrats and isolated individuals within the zone, and estimated a selfing rate per zone and density level. The same procedure as described above was used to compare selfing rates between zones (within density levels), and then to compare the selfing rates of the two density levels. The results of this analysis are presented in Appendix S1.

Results

Saint Bresson

The mean distance to neighbours was small in SB, ranging from 9 cm to 78 cm (mean = 28 cm; sd = 18 cm) which indicated high densities of plants. Focal plants produced between 4 and 46 inflorescences (mean = 16; sd = 12), and the "patch" mean number of inflorescences per plant, including the focal plant, ranged from 2 to 16 (mean = 6; sd = 3). This yielded a mean number of inflorescences per square meter of 312 (sd = 446). The very high standard deviation was due to a patch with extremely close neighbours. Excluding this point, there were 211 inflorescences on average per square meter (sd = 154).

Individual selfing rates estimated in the SB population were low in general, ranging from zero to 0.34 (mean = 0.13; sd = 0.1). There was no effect of the conspecific density, the number of inflorescences of the focal plant, or their interaction on selfing rates of focal plants (density: t = -1.8, p = 0.1, **Figure 2A**; inflorescences of focal: t=-1.05p=0.3, **Figure 2B**; interaction: t = -1.054, p = 0.3). The whole model did not significantly improve the model with just the

intercept (whole model adjusted R^2 = 0.01, p = 0.4). Using the local density of inflorescences instead of the mean distance to the ten closest neighbours did not change this conclusion (whole model adjusted R^2 = -0.08, p = 0.65; **Figure 2C**).

The difference of multilocus and single locus estimates of outcrossing rates, a proxy for biparental inbreeding, was low on average (mean = 0.05; sd = 0.09) and not higher when distance to neighbours was smaller (ρ = 0.03, p = 0.89, **Figure 2D**).

Plant growing among other plants with many inflorescences had more inflorescences themselves (ρ = 0.53, p = 0.02; **Figure 2E**), suggesting the existence of "good patches" and "bad patches" in the population.

Saint Hippolyte

Out of 134 random density points, 84 were empty (**Figure 3**). The mean density of the 50 remaining ones was 22.3 plants.m^{-2} (sd = 43). This confirms that *N. caerulescens* has a patchy distribution in its populations. The low-density group of quadrats had an average of 12.1 plant.m^{-2} (sd = 4.6) and the high density group of quadrats had an average of 118.2 plant.m^{-2} (sd = 69.8). The density of inflorescences per quadrat was varying in the same direction: 26.6 inflorescence.m^{-2} (sd = 11.0) for the low density group and 249.8 inflorescence.m^{-2} (sd = 152.5) for the high density group.

Areas of the mine were significantly genetically differentiated from each other (F_{ZONE} = 0.14; **Table 2**) and even the quadrats within each zone accounted for a small but significant part of the genetic variation ($F_{QUADRAT}$ = 0.06; **Table 2**).

The selfing rate did not significantly differ between quadrats within density levels. (LOW: s_{LOW} = 0.38 (95%CI: 0.35 - 0.45); HIGH: s_{HIGH} = 0.28 (95% CI: 0.21 - 0.34); **Table 3, Figure 4**). The selfing rates of the two density levels were not significantly different either (p = 0.15; **Table 3**), and the constrained selfing rate for both densities was s=0.33 (95%CI: 0.30 - 0.38). Adding isolated individuals to this analysis, and estimating selfing rates within a zone for each density levels rather than quadrats did not change these conclusions (Appendix S1).

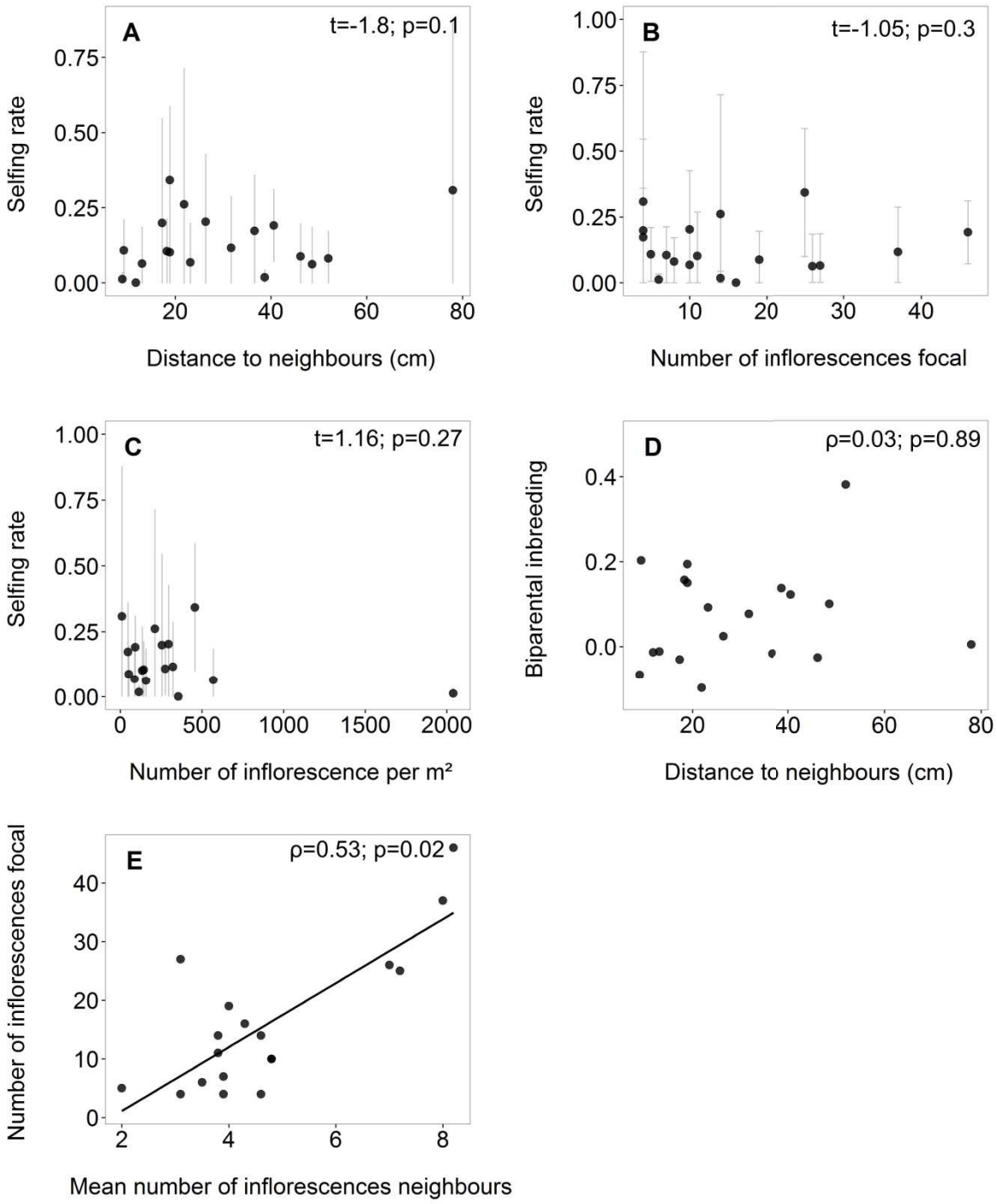

Figure 2. Selfing rate, mean distance to neighbours, number of inflorescences per m² and biparental inbreeding in SB. A) Selfing rates and mean distance to the ten closest neighbours. B) Selfing rates and the total number of inflorescence of the focal. C) Selfing rates and the density of inflorescences in the patch. D) Biparental inbreeding and the mean distance to the ten closest neighbours. E) Number of inflorescences of the focal plant and mean number of inflorescences of the neighbours. Individual selfing rates and biparental inbreeding were estimated using MLTR with the method of moments.

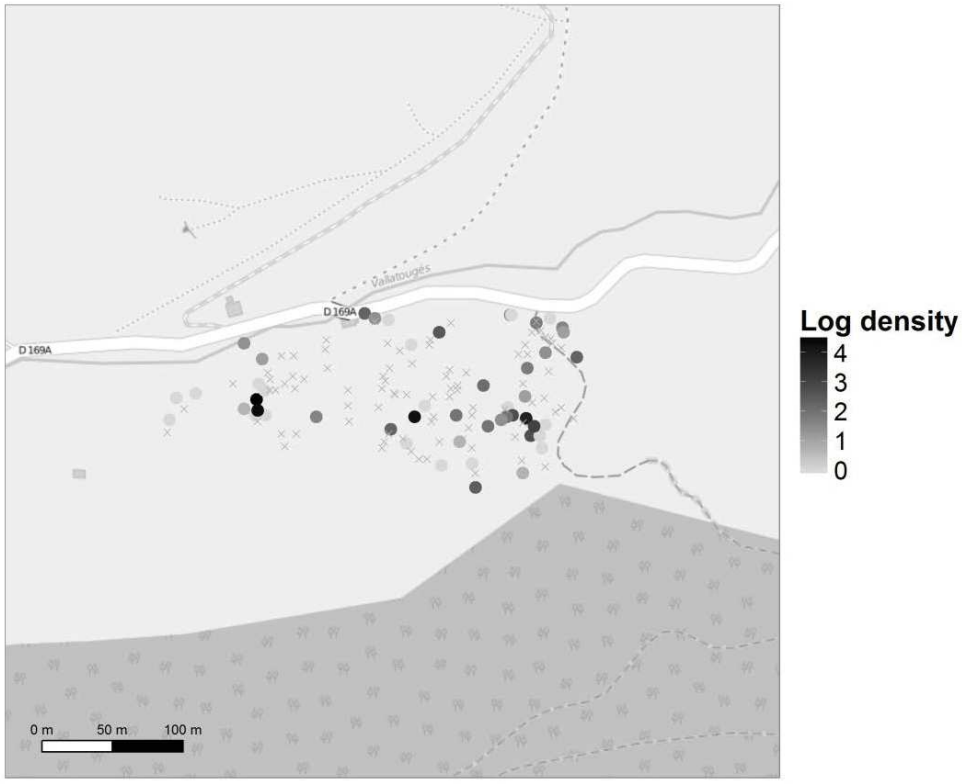

Figure 3. Random density points throughout the HI population. Crosses: empty points. Circles: points with at least one plant. The maximum density that was measured was 90 plants in 0.44 m^2, that is, 203.7 plants.m^{-2}

Table 2. Hierarchical *F*-statistics at different levels of structuration in HI. Analysis was performed with *hierfstat*, and confidence intervals were obtained through 1000 bootstraps at the level just under the tested level. Zones and quadrats include both levels of densities.

Level	***F***	**C.I.**
Between zones	0.14	0.09 – 0.17
Between quadrats within zones	0.06	0.04 – 0.08
Among individuals within quadrats	0.13	0.1 – 0.16

C.I., confidence interval

The analysis that compares models with different and constrained means and variances of selfing rates for each density levels indicates that the best model is the model with the same mean selfing rate in each group and the same variance of selfing rates among quadrats within each density group (**Table 4**, **Figure 5**).

Table 3. Comparison of selfing rates between quadrats within densities, and between densities. Model comparison was performed using Likelihood Ratio Tests on constrained and unconstrained models.

Density	Model	Selfing rate	Log-likelihood	Deviance	D.f.	p
LOW	One selfing rate per quadrat		-548.75	5.98	4	0.2
	One selfing rate for all quadrats	0.38	-551.74			
HIGH	One selfing rate per quadrat		-741.63	9.09	5	0.11
	One selfing rate for all quadrats	0.28	-746.17			
ALL	One selfing rate per density level		-1297.91	2.09	1	0.15
	One selfing rate for the two densities levels	0.33	-1298.96			

D.f.:degrees of freedom; p: p-value

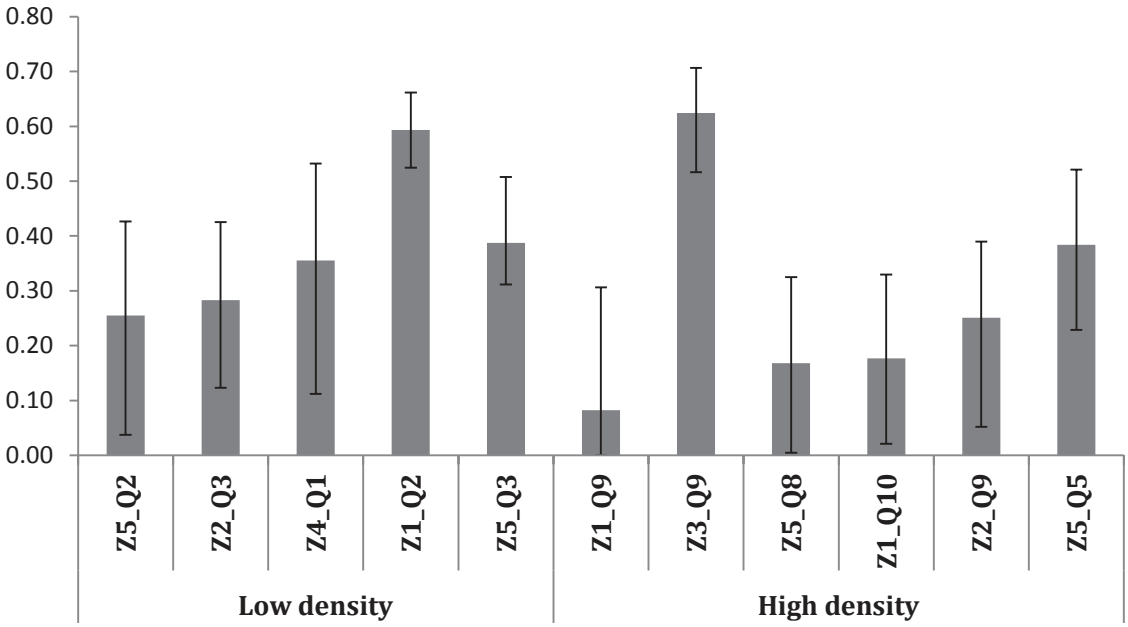

Figure 4. Mean selfing rate of quadrats of low density, and of patches of very-high densities. Error bars represent 95% confidence intervals.

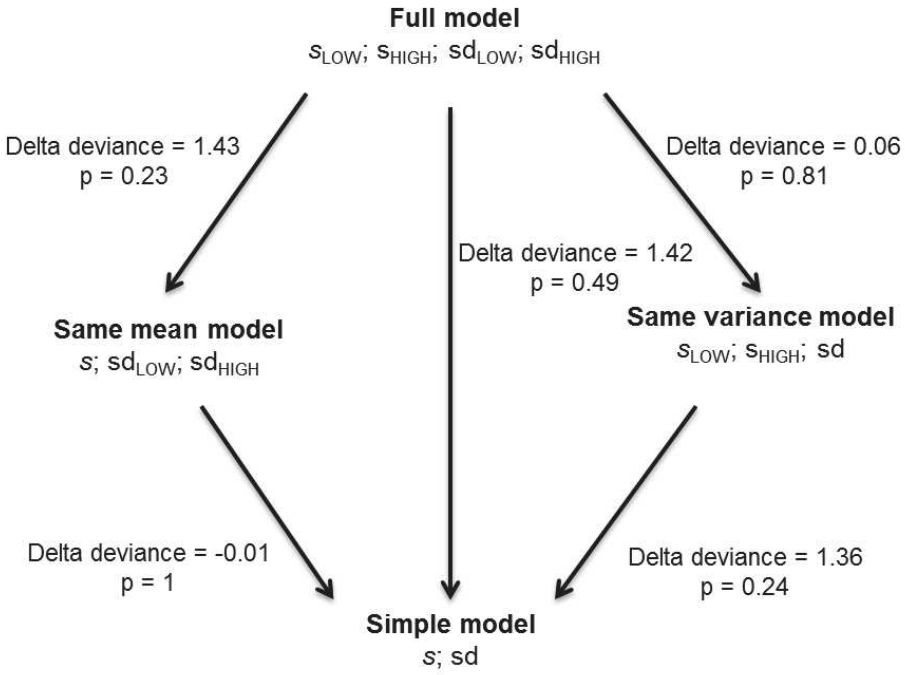

Figure 5. Comparison of models with different constrains on selfing rates and standing deviation of selfing rates in each density levels using Likelihood Ration Tests.

Table 4. Description of the models fitted on selfing rates using the full information of the likelihood profiles.

Modèle	Selfing rate	Standard deviation	Log-likelihood
Full	s_{LOW}=0.38; s_{HIGH}=0.28	sd_{LOW}=0.06; sd_{HIGH}=0.08	-7.51
Same variance	s_{LOW}=0.38; s_{HIGH}=0.28	sd=0.1	-7.54
Same mean	s=0.32	sd_{LOW}=0.09; sd_{HIGH}=0.1	-8.22
Simple	s=0.33	sd=0.09	-8.22

Discussion

We found no significant effect of local density on selfing rates in *Noccaea caerulescens*, in none of the two populations that we investigated. In SB, there was no effect of the floral display on selfing rates either.

The mean selfing rate estimated in SB for this reproductive season is in the same order of magnitude as the constrained selfing rate estimated in this population with the RMES method over four reproductive seasons (s = 0.17; Mousset *et al.* 2016). Similarly, the selfing rates

obtained in HI in the high and low density, or the constrained selfing rate are all consistent with the selfing rates measured in 2012 and 2013 in this population (s_{2012} = 0.34 and s_{2013} = 0.28, Mousset *et al.* 2016). We observed some genetic differentiation between different zones of the mine. This is consistent with the lack of specialized dispersal adaptation of seeds, explosive dispersal of fruits, the pollen dispersal data (Rileys 1956) and previous studies of genetic structure between shaded and non-shaded areas in some mines (Dubois 2005).

Effect of density

The effect of density on selfing rates could be confounded by biparental inbreeding, which can be mistaken for selfing. Some biparental inbreeding is expected in *Noccaea caerulescens* as seeds do not have specialized dispersing structure (Rileys 1956) and fine-scale genetic structure is shown by our analysis in HI. However, biparental inbreeding is low when estimated at the individual level in SB, and at the population level in two other sites (Mousset *et al.* 2016). This suggests that while biparental inbreeding sometimes occurs in *Noccaea caerulescens*, it should not strongly bias the estimation of selfing in SB. Moreover, the power to detect biparental inbreeding was high in this population thanks to a high number of microsatellite markers and the availability of mother's genotypes.

The RMES method that we used to estimate selfing rates in HI, assumes no within-population structure (David *et al.* 2007). However, Wang *et al.* (2012) found little effect of biparental inbreeding on RMES estimations in simulated datasets. In any case, upon finding small-scale genetic structure within HI, we estimated selfing rates at the quadrat level. To evaluate the bias induced by population structure, we carried out supplementary analyses in which we pooled individuals by zones and density levels before estimating selfing rates (Appendix S1). This analysis reached the same conclusions as the ones presented in the results, suggesting that the differentiation between quadrats within zones did not affect our results. We thus have no reason to think that within-quadrat structure, and in particular, biparental inbreeding would have the capacity to modify our conclusions broadly.

Inbreeding depression on germination and early survival might have biased our estimation of selfing rates. If inbreeding depression is high and varies among families, this could blur an existing relationship between density and selfing rates. We however found little inbreeding depression on germination, when measured in the same conditions as the seed germinated here (Mousset et al, in prep).

Assuming that pollinators do modify their behaviour depending on the density of plants, we may have missed the appropriate density range where they do. In the SB population, the density of plants was very high, the largest mean distance to the ten closest neighbours was 78 cm, and the tenth neighbour was always within two meters of the focal plant. By contrast, density varies more widely in several studies that found an effect of density on pollen deposition or on selfing rate (e.g. Karron *et al.* 1995; Franceschinelli and Bawa 2000; Duncan *et al.* 2004; Feldman 2008). For instance, in Karron *et al.* (1995), the "low density" treatment, with all plants of *Mimulus ringens* spaced by 2.4m, represents a level of plant isolation that was not available in our transects. Similarly, in the meadow herb *Salvia pratensis*, higher selfing rates were found in the sparse treatment where plants were separated by 1m (van Treuren *et al.* 1993), or isolated at 20m or 100m from other plants (van Treuren *et al.* 1994). Consequently, we may not have sampled "isolated" plants in SB in that reproductive season at all, or not estimated density at the right scale.

This prompted us to study the relationship between density and mating system in a population with higher density contrasts, HI, and at a slightly larger scale. We believe that the density variation in HI is representative of density variations within and among populations of the region. The estimated selfing rates in the low-density and high-density groups vary consistently with our hypothesis, being higher in low-density patches. However, the moderate difference in selfing rates (0.1), despite differences of orders of magnitude in plant densities, is not detected as significant given our sample size. It is interesting to note that the significant difference in selfing rates between ecotypes in the region detected by Mousset et al (2016), for a sample size twice higher, is larger (0.14) but of the same general magnitude. We therefore cannot definitely exclude that differences in density between polluted and non-polluted sites contribute to the variation in mating system, but the present data does not lend support to the idea that it would be the sole explanation for such variation. Despite estimating density in a larger area in HI, we may still have the wrong spatial scale if the average zone density was sufficient to attract pollinators, which would then forage freely in the zone (even though density varies among quadrats). This hypothesis could also be true of the whole SB mine, which may, from a pollinator point of view, be one large, dense patch of plants in the middle of the forest.

Various pollinators' species may not forage in the same way. We know little about *N. caerulescens*' pollinators in the South of France: bees, bumblebees, small Diptera, and some Lepidoptera visit plants (pers. comm.), but the pollinator community is not characterized. While some literature explores how density modifies Hymenoptera behaviour, much less is known

about Diptera (but see Inouye *et al.* 2015). If several taxa of pollinators pollinate *N. caerulescens* with different foraging behaviours, we might not expect a global relationship between density and selfing rates. What is more, we only considered the density of flowering *N. caerulescens*. But some pollinators have lower constancy than others (i.e., they pollinate different floral types in one pollination bout, see Devaux *et al.* 2014 for a review), which could make both patch attractivity and conspecific pollen carry-over depend on heterospecific density.

Effect of floral display

Even though Thompson (2001) showed that different taxa of pollinators react differently to floral display, many studies found pollinators to be more attracted by plants with larger floral displays (bumblebees: Klinkhamer and de Jong 1990; Robertson and MacNair 1995; Ohashi and Yahara 1998, tachinid fly: Robertson and MacNair 1995 and bees: (Hessing 1988; Robertson and MacNair 1995; Galloway *et al.* 2002). In *N. caerulescens* itself, Riley (1956) noticed that pollinators favoured inflorescences with many open flowers as compared to ones with few expanded flowers. Larger floral display are often associated to higher (geitonogamous) selfing rates (de Jong *et al.* 1993; Harder and Barrett 1995; Galloway *et al.* 2002; Karron *et al.* 2004, 2009; Lau *et al.* 2008; Goodwillie *et al.* 2010). Yet, we found no effect of the number of floral stalks of the focal plant on its selfing rate. One reason may be that it is difficult to disentangle the effect of floral display and density. According to our predictions, isolated plants with large floral displays should self more than plants in dense patches with small floral displays. However, the predictions are less clear for plants in a dense patch and with large floral displays or for isolated plants with small floral displays. The relationship between plant density and floral display in SB gave us little power to detect this interaction.

Our expectations concerning the effect of floral display depend on the type of selfing. If plants do not need pollinators to self (autonomous selfing) and if outcross pollen is not limiting, we expect little effect of the floral display or density on selfing rates, because selfing does not depend on pollinator visitation. If selfing occurs mainly within-flower but with the help of pollinators (facilitated selfing) we expect an increase of the amounts of both selfing and outcrossing with pollinator visitation of the plant, and little effect of density or floral display on their relative proportions. However, selfing can also occur between flowers of a given plant (geitonogamous selfing). Both pollinator behaviour and inflorescence structure should affect the proportion of geitonogamous selfing (see Harder and Barrett 1996 and; Devaux *et al.* 2014 for reviews on pollinator behaviour and geitonogamy). The structure of *N. caerulescens*'

inflorescences is such that peripheral flowers dehisce before central flowers, but many central flowers already have a receptive pistil (Rileys 1956 and personal observations, **Figure 6**). A pollinator walking on the inflorescence from one flower to another can thus very easily perform geitonogamous selfing, even if there is only one inflorescence. This probably weakens the relationship between the number of inflorescences and selfing rates.

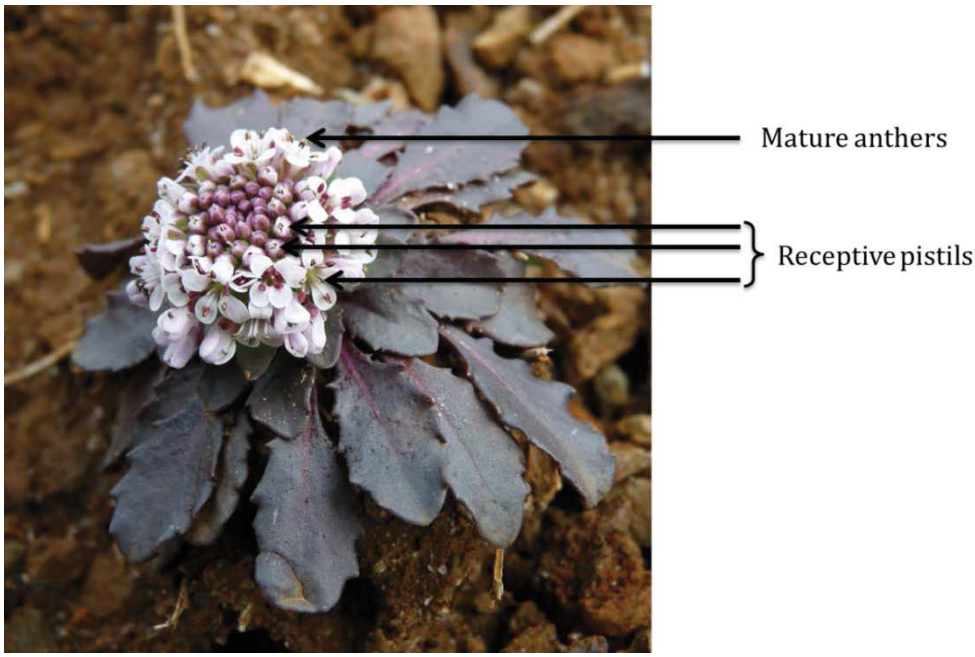

Figure 6. Illustration of the structure of an inflorescence of *Noccaea caerulescens* (Picture credits: M. Mousset). Population: Saint Bresson. The order of magnitude of the width of the inflorescence at its top is of 2 to 4 centimetres.

Summary and perspectives

Our results suggest that if density has an effect on selfing rates at the within-population level, it is small. If density varies from one year to another, we would still expect little impact on selfing rate variation. This is consistent with the absence of temporal variation detected by Mousset et al (2016) in four seasons in SB. Better knowledge on pollinator community and behaviour in *Noccaea caerulescens* is still needed to further uncover the fine mechanics of mating system variation in this species.

Our results suggest that, all other things kept constant, variation in plant density is unlikely to allow large plastic variation of selfing rate. Plastic increase in selfing rate during early colonisation of a new environment was predicted to be advantageous, facilitating adaptation (Peterson and Kay 2015). The present study does not lend support to the idea that plastic

responses of mating system to low density may have similarly facilitated adaptation and the colonisation of new environments in *Noccaea caerulescens*.

Bibliography

Antonovics J. **1968**. Evolution in closely adjacent plant populations V. Evolution of self-fertility. *Heredity* **23**: 219–238.

Assunção A, Bookum W, Nelissen H, Vooijs R, Schat H, Ernst W. **2003**. Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159**: 411–419.

Assunção A, Schat H, Aarts M. **2003**. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159**: 351–360.

Besnard G, Basic N, Christin P-A, Savova-Bianchi D, Galland N. **2009**. *Thlaspi caerulescens* (Brassicaceae) population genetics in western Switzerland: is the genetic structure affected by natural variation of soil heavy metal concentrations? *New Phytologist* **181**: 974–984.

Bosch M, Waser N. **1999**. Effects of local density on pollination and reproduction in *Delphinium nuttallianum* and *Aconitum columbianum* (Ranunculaceae). *American Journal of Botany* **86**: 871–879.

Bosch M, Waser N. **2001**. Experimental manipulation of plant density and its effect on pollination and reproduction of two confamilial montane herbs. *Oecologia* **126**: 76–83.

Cuguen J, Acheroy M, Loutfi AL, Petit D, Vernet P. **1989**. Breeding system differentiation in *Arrhenatherum elatius* populations: evolution toward selfing? *Evolutionary Trends in Plants* **3**: 17–24.

Dauber J, Biesmeijer J, Gabriel D, Kunin W, Lamborn E, Meyer B, Nielsen A, Potts S, Roberts S, Sõber V, Settele J, Steffan-Dewenter I, Stout J, Teder T, Tscheulin T, Vivarelli D, Petanidou T. **2010**. Effects of patch size and density on flower visitation and seed set of wild plants: a pan-European approach. *Journal of Ecology* **98**: 188–196.

David P, Pujol B, Viard F, Castela V, Goudet J. **2007**. Reliable selfing rate estimates from imperfect population genetic data. *Molecular Ecology* **16**: 2474–2487.

Devaux C, Lepers C, Porcher E. **2014**. Constraints imposed by pollinator behaviour on the ecology and evolution of plant mating systems. *Journal of Evolutionary Biology* **27**: 1413–1430.

Doyle J. **1990**. DNA extraction from *Arabidopsis*. *Focus* **12**: 13–15.

Dubois S. **2005**. Étude d'un réseau de populations métallicoles et non-métallicoles de *Thlaspi caerulescens* (Brassicaceae). Structure génétique, démographie et pressions de sélection.

Dubois S, Cheptou P-O, Petit C, Meerts P, Poncelet M, Vekemans X, Lefèbvre C, Escarré J. **2003**. Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* **157**: 633–641.

Dulya O, Mikryukov V. **2015**. Genetic variation and selfing rate in *Lychnis flos-cuculi* along an industrial pollution gradient. *New Phytologist* **209**: 1083–1095.

Duncan D, Nicotra A, Wood J, Cunningham S. **2004**. Plant isolation reduces outcross pollen receipt in a partially self-compatible herb. *Journal of Ecology* **92**: 977–985.

Ellstrand N, Torres A, Levin D. **1978**. Density and the rate of apparent outcrossing in *helianthus annuus* (Asteraceae). *Systematic Botany* **3**: 403–407.

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière Y, Delay B. **2000**. Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. *New Phytologist* **145**: 429–437.

Farris M, Mitton J. **1984**. Population density, outcrossing rate, and heterozygote superiority in Ponderosa Pine. *Evolution* **38**: 1151–1154.

Feldman T. **2008**. The plot thickens: does low density affect visitation and reproductive success in a perennial herb, and are these effects altered in the presence of a co-flowering species? *Oecologia* **156**: 807–817.

Franceschinelli E, Bawa K. **2000**. The effect of ecological factors on the mating system of a South American shrub species (*Helicteres brevispira*). *Heredity* **84**: 116–123.

Galloway L, Cirigliano T, Gremski K. **2002**. The contribution of display size and dichogamy to potential geitonogamy in *Campanula americana*. *International Journal of Plant Sciences* **163**: 133–139.

Goodwillie C, Sargent R, Eckert C, Elle E, Geber M, Johnston M, Kalisz S, Moeller D, Ree R, Vallejo-Marin M, Winn A. **2010**. Correlated evolution of mating system and floral display traits in flowering plants and its implications for the distribution of mating system variation. *New Phytologist* **185**: 311–321.

Grindeland J, Sletvold N, Ims R. **2005**. Effects of floral display size and plant density on pollinator visitation rate in a natural population of *Digitalis purpurea*. *Functional Ecology* **19**: 383–390.

Halimaa P, Blande D, Aarts M, Tuomainen M, Tervahauta A, Kärenlampi S. **2014**. Comparative transcriptome analysis of the metal hyperaccumulator *Noccaea caerulescens*. *Frontiers in Plant Science* **5**: 213.

Harder L, Barrett S. **1995**. Mating cost of large floral displays in hermaphrodite plants. *Nature* **373**: 512–515.

Harder L, Barrett S. **1996**. Pollen dispersal and mating patterns in animal-pollinated plants. In: Lloyd D,,In: Barrett S, eds. *Floral Biology*. Springer US, 140–190.

Hessing M. **1988**. Geitonogamous pollination and its consequences in *Gernium caespitosum*. *American Journal of Botany* **75**: 1324–1333.

Ingrouille M, Smirnoff N. **1986**. *Thlaspi caerulescens* J. & C. Presl. (T. alpestre L.) in Britain. *New Phytologist* **102**: 219–233.

Inouye D, Larson B, Ssymank A, Kevan P. **2015**. Flies and Flowers III: Ecology of foraging and pollination. *Journal of Pollination Ecology* **16**: 115–133.

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O. **2007**. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**: 199–215.

de Jong T, Waser N, Klinkhamer P. **1993**. Geitonogamy: the neglected side of selfing. *Trends in Ecology & Evolution* **8**: 321–325.

Karron J, Holmquist K, Flanagan R, Mitchell R. **2009**. Pollinator visitation patterns strongly influence among-flower variation in selfing rate. *Annals of Botany* **103**: 1379 –1383.

Karron J, Mitchell R, Holmquist K, Bell J, Funk B. **2004**. The influence of floral display size on selfing rates in *Mimulus ringens*. *Heredity* **92**: 242–248.

Karron J, Thumser N, Tucker R, Hessenauer A. **1995**. The influence of population density on outcrossing rates in *Mimulus ringens*. *Heredity* **75**: 175–180.

Klinkhamer P, de Jong T. **1990**. Effects of plant size, plant density and sex differential nectar reward on pollinator visitation in the protandrous *Echium vulgare* (Boraginaceae). *Oikos* **57**: 399–405.

Knuth P. **1908**. *Handbook of Flower Pollination. Volume II. Translated by JR Ainsworth Davis*. Oxford: Clarendon Press.

Koch M, Mummenhoff K, Hurka H. **1998**. Systematics and evolutionary history of heavy metal tolerant *Thlaspi caerulescens* in Western Europe: evidence from genetic studies based on isozyme analysis. *Biochemical Systematics and Ecology* **26**: 823–838.

Kunin W. **1993**. Sex and the single mustard: population density and pollinator behavior effects on seed-set. *Ecology* **74**: 2145–2160.

Kunin W. **1997**. Population size and density effects in pollination: pollinator foraging and plant reproductive success in experimental arrays of *Brassica kaber*. *Journal of Ecology* **85**: 225–234.

Lau J, Miller R, Rausher M. **2008**. Selection through male function favors smaller floral display size in the common morning glory *Ipomoea purpurea* (Convolvulaceae). *The American Naturalist* **172**: 63–74.

Lefèbvre C. **1970**. Self-fertility in maritime and Zinc mine populations of *Armeria maritima* (Mill.) Willd. *Evolution* **24**: 571–577.

Levin D. **2010**. Environment-enhanced self-fertilization: implications for niche shifts in adjacent populations. *Journal of Ecology* **98**: 1276–1283.

Lombi E, Zhao F, Dunham S, McGrath S. **2000**. Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. *New Phytologist* **145**: 11–20.

Meerts P, Van Isacker N. **1997**. Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133**: 221–231.

Mousset M, David P, Petit C, Pouzadoux J, Hatt C, Flaven E, Ronce O, Mignot A. **2016**. Lower selfing rates in metallicolous populations than in non-metallicolous populations of the pseudometallophyte *Noccaea caerulescens* (Brassicaceae) in Southern France. *Annals of Botany* **117**: 507–519.

Murawski D, Hamrick J. **1991**. The effect of the density of flowering individuals on the mating systems of nine tropical tree species. *Heredity* **67**: 167–174.

Murawski D, Hamrick J. **1992**. The mating system of *Cavanillesia platanifolia* under extremes of flowering-tree density: a test of predictions. *Biotropica* **24**: 99–101.

Mustajärvi K, Siikamäki P, Rytkönen S, Lammi A. **2001**. Consequences of plant population size and density for plant–pollinator interactions and plant performance. *Journal of Ecology* **89**: 80–87.

Ohashi K, Yahara T. **1998**. Effects of variation in flower number on pollinator visits in *Cirsium purpuratum* (Asteraceae). *American Journal of Botany* **85**: 219–224.

Peterson M, Kay K. **2015**. Mating system plasticity promotes persistence and adaptation of colonizing populations of hermaphroditic angiosperms. *The American Naturalist* **185**: 28–43.

R Core Team. **2014**. R: A language and environment for statistical computing.

Rileys R. **1956**. The influence of the breeding system on the genecology of *Thlaspi Alpestre* L. *New Phytologist* **55**: 319–330.

Ritland K. **2002**. Extensions of models for the estimation of mating systems using n independent loci. *Heredity* **88**: 221–228.

Robertson A. **1992**. The relationship between floral display size, pollen carryover and geitonogamy in *Myosotis colensoi* (Kirk) Macbride (Boraginaceae). *Biological Journal of the Linnean Society* **46**: 333–349.

Robertson A, MacNair M. **1995**. The effects of floral display size on pollinator service to individual flowers of *Myosotis* and *Mimulus*. *Oikos* **72**: 106–114.

Thompson J. **2001**. How do visitation patterns vary among pollinators in relation to floral display and floral design in a generalist pollination system? *Oecologia* **126**: 386–394.

van Treuren R, Bijlsma R, Ouborg N, van Delden W. **1993**. The significance of genetic erosion in the process of extinction. IV. inbreeding depression and heterosis effects caused by selfing and outcrossing in *Scabiosa columbaria*. *Evolution* **47**: 1669–1680.

van Treuren R, Bijlsma R, Ouborg N, Kwak M. **1994**. Relationships between plant density, outcrossing rates and seed set in natural and experimental populations of *Scabiosa columbaria*. *Journal of Evolutionary Biology* **7**: 287–302.

Vaquero F, Vences F-J, Garcia P, Ramirez L, Perez de la Vega M. **1989**. Mating system in rye: variability in relation to the population and plant density. *Heredity* **62**: 17–26.

Wang J, El-Kassaby Y, Ritland K. **2012**. Estimating selfing rates from reconstructed pedigrees using multilocus genotype data. *Molecular Ecology* **21**: 100–116.

Watkins L, Levin D. **1990**. Outcrossing rates as related to plant density in *Phlox drummondii*. *Heredity* **65**: 81–89.

Contributors

Several people helped with the planning and the realisation of the two studies described in this manuscript:

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I personally was involved in all of these steps.

Appendix

Appendix S1. Comparison of selfing rates between zones within densities, and between densities. Model comparison was performed using Likelihood ratio tests on constrained and unconstrained models.

Density	Model	Selfing rate	Log-likelihood	Deviance	D.f.	p-value
LOW	One selfing rate per zone		-668.4	3.1	4	0.53
	One selfing rate for all zones	0.39	-669.9			
HIGH	One selfing rate per zone		-766.4	5.8	3	0.12
	One selfing rate for all zones	0.29	-769.2			
ALL	One selfing rate per density		-1440.3	2.35	1	0.125
	One selfing rate for the two densities	0.346	-1439.1			

D.f. degrees of f

CHAPITRE 3

INBREEDING DEPRESSION IN *NOCCAEA CAERULESCENS*

Abstract

Inbreeding depression, the relative decrease of fitness of inbred compared to outbred individuals is a strong determinant of mating system evolution. It is highly variable among populations but the factors that control its variation are not well understood. In particular, interactions between inbreeding depression and environment remain unclear. Here, we test the effect of the history of adaptation to a given environment on the inbreeding depression of populations placed in a similar or new environments using a common garden experiment. We use the pseudometallicolous plant *Noccaea caerulescens*, which can grow on toxic and non-toxic soils. We detect moderate amounts of inbreeding depression in this mixed mating plant. Inbreeding depression was sometimes higher in the metallicolous ecotype, but there was little effect of toxicity on inbreeding depression, and no interaction between toxicity, the history of adaptation to toxicity and inbreeding depression. If these low levels of inbreeding depression are similar in natural conditions, our results suggest that inbreeding depression does not constrain the evolution of higher selfing rates in this species.

Introduction

Inbreeding depression, the relative decrease of fitness of inbred compared to outbred individuals is a major driver of mating system evolution (e.g. Lande and Schemske 1985; Schemske and Lande 1985; Charlesworth and Charlesworth 1987; Goodwillie *et al.* 2005; Charlesworth 2006). It has important demographic consequences (e.g. Frankham 2005; O'Grady *et al.* 2006; Liao and Reed 2009) and may affect the processes of local adaptation (Epinat and Lenormand 2009) and the colonization of new habitats (in which selfing may prove beneficial Peterson and Kay 2015). Inbreeding depression varies greatly among species, populations, traits (Schemske and Lande 1985; Husband and Schemske 1996; Keller and Waller 2002; Crnokrak and Barrett 2002; Winn *et al.* 2011; Angeloni *et al.* 2011) as well as among environments (Byers and Waller 1999; Crnokrak and Roff 1999; Keller and Waller 2002; Armbruster and Reed 2005; Fox and Reed 2011). Stress has traditionally been one of the main factors sought to explain some of this environmental variation of inbreeding depression (Armbruster and Reed 2005; Fox and Reed 2011), with a stressful environment defined as an environment where the mean fitness of a population is reduced compared to another, more benign, environment. However, some authors have argued that the history of selection may better explain variation in inbreeding depression than stress (Ronce *et al.* 2009; Agrawal and Whitlock 2010; Long *et al.* 2013). Alternatively, levels of inbreeding depression might simply reflect levels of phenotypic variation (the opportunity for selection, Waller *et al*. (2008)) This question is not settled, and mixed mating populations evolving in heterogeneous habitat are good systems to study what structures the variation of inbreeding depression in natural populations. *Noccaea caerulescens* is a mixed-mating, heavy-metal tolerant plant species which has repeatedly colonized stressing environments (mines or mine wastes) and harbours distinct ecotypes with different degrees of adaptation to metal pollution. Using a common garden experiment, we investigated the importance of stress and the history of selection on the variation of inbreeding depression in this species.

The current consensus is that inbreeding depression is mostly due to the increased expression of deleterious mutations in inbred individuals (Charlesworth and Charlesworth 1999; Charlesworth and Willis 2009; Fox *et al.* 2008) with the occasional involvement of overdominant loci (e.g. Kärkkäinen *et al.* 1999). As the relative importance of recessive deleterious mutations, overdominant loci or other mechanisms is in general unknown for non-model species, the precise genetic architecture of inbreeding depression remains unknown for most organisms (Carr and Dudash 2003). Any parameter affecting the distribution of effects of

deleterious alleles on fitness, their frequency, and their dominance can lead to variation in inbreeding depression.

Early theoretical work predicted that the expression of the most deleterious recessive mutations with inbreeding or selfing should be followed by purging of these mutations (Schemske and Lande 1985; Charlesworth and Charlesworth 1987). There is some empirical evidence for purging with inbreeding (Willis 1999; Crnokrak and Barrett 2002; Fox *et al.* 2008; Charlesworth and Willis 2009) but also for its absence (Husband and Schemske 1996; Byers and Waller 1999; Carr and Dudash 2003). In a meta-analysis, Winn *et al.* (2011) found that outcrossing and mixed mating species had similar levels of inbreeding depression, suggesting that purging in mixed mating populations was not much stronger than in outcrossed populations. They argue that it could be due to high selective interference among loci (Lande *et al.* 1994; Lande and Porcher 2015). Peterson and Kay (2015) found in their simulations that after colonisation of a new habitat, genetic load in outcrossing and mixed mating populations was indeed similar. Purging efficiency depends on the genetic architecture of inbreeding depression, and on the history of inbreeding of each considered population (Crnokrak and Barrett 2002; Frankham 2005; Charlesworth and Willis 2009). It also depends on life history stages (Husband and Schemske 1996; Angeloni *et al.* 2011).

Small effective size (N_e) is another factor that explains some of the variation of inbreeding depression, as genetic drift increases in small populations. Firstly, small N_e allows the fixation of slightly deleterious alleles in populations (called drift load), thus reducing the fitness of both inbred and outbred individuals. While the fitness of the population is decreased compared to populations with larger N_e, inbred and outbred individuals become more similar, and inbreeding depression is expected to decrease (Bataillon and Kirkpatrick 2000; Glémin 2003; Roze 2015). Secondly, panmictic mating in a finite population leads to variation of homozygosity, opening the door for purging of deleterious alleles by drift (even though it should be less efficient than purging through non-random mating described above, Glémin 2003). This should also reduce inbreeding depression is small populations. To summarize, smaller N_e should lead to reduced inbreeding depression compared to large N_e. Indeed, lower inbreeding depression was observed in population with smaller effective size (Lohr and Haag 2015) or even with small size compare to large size (see Angeloni et al. 2011 for a meta-analysis).

Variation among environments

Inbreeding depression often varies among environments (e.g. Byers and Waller 1999; Keller and Waller 2002). Meta-analyses find that in many cases, inbreeding depression increases with

environmental stress (Armbruster and Reed 2005; Fox and Reed 2011), but not always. The magnitude of stress might be a crucial factor (e.g. Fox and Reed 2011; Enders and Nunney 2012; Schou *et al.* 2015). Some authors point out that stress is often confounded with lack of adaptation to the test conditions (Long *et al.* 2013). We will shortly describe several mechanisms that could account for inbreeding depression varying among both populations and environments.

An environment of low quality that decreases the fitness of all individuals, irrespective of their genetic background or history can affect inbreeding depression in several ways. Inbreeding depression is traditionally defined as a relative decrease of fitness, and not as an absolute difference in fitness (for discussion, see Moorad and Wade 2005; Cheptou and Donohue 2011). An increase of inbreeding depression can occur when inbred individuals are more sensitive to stress and their fitness declines more than the fitness of outbred individuals in the stressing environment (**Figure 1A-B**). Higher sensibility to stress may happen for instance if inbreeding leads to the expression of genetic load affecting general cellular functions, such as DNA repair (reviewed in Reed *et al.* 2012). Several studies comparing gene expression in inbred and outbred individuals found that genes involved in the stress pathways are overexpressed in inbred compared to outbred individuals of *Drosophila melanogaster* and *Arabidopsis lyrata* (Kristensen *et al.* 2010 and references therein; Menzel *et al.* 2015). Furthermore, Ayroles *et al.* (2009) found that genes involved in the metabolism, defence and stress pathways are differentially expressed between low and high inbreeding depression lines. This suggests that inbreeding depression acts as a form of "genetic" stress. If inbreeding itself activates the metabolic and stress response pathways (be it mutations in genes involved in it, or by compensation), inbreeding in a stressing environment might be more difficult to compensate for. A variant hypothesis is that deleterious alleles could become even more deleterious in a stressful environment, which would lead to higher inbreeding depression in such an environment. This could happen if stressed organisms have lower capacities to compensate for the effect of deleterious mutations (e.g. lower immunity, less efficient repair mechanism etc.). There is mixed support for this in the literature (Kishony and Leibler 2003; Martin and Lenormand 2006; Agrawal and Whitlock 2010; Plough 2012; Wang *et al.* 2014). Conversely, lower inbreeding depression in the stressing environment may occur when outbred individuals are more able to exploit the non-stressing environment (**Figure 1C**), and their fitness decline faster with stress than inbred individuals, which have a low fitness in all environments (Cheptou and Donohue 2011).

A distinct, but related hypothesis is the phenotypic variability hypothesis (Waller *et al.* 2008), which states that inbreeding depression is a form of selection, and is thus limited by the ratio of fitness variation to the squared average fitness within the inbred and outbred groups of the population (Crow 1958). This ratio, representing the opportunity for selection, is equal to square the coefficient of variation (hereafter CV^2) for a given trait. If variance of fitness is lower in a given environment, inbreeding depression will be more constrained and thus potentially smaller than in an environment where CV^2 is larger (**Figure 1D**). This hypothesis is not independent from the stress hypothesis. Some support for the CV^2 hypothesis was found in several studies (Waller *et al.* 2008; Fox and Reed 2011; Long *et al.* 2013).

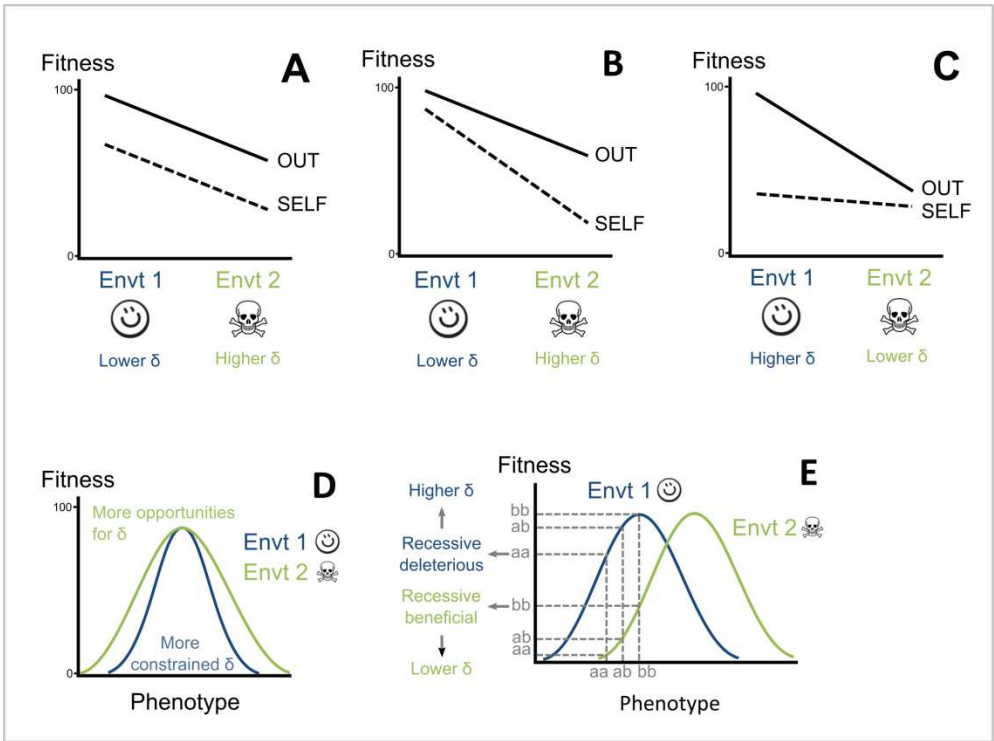

Figure 1. Different types of interaction between inbreeding and environmental stress, expressed through population fitness. A) The environment with a low quality (Envt2) affects the considered populations similarly. B) The stressing environment (Envt2) affects inbred individuals more than outbred ones. C) The stressing environment (Envt2) affects inbred individuals more strongly than outbred individuals. D) Variance of effects increasing under stressful conditions. E) Effect of the distance to the optimum on the recessivity of mutations on fitness, in a quantitative genetics framework with Gaussian fitness. δ: inbreeding depression. Green: represent the stressing environment. Blue: represent the benign environment.

Historical effects

Beside environmental effects previously described, one need to take into account the history of past selection pressures and how adapted or far from the optimum (maladapted) a given

population is to understand the segregating variance and how the distribution of expressed mutations is affected by environmental change.

Heterogeneous selection pressure (whether temporal or spatial variation) is expected to allow maintenance of alleles with conditionally deleterious effects. These alleles may be neutral or even beneficial ("environmental antagonism") in some environments, but deleterious in some other environments. Such alleles are expected to segregate at higher frequencies than unconditionally recessive deleterious alleles in heterogeneous environments (Epinat and Lenormand 2009; Agrawal and Whitlock 2010; Long *et al.* 2013). Their frequency in a given population depends strongly on the pattern of exposure to the environment where they are deleterious (Cheptou and Donohue 2011; Long *et al.* 2013). Alleles that will be most difficult to purge are the ones with recessive effects in the environments where they are deleterious (Gillespie 1973). These also generate inbreeding depression. If stress is confounded with an environment to which the populations is not adapted, purging of these mutations may be limited (Agrawal and Whitlock 2010; Long *et al.* 2013). Inbreeding depression should therefore be higher in an environment to which the population was not exposed in the past, or when there is temporally or spatially heterogeneous selection reducing the purging of conditionally deleterious recessive alleles (Long *et al.* 2013).

Another mechanism that may generate inbreeding × environment interaction is that the dominance of alleles varies among environments (Manna *et al.* 2011). This possibility was modelled by Ronce *et al.* (2009) in a quantitative genetic model. In this model, alleles had additive effects on the phenotype, but there was a Gaussian relationship between phenotype and fitness. The curvature of this function created dominance effects of alleles on fitness (**Figure 1D**). This model predicts that a random mating population, adapted to a given environment (at the optimum) and experiencing a brutal environmental change, will have a lower inbreeding depression in the new environment than in the environment to which it is adapted. This occurs because near the phenotypic optimum, the curvature causes deleterious mutations to be recessive on average (thus participating to inbreeding depression) whereas far from the optimum, the inverse curvature causes deleterious mutations to be dominant on average (and thus contributing to outbreeding depression). Consistent with theoretical predictions, stress increased the dominance of deleterious mutations in a study investigating viability QTLs (Plough 2012). Another way to understand the results of the model is that as one generation of selfing increases genotypic variance, selfing at the optimum creates mostly less adapted genotypes. On the other hand, when the population is very maladapted, increasing phenotypic variance increases mean fitness. When stress represents an environment to which a given population is

not yet adapted, this model predicts lower inbreeding depression when the population is maladapted than when it is close to the optimum. This prediction was not supported by results from Long *et al.* (2013), who found higher inbreeding depression in some new environments rather than the reverse.

In summary, the history of selection, and potentially adaptation, of the considered populations are of high interest to understand environment-dependant inbreeding depression. However, predictions depend on what mostly makes up the segregating genetic load and the type of selection applying. On the one hand, one could predict that an adapted population under stabilizing selection will have lower inbreeding depression in its "home" environment than in an environment in which it is maladapted (and thus potentially stressed). On the other hand, the opposite is predicted if dominance changes (Ronce *et al.* 2009; Peterson and Kay 2015). This prediction might explain why the few studies that investigated interactions between past selective history and the testing environment found mixed results (Wool and Sverdlov 1976; Schmitt and Gamble 1990; Swindell and Bouzat 2006; Murren and Dudash 2012; Long *et al.* 2013; Hereford 2014).

We here test whether inbreeding depression depends on history of adaptation and on the environment where it is measured, using the pseudometallicolous species, *Noccaea caerulescens*. Populations of *N. caerulescens* are tolerant to high concentrations of heavy metal such as Lead, Zinc or Cadmium, with greater tolerance in metallicolous populations than in non-metallicolous populations (Meerts and Van Isacker 1997; Escarré *et al.* 2000; Assunção *et al.* 2003; Dechamps *et al.* 2007; Jiménez-Ambriz *et al.* 2007). Reciprocal transplants in natural populations further suggest that metallicolous populations are locally adapted, being superior to non-metallicolous populations in polluted sites, while the pattern is less clear for non-metallicolous populations (Dechamps *et al.* 2008). Ecotype differentiation explains little of the structuration of neutral diversity (Koch *et al.* 1998; Dubois *et al.* 2003; Jiménez-Ambriz *et al.* 2007; Mousset *et al.* 2016). *Noccaea caerulescens* has a mixed mating system (Dubois *et al.* 2003; Besnard *et al.* 2009), with higher selfing rates and lower effective population sizes in non-metallicolous populations than in metallicolous populations in the South of France (Mousset et al. 2016; 40% selfing rate versus 25%). This species is a good model to test the predictions on the interaction of inbreeding, adaptation, and stress because of the existence of two ecotypes, with some evidence of differential adaptation to stressful environment, and of the possibility to perform control crosses.

We used a common garden experiment to test whether 1) there is inbreeding depression in *N. caerulescens*, 2) inbreeding depression depends on soil toxicity, and 3) effect of soil toxicity on

inbreeding depression varies among ecotypes with different history of exposure to toxic soils. As Zinc (Zn) concentration differs between the natural metalliferous and non-metalliferous soil on which the metallicolous and non-metallicolous ecotypes evolved, we experimentally manipulated it. We can formulate different sets of expectations.

Firstly, as non-metallicolous populations have higher selfing rates, and lower effective population sizes than metallicolous populations, inbreeding depression should be lower in non-metallicolous populations.

Secondly, as individuals coming from the non-contaminated habitat have a lower fitness when grown on a soil with high Zn concentrations (Dechamps *et al.* 2007; Jiménez-Ambriz *et al.* 2007), toxicity should affect inbreeding depression, by either representing a stressful environment, and/or new environment. Following the theoretical predictions of Ronce *et al.* (2009), non-metallicolous populations should suffer more from inbreeding depression on toxic soil than on non-toxic soil. Following most other hypotheses, non-metallicolous populations should have higher inbreeding depression on the toxic soil.

Thirdly, if toxic and non-toxic soils are similar from a metallicolous plant point of view (Dechamps *et al.* 2007; Jiménez-Ambriz *et al.* 2007), we expect little effect of toxicity on inbreeding depression in metallicolous plants. If, however, some conditionally deleterious mutations are expressed on the non-toxic soil, which represent a new environment for metallicolous populations, one expects higher inbreeding depression on non-toxic soil. Alternatively, fine scale spatial heterogeneity of soil contamination in the metalliferous sites could lead to the maintenance of mutations deleterious on both non-toxic and toxic soil. In that case, inbreeding depression should be high in the metallicolous ecotype, but toxicity should not affect it much.

Material & Methods

Noccaea caerulescens (J. Presl & C. Presl F.K. Mey) is a metallicolous *Brassicaceae* found in Europe, from Mediterranean regions to Czechoslovakia and Scandinavia (Ingrouille and Smirnoff 1986; Koch *et al.* 1998). It is usually annual to perennial and flowers in spring (Dubois 2005). It is self-compatible and it partly self-fertilizes in natural populations (Koch *et al.* 1998; Dubois 2005; Besnard *et al.* 2009; Mousset *et al.* 2016).

Experiment

Studied populations

All populations involved in the experiment are localised in the South of France, in the Cevennes Mountains or on the Larzac Causses (**Table 1, Figure 2**) at close distance to the Ganges and Saint-Laurent-Le-Minier towns. The populations Avinières (AV), Malines (MA), Moyen-Age (MG), Saint Bresson (SB) and Saint Hippolyte (HI) grow on wastes of former mines exploited sometimes as soon as the Middle Age, and as late as the end of the twentieth century (Dubois 2005). The soils are heavily contaminated, with high concentrations of Lead, Zinc and Cadmium. Baraquette (BQ), Coulet (CO), Navacelles (RT) and Saint Baudille (BD) are non-metallicolous populations growing on limestone, relatively close to the metallicolous populations (**Figure 2**).

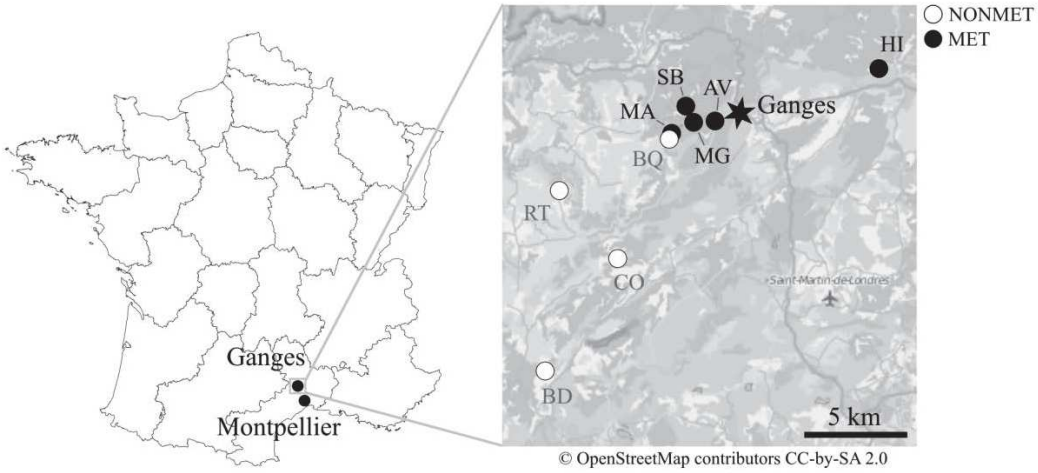

Figure 2. Geographical location of the nine natural populations *of Noccaea caerulescens* sampled in the South of France in 2012. Grey dots: non-metallicolous populations; black dots: metallicolous populations. AV, Avinières; BD, Saint Baudille; BQ, Baraquette; CO, Coulet; HI, Saint Hippolyte; MA, Malines; MG, Moyen-Âge; RT, Navacelles Route; SB, Saint Bresson. Background maps: © OpenStreetMaps Contributors.

Although geographically close, between 1 and 38 km, the studied populations are genetically differentiated, suggesting little gene flow between them (Dubois *et al.* 2003; Mousset *et al.* 2016). They have a mixed mating system and metallicolous populations have lower selfing rates than non-metallicolous populations (Mousset *et al.* 2016). Effective population size was found to be small in non-metallicolous populations, and moderate in metallicolous populations (Mousset *et al.* 2016).

Table 1. Name, and location of the sampled natural populations of *Noccaea caerulescens*. Concentrations of trace elements can be found in publications listed in the notes, when available.

Ecotype	Population	Latitude (N)	Longitude (E.)	Location	Altitude
MET	Avinières (AV) [2,3]	43.932138	3.662831	Saint-Laurent-le-Minier	250
	Saint Hippolyte (HI)	43.971409	3.833329	Saint-Hippolyte-du-Fort	248
	Malines (MA) [2,3]	43.922939	3.617699	Saint-Laurent-le-Minier	550
	Moyen-Age (MG) [1,2,3]	43.931157	3.640581	Saint-Laurent-le-Minier	355
	Saint Bresson (SB) [2]	43.943303	3.632505	Pommiers	410
NONMET	Saint Baudille (BD)	43.744648	3.4861	Montpeyroux	820
	Baraquette (BQ) [4]	43.918536	3.615228	Saint-Laurent-le-Minier	730
	Coulet (CO)	43.829053	3.561568	Saint-Maurice-Navacelles	468
	Navacelles 'Route' (RT) [4]	43.880013	3.500879	Saint-Maurice-Navacelles	619

MET, metallicolous; NONMET, non-metallicolous

[1] Also known as Petra Alba in Escarré *et al.* (2011)

[2] Dubois *et al.* (2003)

[3] Escarré *et al.* (2011)

[4] Dubois (2005)

Generation F_0

We collected rosettes of medium size in five metallicolous and four non-metallicolous populations during October 2012 (Table 1), and transferred them to the experimental garden "Plateforme Terrains d'Expériences" of the LabEx CeMEB, Montpellier, where the whole experiment was performed. When possible, we picked plants growing more than one meter apart, in order to reduce the probability of sampling closely related individuals. In some non-metallicolous populations it was occasionally impossible. Individuals were transplanted in 1l pots with either soil collected in their respective natural populations (for metallicolous populations) or a sterile mix of 1/3 sand 1/3 potting soil and 1/3 regular common garden soil (for non-metallicolous soil, where there was too little substrate to collect from). We randomized pots on tables outside and watered plants when needed. During late spring, we treated all plants against thrips and spider mites to avoid uncontrolled pollinations.

Controlled crosses

Upon start of their flowering, we transferred individuals in a temperate, pollinator-free greenhouse. We performed self-fertilization ("SELF" treatment) and within-population ("OUT" treatment) crosses on plants with at least two floral stalks. We castrated flowers assigned to either treatment just before the opening of the anthers to avoid within-flower self-fertilization, and to reduce differences in resource allocation among type of crosses. At the same moment, the stigma was receptive (Rileys 1956), and we performed pollination by cutting the base of an anther and making contact between the anther and the stigma. For within-population outcrossing, we varied pollen donors whenever possible. Only one type of cross was performed on a given floral stalk and unused flowers were always removed. We collected fruits at maturity just before dehiscence, counted seeds produced by each individual for each cross type, and weighed them using a precision scale (Scaletec SPB33). We took pictures of all seeds using a Wild/Leica M3Z Stereo Microscope equipped with an AVT Oscar-830 camera (Allied Vision). We analysed all snapshots using Image J (version 1.46, http://imagej.nih.gov/ij/index.html) to obtain seed count, as well as the section area of each seed. Mean seed mass was derived for each cross within mother by dividing the total mass of seeds collected by the seed count.

In order to verify the quality of the manual crossings performed on the F_0 plants, we genotyped sixteen families (mother and offspring of both cross types) of the CO population using microsatellite markers. For each of these mothers, we sowed the seeds of each cross type in September 2013 in 1l pots filled with the previously described soil mix. We collected and oven dried (at 56°C during 48h) leaves of available seedlings. We genotyped sixteen mothers, with one or two offspring for each type of cross (24 selfed and 29 outcrossed individuals), following the methods described in Mousset *et al.* (2016). In the sample of supposedly outcrossed individuals, no multiloci genotype was incompatible with the maternal genotype. We used MLTR (Ritland 2002) to estimate outcrossing rate in this group, using Newton-Raphson algorithm assuming different pollen and ovules allele frequencies. Outcrossing rates ranged from 0.92 to one, depending on initial values for model parameters (outcrossing rate: 0.5 or 0.9), which mean an error rate between 0 and 8%. In the supposedly selfed sample, two individuals were clearly incompatible with selfing. A third one gave conflicting results but we could not rule out a contamination during the genotyping. This yielded an error rate between 8% and 12% for selfed individuals. Overall, for the two cross types, the maximal error rate was of the order of 10%.

F_1 generation

Beginning in November 2013, we sowed between 12 and 70 seeds of each F_0 plant and cross type in rectangular containers filled with non-contaminated vermiculite (a neutral substrate).

Boxes were randomized across two tables in the temperate greenhouse (day: 23°C; night: 16°C) and watered to keep the substrate humid. On the 27th and the 28th of November, that is, 21 days after sowing, we recorded the number of living seedlings.

Between the 4th and the 11th of December, we transferred 3586 individuals into individual 1l pots filled with the previously mentioned sterile mix. The soil of 1992 of these pots had been saturated with a 0.10 $mol.L^{-1}$ zinc solution (Zinc heptahydrate, Labover) in order to obtain 1000 $mg.kg^{-1}$, in the "TOX" treatment, a concentration similar to concentrations in Jiménez-Ambriz et al., 2007. We left the remaining 1594 other pots uncontaminated ("NONTOX" treatment). When possible, we transferred six individuals per family and cross type on each toxicity treatment, except for the non-metallicolous plants on toxic soil, where we transferred nine. This original disequilibrium was meant to compensate for the higher mortality rate observed in Jiménez-Ambriz et al., (2007), and obtain an approximately balanced design at the end of the life cycle. Pots were randomized between two glasshouses (day: 25°C, night: 15°C, watered when needed). In order to collect and recycle the polluted water, the two levels of toxicity were separated on twelve different tables, which were later included in the analysis as a random blocking factor. Families were randomized across tables. Tables were rotated within each glasshouse once.

We assessed juvenile mortality between the 16th and the 19th December and replaced dead seedlings whenever biological material was available. From beginning of January 2014 onwards, the greenhouse temperature passively tracked outside temperatures but was not allowed to freeze. On the 6th of February, we moved all plants outside in the experimental garden so that they could vernalize, and we treated them against slugs and snails. On the 7th of March, plants were transferred to their final location and re-randomized. The tables were again included in the model as blocking factors. Plants were watered twice a day until autumn 2014.

Trait measurements

All measured traits and the type of analysis used are presented in **Table 2**. We analysed survival just before flowering (on the 19th of March), but we measured it once every month on average. From the 25th to the 31th of Mars, we estimated rosette size as the area of an ellipse where $A = \pi \times (\text{largest diameter}/2 \times \text{perpendicular diameter}/2)$. From the 19th of March onward, we documented flowering status every week, and eventually obtained the probability of flowering (probability of producing at least one open flower given that plants survived until the 19th of March). We collected all inflorescences at fruit maturity.

Table 2. Type of analysis performed on each trait.

Trait	Type of analysis	Transformation	Distribution	Link	Sample size
Seed area	LMM	Logarithm	Normal	Identity	39549
Germination probability	GLMM		Binomial	Logit	13468
Rosette area	LMM	Logarithm	Normal	Identity	2787
Survival	GLMM		Binomial	Logit	3481
Probability of flowering given survival	GLMM		Binomial	Logit	2789
Number of flowering stalks	GLMM		Poisson	Logarithm	2280
Number of fruits per cm	LMM	Logarithm	Normal	Identity	2265
Floral stalk height	LMM	Logarithm	Normal	Identity	2277
Number of seeds per fruit (for plants with more than five closed fruits)	LMM		Poisson	Logarithm	1980
Number of produced seeds	ASTER				2884

We used a subsample of plants to determine which reproductive traits we materially could and would focus on. On 72 plants, from all populations, we measured, for all inflorescences: the number of flower pedicles, the number of mature fruits and the length of inflorescences. Mean inflorescence size of all inflorescence on a plant was well predicted by the mean inflorescence size measured on only four randomly chosen inflorescences (R^2=0.94; p=2.2e-16). The total number of mature fruits produced by the plant was well predicted by the estimated number of fruits measured as the mean number of fruits on four inflorescences × the number of inflorescences (R^2=0.92; p=2.2e-16). Finally, the total number of flowers produced by the plant was relatively well predicted by the sum of all inflorescence length (R^2=0.84; p=2.2e-16). We therefore only investigated the total number of inflorescence, and, for four randomly chosen inflorescences, we estimated the number of fruits per inflorescence and the average length of an inflorescence, from the base of the first flower peduncle to the last, and the number of mature fruits. We then recorded the number of seeds in ten randomly chosen, closed fruits, and weighed these seeds. Only plants with more than five closed fruits were kept in the following analysis, to avoid bias when nearly all good fruits had already opened when we collected floral stalks. These measurements allowed us to approximate the number and mass of seeds produced by plants that flowered, which are our best approximation of female reproductive output.

Statistical analysis

Inbreeding depression

We computed population level inbreeding depression for the toxic and non-toxic environments for each trait representing one aspect of individual performance. Inbreeding depression was estimated as the relative decrease of performance in inbred relative to outbred individuals. While inbreeding depression at the level of family could be interesting to understand its evolution (e.g. Moorad and Wade 2005), we focused on population level inbreeding depression as a classical driver of mating system evolution, and therefore used mean population values in the above calculation. The trait population means were computed from family means to reduce the effect of sample size differences among families. Confidence intervals were generated using 1000 bootstraps with within-families resampling. Ecotype means were calculated as the average of population inbreeding depressions, and their confidence intervals were generated using the same bootstraps as for population inbreeding depression.

Sequential trait analysis

In a first analysis, we considered traits sequentially, conditioned on the previous stage (**Table 2**). We log-transformed continuous traits (seed size, rosette area, mean number of fruits per cm, height of floral stalks) and analysed them with a General Linear Mixed Model (LMM). We used the log-transformation so that significant differences between cross types in the model are multiplicative on the natural scale instead of additive, i.e. in a form closer to the classical inbreeding depression estimate (Moorad and Wade 2005; Cheptou and Donohue 2011). We analysed binomial traits (probability of germination, survival and probability of flowering) using Generalized Linear Mixed Models (GLMMs) with a binomial error and a logit link. We analysed count variables (number of inflorescences and the number of seeds per fruits) using GLMM with a Poisson error and a logarithm link. Following our experimental design, all models included the following fixed factors:

- **ecotype** (MET or NONMET),
- **cross type** (SELF or OUT),
- soil **toxicity** (TOX or NONTOX),
- the 3 simple interactions between them :
    - **ecotype × cross type** (which tests whether inbreeding depression depends on the ecotype),
    - **ecotype × toxicity** (which tests for adaptation to soil toxicity) and

    - **cross type × toxicity** (which tests whether inbreeding depression depends on stress).
- the three-way interaction among them: **ecotype × cross type × toxicity** (which testes whether the effect of soil toxicity on inbreeding depression depends on the history of adaptation, i.e. the ecotype
- **glasshouse** (for traits measured after transplantation): because there were only two glasshouses, we included them as a fixed factor.

All models also included, by default, population and family as random intercept factors.

In addition to this core model, we tested the following random factors: **blocks** inside the glasshouse and in the experimental garden as random intercepts, as well as population and families interactions with cross type and toxicity (as random slopes, with estimation of correlations between slopes and intercept). Due to convergence problems, we often had to simplify the structure of the models, by suppressing random effects associated with the smaller variance, until the model converged. Nonsignificant random factors were removed based on Likelihood Ratio Tests (LRT) using a 10% type 2 error threshold.

The significance of fixed effects was also assessed using LRT on nested models. When an interaction was significant, we split the dataset according to one of the factors to test main effects of the other factor (in general, dataset was split by ecotype, except when ecotype was not involved in the interaction). All analyses were performed in R (R Core Team 2014) using the lme4 package (Bates *et al.* 2015).

Aster analysis

We performed an unconditional aster analysis to properly model the dependence structure among traits (Geyer *et al.* 2007; Shaw *et al.* 2008) to analyse our best proxy for female fitness: the estimated number of produced seeds. This analysis is based on a precise description of the traits, their dependence and their distributions given the dependant trait (called a "graph"). It allows us to use suitable probability distribution for each trait, and in particular total fitness, without data loss.

The graph we used was simple and identical for all individuals; it included survival until the beginning of the flowering season ("survival", as a Bernouilli variable), whether the individual reproduced and produced inflorescences ("reprod", as a Bernouilli variable), and an aggregated measure of total seed production (number of inflorescence × mean number of fruits per inflorescence × mean number of seeds per fruit with a Negative Binomial distribution). Because

mixed effect aster models behave less well than non-mixed aster models (Geyer *et al.* 2013), we only performed a fixed effect analysis, including the previously described factors: ecotype, cross type, toxicity, and all one-way and two-ways interactions. These factors are acting on the last "node" i.e. total seed production, and were tested using Likelihood Ratio Tests.

Stress and coefficient of variation

We eventually tested two classical factors sometimes found to structure the variation of inbreeding depression: stress and CV^2, focusing on variation at the population, environment and trait level. Stress was computed as the relative decrease of fitness of a given population when placed in a "stressful" environment compared to a "benign" environment. The "stressful" and "benign" environments were not a-priori defined, but depended on the mean population outbred fitness. Stress was computed on outbred means. The coefficient of variation was calculated for each population and each toxicity level, based on family means, and thus represents a "genetic" coefficient of variation. We used simple linear regression to study the relationship between CV^2 and inbreeding depression at the population level (pooling the two toxicity levels), and to study the relationship between stress and the ratio of inbreeding depression between the two environments.

Results

The results of general and generalized linear models on different traits are provided in **Table 3**, and the predictions from the models are provided in **Figure 3**. Estimates of inbreeding depression are provided in **Figure 4**, Appendix S1 and S2.

Plants of both ecotypes had a high germination rate: 93% ± 0.003 (mean ± standard error of mean) and 94% ± 0.002 of the seeds, respectively, of the metallicolous and non-metallicolous ecotype germinated and survived up to 21 days. Just before transplantation, mortality increased in the germination trays but was not quantified. Ninety percent (± 0.008) of metallicolous plants and 92% ± 0.006 of non-metallicolous plants survived their transplantation and the first two weeks on non-toxic and toxic soil. Respectively 85% ± 0.9 and 77% ± 0.9 of metallicolous and non-metallicolous plants survived until mid-March (four months and 13 days, or 133 days after sowing). Most plants alive at this date flowered (MET: 0.89 ± 0.009 and NONMET: 0.95 ± 0.005). Flowering plants produced an average of 14.8 ± 0.33 and 11.3 ± 0.2 floral stalks in the metallicolous and non-metallicolous ecotypes respectively, with 0.7 ± 0.2 and 0.7 ± 0.1 fruits for every cm of floral stalk. Each fruit contained an average of 3.8 ± 0.1 and 4.3 ± 0.62 seeds (full

mature fruits). By the end of October, 8% of the plants were still alive (265 plants). These plants did not survive the following winter.

Metallicolous plants have higher tolerance for Zn toxicity

We expected plants from the metallicolous ecotype, which evolved on Zn-rich, toxic soils, to be more tolerant to soil pollution compared to the non-metallicolous ecotype. In our experiment, a significant interaction between ecotype and toxicity indicated that, indeed, plants from different ecotypes differ in their sensitivity to soil contamination. We detected such an interaction for survival probability and rosette area (**Table 3**, **Figure 3** and **Figure 4**). The survival of non-metallicolous plants was negatively affected by high Zinc concentrations (χ^2=94; p<10^{-16}), while toxicity had no effect in the metallicolous ecotype (χ^2=2.2; p=0.13). Whereas survival of both ecotypes was similar on non-toxic soil, survival of the metallicolous ecotype was higher than that of non-metallicolous ecotype on toxic soil (**Figure 3**). Plants of both ecotypes were smaller on toxic soil than on non-toxic soil (MET: χ^2= 9.74; p=0.0018; NONMET: χ^2= 16.99; p<10^{-4}), but metallicolous plants were less affected than non-metallicolous plants, again consistent with local adaptation of the metallicolous ecotype to the toxic environment (Figure 3). A significant interaction between ecotype and toxicity was also found when integrating life history traits in a single fitness measure: the lifetime female reproductive success, hereafter "total number of seeds", declined with toxicity in both ecotypes (MET: χ^2= 4.83, p=0.03; NONMET: χ^2= 46.26; p<10^{-10}), but more in non-metallicolous plants than in metallicolous plants (**Table 3**, **Figure 3**). This interaction is mainly driven by survival, and is not significant for reproductive traits when analysed separately.

Toxicity had two other effects (**Table 3**, **Figure 3**). First, it reduced similarly the number of floral stalks in the metallicolous and the non-metallicolous ecotypes. Secondly, it affected the probability to reproduce, but its effect depended on cross type (χ^2= 4.7; p=0.03).

Independently of toxicity, plants of the two ecotypes differed for several traits. The metallicolous ecotype had smaller seeds, a lower probability of germination, a marginally lower probability of flowering given survival, and a marginally lower seed production per fruit than the non-metallicolous ecotype (**Table 3**, **Figure 3**, Appendix S3).

Populations varied in probability of survival, probability of flowering, number of inflorescences, height of inflorescences, number of fruits per cm and number of seeds per fruit (**Figure 3**). Additionaly, there was an interaction between population and toxicity for plant size, suggesting that populations within the same ecotype differ in their growth response to toxicity (**Table 3**).

Table 3. Test of fixed and random factors with LMM and GLMM. Blank cells represent factors that were not included in the final model. Dashes represent factors included in the model but not tested directly, due to their presence in a statistically significant interaction.

Trait		Ecotype	Cross type	Toxicity	Ecotype * Cross type	Ecotype * Toxicity	Cross type * Toxicity	Ecotype * Cross type * Toxicity	Greenhouse	Population	Population * Cross type	Population * Toxicity	Population * Cross type * Toxicity	Family	Family * Cross type	Family * Toxicity	Family * Cross type * Toxicity	Block_greenhouse	Block_February	Block_final
Seed area	χ^2	17.43	36.55		0.93					13.6				-	1068.8					
	p-value	**$<10^{-4}$**	**$<10^{-9}$**		0.33					**0.0002**				-	**$<10^{-15}$**					
Germination probability	χ^2	5.71	3.47		1.92					2.28				-	119.6					
	p-value	**0.019**	*0.062*		0.17					0.13				-	**$<10^{-16}$**					
Rosette area	χ^2	-	-	-	6.56	15.11	0.59	0.49	9.11	-		6.77		82.91				34.33		
	p-value	-	-	-	**0.01**	**0.0001**	0.44	0.48	**0.003**	-		**0.03**		**$<10^{-15}$**				**$<10^{-6}$**		
Survival probability	χ^2	-	-	-	3.42	28.3	1.96	2.3	45.17	6.19	5.15			-	102.8					
	p-value	-	-	-	*0.06*	**$<10^{-7}$**	0.16	0.13	**$<10^{-9}$**	**0.01**	**0.08**			-	**$<10^{-15}$**					
Probability of flowering given survival	χ^2	3.37	-	-	0.017	1.38	4.69	0.13	8.79	14.7				7.71						5.53
	p-value	*0.07*	-	-	0.9	0.24	**0.03**	0.72	**0.003**	**0.0001**				**0.005**						**0.02**
Number of floral stalks	χ^2	1.74	11.88	7.82	0.59	0.08	2.3	0.001	3.13	49.78				-	-	-	154.08	196.44		152.21
	p-value	0.19	**0.0006**	**0.005**	0.44	0.78	0.13	0.97	0.077	**$<10^{-11}$**				-	-	-	**$<10^{-15}$**	**$<10^{-15}$**		**$<10^{-15}$**
Inflorescence height	χ^2	2.4	0.2	0.19	0.37	0.02	0	0.45	1.32	101.13				94.19					8.69	31.5
	p-value	0.12	0.65	0.66	0.54	0.89	1	0.5	0.25	**$<10^{-15}$**				**$<10^{-15}$**					**0.003**	**$<10^{-7}$**
Number of fruits per cm	χ^2	-	-	-	2.7	1.24	0.6	0.18	0.38	29.03				88.94						7.91
	p-value	-	-	-	*0.1*	0.27	0.44	0.67	0.54	**$<10^{-7}$**				**$<10^{-15}$**						**0.005**
Number of seeds per fruit	χ^2	3.59	8.2	1.79	0.13	0.07	0.13	1.07	0.06	26.69				7.99						
	p-value	*0.06*	**0.004**	0.18	0.72	0.79	0.72	0.3	0.81	**$<10^{-6}$**				**0.005**						
Total number of produced seeds	χ^2				1.64	4.92	0.37	1.36	0.09											
	p-value				0.2	**0.03**	0.54	0.24	0.76											

χ^2: Chi-square; p-value: associated with a Likelihood Ratio Test

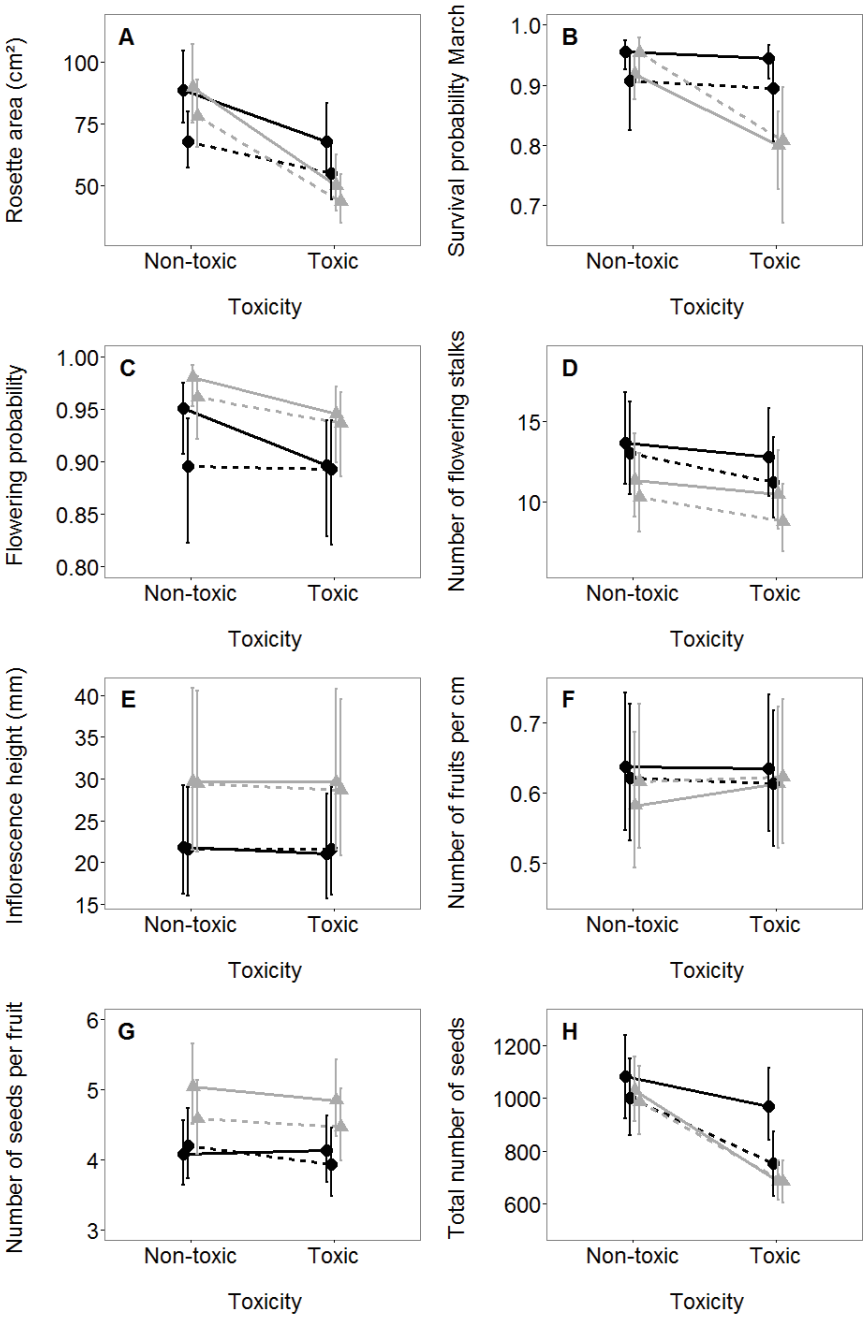

Figure 3. Predictions of values of different traits for the two cross types, ecotypes, and toxicity levels. Error bars represent approximate 95% confidence intervals. Black circles: metallicolous ecotype; grey triangles: non-metallicolous ecotype. Continuous lines: outcross averages; dashed lines: selfed averages. A) Rosette area. B) Probability of survival. C) Probability of flowering given survival. D) Number of inflorescences. E) Inflorescence height. F) Number of fruits per cm of floral stalk; G) Number of seeds per fruit. H) Total number of seeds produced.

Limited inbreeding depression in *Noccaea caerulescens*

Noccaea caerulescens suffered from inbreeding depression for most traits, at least in one ecotype, or in one environment as indicated by a significant effect of cross type or interactions involving cross type. However, the magnitude of detected inbreeding depression was typically small (**Table 3, Figure 4**, Appendix S1, Appendix S2).

Before transplantation, the effect of cross type was significant for seed size and marginally significant for germination (although very small; Appendix S3), with selfed seeds being slightly smaller and selfed seedling surviving less well. It should be noted that these two traits are not independent: including seed size as a covariate in the models studying germination is sufficient to explain the differences between cross types and ecotypes observed in germination (cross type: χ^2= 1.65; p = 0.2; ecotype: χ^2 = 1.2; p = 0.27).

After transplantation of plants on toxic or non-toxic soil, cross type affected the number of inflorescences and the number of seeds within a fruit, with selfed plants having respectively fewer inflorescences and fewer seeds per fruit than outcrossed plants (**Table 3**, **Figure 4**, Appendix S1, Appendix S2). It also affected rosette area, survival and flowering probability, but with effects depending on ecotype or soil toxicity (see below).

Population inbreeding depression for different traits ranged from δ=-0.83 (95% CI: -1.48 – -0.36) in AV (on the total number of seeds) to δ=0.55 (95% CI: 0.16 – 0.79) in MA (Appendix S1, Appendix S2). On average, the mean inbreeding depression (averaged across populations and toxicities) was highest for rosette size (δ=0.17 ± 0.005) and smallest for the number of fruits per centimetre (δ=-0.014 ± 0.004).

The effect of cross type on total seed production, our most integrative fitness measure, was however not significant (**Table 3**). Inbreeding depression for this trait was in the range of magnitude observed for other traits, and only in the metallicolous ecotype on toxic soil did the confidence intervals not cover zero (**Figure 4**, Appendix S1, Appendix S2).

There was no clear temporal pattern in the magnitude of inbreeding depression along the life cycle (Appendix S2). Early traits such as germination and early survival had very

small inbreeding depression, and some later traits, such as the number of seeds per fruit, had virtually no inbreeding depression (**Figure 4,** Appendix S3). For traits measured after transplantation, and until flowering, the magnitude of inbreeding depression tended to be higher in the metallicolous ecotype, with non-metallicolous populations sometimes suffering from outbreeding depression. After flowering, however, the pattern was not clear, with confidence intervals of both ecotypes often overlapping (**Figure 4**).

Inbreeding depression does not depend on local adaptation to the test environment.

The interaction between cross type, ecotype and soil toxicity was never statistically significant (**Table 3**). This means that the relationship between inbreeding depression and toxicity, if it existed, never depended on the ecotype, i.e. on the history of adaptation of populations to a given level of pollution.

In general, inbreeding depression does not depend on soil toxicity

In our models, the only case of interaction between the effect of cross type and the environment occurred for the probability of flowering given survival: selfed plants had a lower probability of flowering than outcrossed plants in the non-toxic environment, but not in the toxic environment (NONTOX: χ^2=10.11, p=0.001; TOX: χ^2= 0.11, p=0.73, **Table 3**, **Figure 3**). This pattern was particularly visible in metallicolous plants: the metallicolous ecotype had significant inbreeding depression on non-toxic soil, and virtually no inbreeding depression on toxic soil (**Figure 4**).

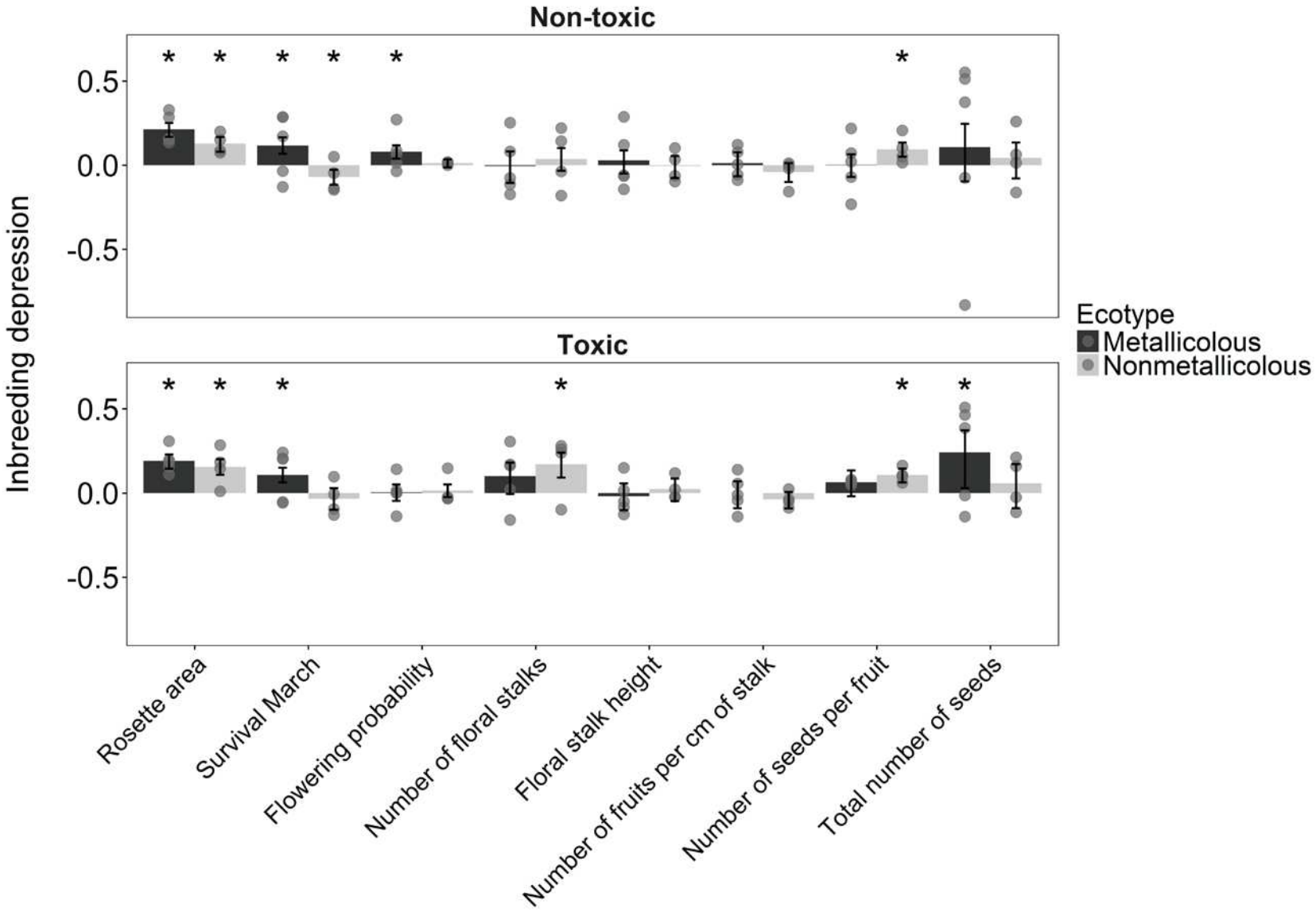

Figure 4. Inbreeding depression in *Noccaea caerulescens* for several traits on non-toxic and toxic soils. Error bars: approximate 95% bootstrap confidence intervals. Points: population average based on family means.

Inbreeding depression sometimes depends on ecotype

There was a significant interaction between cross type and ecotype for rosette size, and a marginal one for survival and for the number of fruits per centimetre (**Table 3**, **Figure 3**). Selfed plants were smaller than outcrossed plants in both ecotypes (MET: χ^2=79.3, $p<10^{-16}$; NONMET: χ^2= 26.5; $p<10^{-07}$) even though the difference was larger in the metallicolous ecotype. These results agree with direct estimations of inbreeding depression, which are generally higher in the metallicolous ecotype (**Figure 4**). In the case of survival probability and number of fruits per centimetres, where the ecotype × cross type interaction was marginally significant, the effect of cross type is opposite in the two ecotypes: selfed plants appear to die more, and have fewer fruits than outcrossed plants in the metallicolous ecotype, and to survive better and have more fruits than outcrossed in the non-metallicolous ecotype, but the comparisons of selfed and outcrossed plants are not significant in either ecotypes (**Figure 3**). Additionally, this cross type × ecotype interaction was strongly significant for survival in mid-December (results not shown). This interaction is consistent with our measurements of inbreeding depression, even though for the number of fruits per cm, the confidence intervals cover zero in all cases (**Figure 4**). Despite higher predicted inbreeding depression for our integrative measure of fitness in the metallicolous ecotype (**Figure 4**), we found no significant interaction between cross type and ecotype in our Aster analysis (**Table 3**).

Does inbreeding depression depend on stress or CV?

While there was barely any interaction between populations and cross type in the statistical models (**Table 3**), the computation of inbreeding depression and its confidence interval suggest some moderate variation of inbreeding depression among populations (Appendix S1 and S2).

Stress was one potential factor influencing inbreeding depression. It was estimated as the relative decrease of fitness between the most benign environment and the harshest environment in the outcrossed individuals, at the population level. It ranged from 0.001 in AV for survival, to 0.43 in CO for plant size. The change of inbreeding depression between environments did not depend on stress levels experienced by populations, for nearly all

traits (in the case of flowering probability, it was marginally significant (**Figure 5, Table 3, Table 4**).

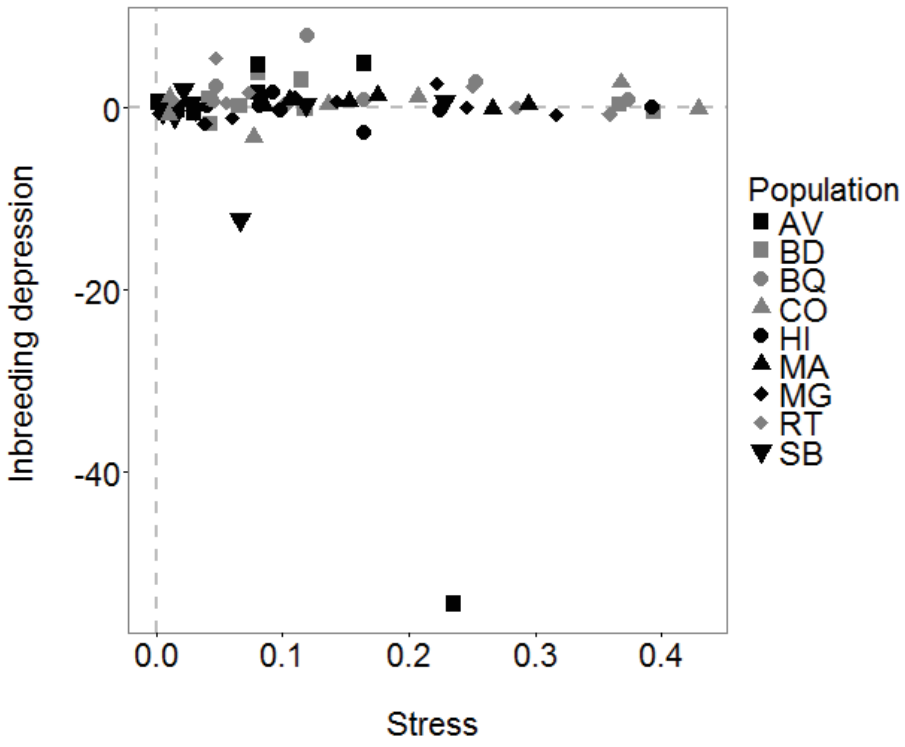

Figure 5. Ratio of inbreeding depression between the non-toxic and toxic environments, depending on stress, for all traits and populations. Stress was computed as the relative decrease of fitness of a given population when placed in a "stressful" environment compared to a "benign" environment, on outbred means. Black symbols: metallicolous populations; grey symbols: non-metallicolous populations. The black square with a very low ratio represent the AV population for the total number of seeds. It is very low because there was negative inbreeding depression in the non-toxic environment, and a nearly null inbreeding inbreeding depression in the toxic environment.

Table 4. Effect of stress on the ratio of inbreeding depression between the toxic and non-toxic environments in *Noccaea caerulescens*.

Trait	R^2	p-value
Rosette area	0.0004	0.96
Survival probability	0.09	0.45
Probability of flowering given survival	0.33	0.18
Number of floral stalks	0.39	0.07
Inflorescence height	0.08	0.45
Number of fruits per cm	0.07	0.5
Number of seeds per fruit	0.29	0.14
Lifetime female fitness	0.05	0.55

Inbreeding depression depended on the coefficient of variation for only three traits: plant size, survival and flowering probability (Table 5). The global variation of inbreeding depression at the population × toxicity level was weakly related to the CV^2 (R^2=0.05, p=0.007). More importantly, one can visually see an abrupt absolute increase of the variation of inbreeding depression with the coefficient of variation (cone-shaped pattern) which is consistent with the idea that inbreeding depression is limited by available variation (Figure 6). Populations from the metallicolous ecotype harboured slightly more between-families variation than populations from the non-metallicolous ecotype (F=5.13, p=0.03).

Table 5. Effect of the squared coefficient of variation and of toxicity on inbreeding depression in *Noccaea caerulescens.*

Trait	R^2	p-value
Rosette area	0.20	0.06
Survival probability	0.39	0.01
Probability of flowering given survival	0.23	0.04
Number of floral stalks	0.02	0.57
Inflorescence height	0.00005	0.98
Number of fruits per cm	0.002	0.88
Number of seeds per fruit	0.01	0.65
Lifetime female fitness	0.07	0.29

Discussion

Inbreeding depression was small in the nine studied populations of *Noccaea caerulescens* and did not depend on the interaction between the history of selection and the testing environment. In traits for which inbreeding depression differed between ecotypes, the metallicolous ecotype had higher inbreeding depression. Toxicity affected inbreeding depression only in one trait.

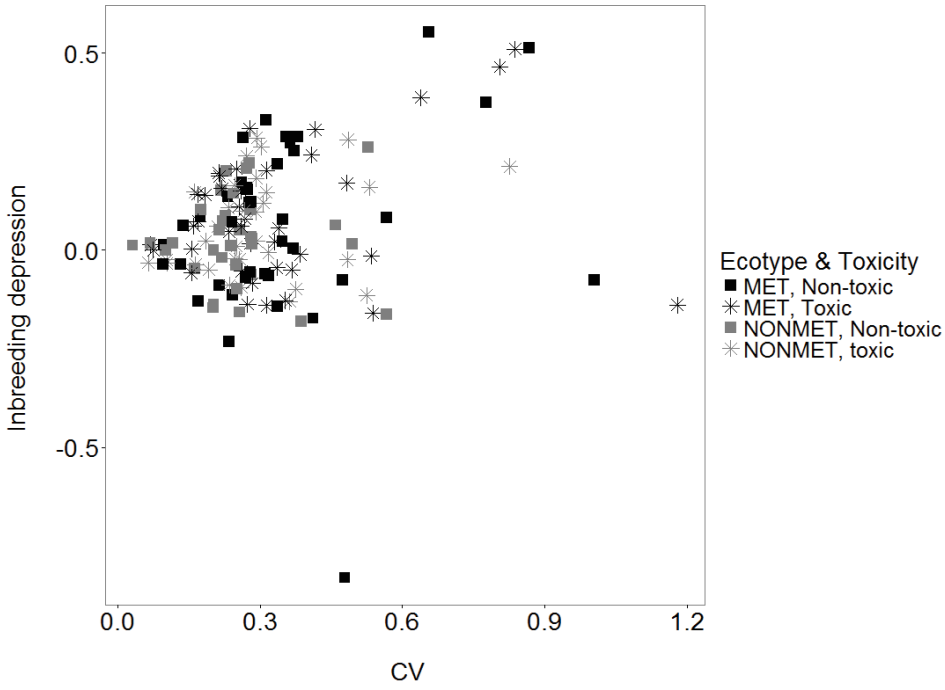

Figure 6. Inbreeding depression and the genetic coefficient of variation. Black symbols: metallicolous populations; grey symbols: non-metallicolous populations. Squares: non-toxic environment; Star: toxic environment.

Adaptation of metallicolous plants to contaminated soil

A strong assumption of our study is that metallicolous plants are locally adapted to mine environments (Dechamps *et al.* 2008). Different response of metallicolous and non-metallicolous plants growing on low and high Zn-rich soils have been previously observed

in two common garden experiments (Dechamps *et al.* 2007; Jiménez-Ambriz *et al.* 2007). We found such interaction for rosette size, survival, and for the integrative measure of fitness. There however were differences among the three experiments in the traits toxicity affected. Neither us nor Dechamps *et al.* (2007) found an interaction between ecotype and cross type for the number of inflorescences, while Jiménez-Ambriz *et al.* (2007) did, in a set of populations partially overlapping ours. For this trait, toxicity affected the number of inflorescences negatively in both ecotypes in our study, contrary to previous experiments. The differences among studies found in traits that depend on pollination service may be explained by an interaction between the environment of the experiment and the effect of toxicity in each ecotype. In general, the differences in how toxicity affects reproductive traits and ecotype in the three experiments suggest that there is variability for these traits in natural populations, but that it is not always sampled, or expressed.

The higher fitness of metallicolous plants in mine environments can be the product of genetic factors and maternal effects. Our experiment cannot disentangle these effects. Indeed, the F_0 plants germinated and grew in natural populations before we transferred them in the common garden during the autumn. A generation of plants placed in the same environment is necessary to estimate the genetic contribution of inbreeding depression, but crossing maternal environments would have been intractable, and using only one common environment arbitrarily would have greatly complicated the interpretation of results. We controlled for part of potential maternal effects by including a family random factor in the models and by adding seed size as a covariate in the analysis of early traits. Although seed size had a significant effect on germination, its inclusion in a model for survival in December was not significant (results not shown).

Small to moderate inbreeding depression

We found significant but often low levels of inbreeding depression in at least one of the ecotype or toxicity levels for most of the traits that we measured. There was no obvious pattern of variation of inbreeding depression along the life cycle, such as is often found (Husband and Schemske 1996; Winn *et al.* 2011; Angeloni *et al.* 2011), and the effects of tested factors on inbreeding depression and their interactions were remarkably trait-dependant.

We used two approaches to estimate the presence of inbreeding depression. On the one hand, general(ized) linear mixed models can accommodate complex designs, including blocking factors, but does not analyse inbreeding depression in its traditional definition. On the other hand, bootstrapping the measure of inbreeding depression provides a direct measure, but does not allow global hypothesis testing.

We may have underestimated inbreeding depression in the early stages. Some seedlings died between our assessment of germination/early survival and between plants transplantation (in the timespan of about one week), but they could not be quantified. Some of this mortality may have been structured by cross type. If this mortality was the result of lethal mutations expressed during seedling development, we find it difficult to conceive that it should occur at this exact stage, given the very high germination rates of the three previous weeks.

Inbreeding depression often depends on the environment (Byers and Waller 1999; Crnokrak and Roff 1999; Keller and Waller 2002; Armbruster and Reed 2005; Fox and Reed 2011). The magnitude of inbreeding depression that we measured in the common garden may therefore differ from the one expressed in the field. The environment of the common garden is, in several ways, more benign than natural conditions. In particular, plants were watered every day, in individual pots and the potting soil is likely richer than most natural growing substrates. It may lead to lower inbreeding depression than in the field. We detected moderate but significant amounts of selfing in natural populations, sampling individuals at the adult stage (Mousset *et al.* 2016). This indicates that inbreeding depression in natural populations is not strong enough to totally erase signal of selfing in the adults, as happen with very strong selective interference among recessive deleterious loci (Lande *et al.* 1994; Winn *et al.* 2011).

Inbreeding depression, when detected, was small to moderate (δ=-0.014 ± 0.004 for the number of fruits per cm and δ=0.17 ± 0.005 on average for rosette size). This estimation is small compared to the mean 50% inbreeding depression that Winn *et al.* (2011) find in outcrossers and mixed mating plants. Several non-exclusive hypotheses may explain this small value. A first hypothesis, derived from the results that stress often increase inbreeding depression, is that the environment was too benign in our experiment (see above). A second hypothesis is that higher inbreeding purged inbreeding depression

in the past. High selfing rates (higher than 0.8) are associated with lower inbreeding depression in empirical data (Winn *et al.* 2011) and in theoretical models, including single and multiloci approaches (Lande and Schemske 1985; Glémin 2003; Roze 2015). Mousset *et al.* (2016) found neither high selfing rates, neither variation of mating system in two reproductive seasons, but this does not disqualify higher selfing rates in the past, whether constant or occasional. Another hypothesis is based on population effective size. Small effective population size could lead to the fixation of part of the segregating load. This would reduce the relative difference between inbred and outbred individuals (Bataillon and Kirkpatrick 2000; Glémin 2003; Angeloni *et al.* 2011; Lohr and Haag 2015). Current effective size in the studied populations of *Noccaea caerulescens* tend to be small to moderate. Furthermore, bottlenecks during the colonization of these populations cannot be ruled out. This could have led to the purging and fixation of some part of inbreeding depression.

Inbreeding depression for seed traits (number, weight) is not uncommon in Angiosperms (Husband and Schemske 1996; Winn *et al.* 2011; Sletvold *et al.* 2013). Inbreeding depression on seed size could result either from different allocation of maternal resources between selfed and outcrossed seeds, or of the expression of early lethal genes in the embryo. Distinguishing these two hypotheses is impossible at this stage, and not of high interest given the minute amount of inbreeding depression detected in this trait. Inbreeding depression for germination was very low, and fully explained by these tiny differences in seed size in selfed and outcrossed seeds. This result was interesting as some inbreeding depression is expected for this trait in outcrossers, but not in selfers (Husband and Schemske 1996; Winn *et al.* 2011; Angeloni *et al.* 2011). This suggests that inbreeding depression is either not expressed or was efficiently purged for this stage in this mixed mating species.

Inbreeding depression was the strongest for rosette size in all combinations of treatments. As this trait is influenced by many genes, it offers many more opportunities for negative effects to accumulate, and inbreeding depression for growth-related trait is common (Husband and Schemske 1996; Winn *et al.* 2011). For survival, where the same argument should have applied, inbreeding depression was however significantly positive only in the metallicolous ecotype. Similarly, inbreeding depression for reproductive traits was small, even though these are usually subject to high inbreeding depression (Husband

and Schemske 1996; Winn *et al.* 2011; Angeloni *et al.* 2011). It could be possible that some genes affect both survival and fecundity, and that the selective death of individuals with deleterious alleles would leave survival individuals with less deleterious mutations.

Inbreeding depression and adaptation to the test environment

Inbreeding depression was equivalent when the test environment matched the history of selection and when it did not. Whether the interaction between history and current environment is small and undetectable with such low inbreeding depression levels, or whether it does not exist, is difficult to say.

The few studies that have similarly attempted to disentangle the effect of local adaptation on inbreeding depression have yielded equivocal results. Hereford (2014) found differences between selfed and outcrossed individuals of *Diodia teres* populations when individuals grew in a new habitat outside of their range, but none in their own habitat or in a new habitat within their range. Smaller sampling in the "home" habitat however raised doubts on the power to detect inbreeding depression in this sample. Wool and Sverdlov (1976) found that inbred individuals of *Tribolium castaneum* that evolved in a variable and stressing environment had higher fitness when tested in a constant, optimal environment than inbred individuals who evolved in this same, constant optimal environment. Outbred individuals were generally performing similarly in the two environments. Inbreeding depression seemed higher for populations that evolved in the constant environment (regrettably, this observation was not tested), which matched the testing environment, than in population raised in variable environment, which did not match the testing environment. Long *et al.* (2013) found that in *Drosophila melanogaster*, inbreeding depression was higher in populations with a history of heterogeneous selection. They however found mixed support for the impact of history of selection in constant environment. In one test environment, populations that were adapted to this test environment had lower inbreeding depression than populations that had not evolved in this environment, but there were no differences between them in another test environment. Mixed results are, again, found in Swindell and Bouzat (2006), where *Drosophila melanogaster* evolving in benign, high temperature and high competition environments were tested for inbreeding depression in the benign environment. Inbreeding depression of lines that evolved in competition had no inbreeding depression,

while inbreeding depression of the other lines was higher (and similar to each other). While the results from Long *et al.* (2013) emphasized that testing environments are all not equivalent, results from Swindell and Bouzat (2006) emphasize that stressors in the history of population are not all equivalent either. Murren and Dudash (2012) also found mixed results: the patterns of interactions between the origin of a population and the testing environment differed between traits, and for one trait, between populations. Finally, Schmitt and Gamble (1990) found, that in a plant population, fitness decreased with the distance to parental plants, while inbreeding depression increased. This effect was occurring at a very small scale: the largest studied distance was 12 meters. To summarize, several experiments investigated one aspect or another of the interaction between the historical environment, or historical selection pressures, and the environment where inbreeding depression is tested. However, there is no general, consistent effect of this interaction, which, when it exists, depends on the studied traits, the type of selective pressure, and the testing environment itself.

Inbreeding depression and toxicity

We did not observe a strong effect of toxicity on inbreeding depression in our experiment. Only inbreeding depression for flowering probability depended on toxicity, with inbreeding depression being higher in the non-toxic environment. Toxicity yet had a significant main effect on the traits, and the stress experienced by populations represented up to about 43% reduction of fitness component in our experiment (mean=0.13, sd=0.12). We did not quantify the toxicity at the end of the experiment, but the effect of toxicity on flowering suggests that plants felt the effects of the Zinc late in the life cycle. Meta-analyses suggest that the effect of low stress on inbreeding depression is weak and inconsistent, while larger stress (usually above 25%), positively correlates with inbreeding depression differences (Fox and Reed 2011). In our study, a dozen of populations were in a situation of stress higher than 25%, and the stress did not explain inbreeding depression ratio in this subset either. It is possible that the constitutive tolerance to heavy metals in *Noccaea caerulescens* makes Zn concentration a poor choice of stressor. This would occur if enough exposition of plants to toxicity occurred at a regional scale so that the genes involved in toxicity tolerance have been mostly purged of their recessive deleterious alleles in both metallicolous and non-metallicolous populations (which does not necessarily precludes variability of tolerance, as long as this variability is

not based on recessive alleles). Alternatively, fitness on contaminated soil may depend on a few major genes, with few recessive alleles. In this case, these genes would contribute little to inbreeding depression in either environment.

Inbreeding depression and ecotype

Where ecotype had an effect (or a marginal effect) on inbreeding depression, the metallicolous ecotypes had higher inbreeding depression than the non-metallicolous ecotype. This difference is consistent with the hypothesis that higher selfing rates and probably smaller effective sizes in the later would expose more of the load to selection (Mousset *et al.* 2016). Beside higher purging or fixation of the load, one can offer several non-exclusive hypotheses.

Firstly, metallicolous populations have more heterogeneous toxicity levels. The little data on within-mine toxicity variation (Dubois 2005; Escarré *et al.* 2011) suggest that mines have high heterogeneity of metal concentrations. This would generate heterogeneous selection pressures within mines. This heterogeneous selection could prevent the purge of antagonistic recessive mutations. This would be a reason to expect higher inbreeding depression in the metallicolous ecotype (Long *et al.* 2013). In other words, metallicolous populations may have a larger genetic variance, which would provide more opportunities for selection. Jiménez-Ambriz (2006) found a trend for higher additive genetic variance in a metallicolous population than in a non-metallicolous population. Furthermore, in our experiment the coefficient of variation was slightly higher in metallicolous populations than in the non-metallicolous populations. This slightly higher opportunity for selection could participate to the observed differences between ecotypes.

Secondly, heavy metal tolerance may be costly: metallicolous populations could be more sensitive to genetic stresses than non-metallicolous populations. The number of seeds produced in the field for metallicolous and non-metallicolous ecotype did not differ in the South of France (Jiménez-Ambriz *et al.* 2007) and Dechamps *et al.* (2007) found no cost of metal tolerance in the metallicolous ecotype. In a controlled experiment, Jiménez-Ambriz *et al.* (2007) found an interaction between ecotype and toxicity, but no main ecotype effect. In natural conditions, metallicolous populations suffered more from herbivory than non-metallicolous populations in a non-metallicolous site (Dechamps *et al.* 2008). These results suggest that the cost to higher tolerance might be small and, not

expressed in all environments, probably because the species is anyway constitutively tolerant to metal pollution.

Thirdly, non-genetic effects could also be involved, such as epigenetics marking. Vergeer *et al.* (2012) found that inbreeding modified epigenetic marking and that demethylation reduced or suppressed inbreeding depression depending on the traits. Since both inbreeding depression and epigenetic marks depend on environment, several authors have argued that epigenetic modification may explain at least some of the environmentally variable inbreeding depression (Biémont 2010; Cheptou and Donohue 2013). There might be non-genetic effects involved in the differences between ecotypes.

The interaction between population and cross type in our models was nearly never significant. Despite this, there seem to be some inbreeding depression variation between populations, especially among populations of the metallicolous ecotype when we separately estimate inbreeding depression in each population. This variation has not been studied so far, and will be the object of further analyses. What we know is that it is not entirely explained by stress, nor by the coefficient of variation. It seems that the CV^2 is a limiting factor for the occurrence of inbreeding depression in our populations, but that a sufficient amount of variation is rapidly reached.

Perspectives

The low magnitude of inbreeding depression in *Noccaea caerulescens* raises questions about the history of the species. Did populations experience bottlenecks in the past? Were selfing rates larger? Both events are likely in a species colonizing heterogeneous environments. Such a low inbreeding depression, coupled with the capacity to self-fertilize could have been advantageous during colonization of new habitats (Baker 1955; Levin 2010; Pannell 2015; Cheptou 2012). Low inbreeding depression could also facilitate the transition towards much higher selfing rates. This of course would depend on the details of *Noccaea caerulescens*' pollination. Currently, we do not know how the pollen is transferred. If plants self autonomously, that is, without the help of any vector, this should facilitate colonization of new habitats. It should also select for more selfing. If this process is happening now, we are obviously far from the equilibrium. If, on the other hand, selfing mainly occurs within flower or between flowers when pollinators visit the plant, the advantage of selfing in the colonisation process would be less high (plants are still

dependent on pollinators). It is conceivable that selfing is only a passive side product of pollinator's visitation. In this case, one would not expect mating system to evolve towards very high selfing rates, because the capacity to self is intimately linked with the capacity to outcross. Alternatively, the capacity for autonomous self-fertilisation could be selected for.

Conclusion

Different underlying mechanisms lead to contradictory predictions concerning the interaction between history of adaptation and inbreeding depression. In a common garden experiment, we found low levels of inbreeding depression in *Noccaea caerulescens*, with a trend for higher inbreeding depression in the less inbred metallicolous ecotype. We found no general effect of past adaptation to the test environment.

Bibliographie

Agrawal A, Whitlock M. **2010**. Environmental duress and epistasis: how does stress affect the strength of selection on new mutations? *Trends in Ecology & Evolution* **25**: 450–458.

Angeloni F, Ouborg N, Leimu R. **2011**. Meta-analysis on the association of population size and life history with inbreeding depression in plants. *Biological Conservation* **144**: 35–43.

Armbruster P, Reed D. **2005**. Inbreeding depression in benign and stressful environments. *Heredity* **95**: 235–242.

Assunção A, Bookum W, Nelissen H, Vooijs R, Schat H, Ernst W. **2003**. Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159**: 411–419.

Ayroles J, Hughes K, Rowe K, Reedy M, Rodriguez-Zas S, Drnevich J, Cáceres C, Paige K. **2009**. A Genomewide assessment of inbreeding depression: gene number, function, and mode of action. *Conservation Biology* **23**: 920–930.

Baker H. **1955**. Self-compatibility and establishment after "long-distance" dispersal. *Evolution* **9**: 347–349.

Bataillon T, Kirkpatrick M. **2000**. Inbreeding depression due to mildly deleterious mutations in finite populations: size does matter. *Genetics Research* **75**: 75–81.

Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**.

Besnard G, Basic N, Christin P-A, Savova-Bianchi D, Galland N. 2009. *Thlaspi caerulescens* (Brassicaceae) population genetics in western Switzerland: is the genetic structure affected by natural variation of soil heavy metal concentrations? *New Phytologist* **181**: 974–984.

Biémont C. 2010. Inbreeding effects in the epigenetic era. *Nature Reviews Genetics* **11**: 234–234.

Byers D, Waller D. 1999. Do plant populations purge their genetic load? Effects of population size and mating history on inbreeding depression. *Annual Review of Ecology and Systematics* **30**: 479–513.

Carr D, Dudash M. 2003. Recent approaches into the genetic basis of inbreeding depression in plants. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **358**: 1071–1084.

Charlesworth D. 2006. Evolution of plant breeding systems. *Current Biology* **16**: 726–735.

Charlesworth D, Charlesworth B. 1987. Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics* **18**: 237–268.

Charlesworth B, Charlesworth D. 1999. The genetic basis of inbreeding depression. *Genetics Research* **74**: 329–340.

Charlesworth D, Willis J. 2009. The genetics of inbreeding depression. *Nature Reviews Genetics* **10**: 783–796.

Cheptou P-O. 2012. Clarifying Baker's Law. *Annals of Botany* **109**: 633–641.

Cheptou P-O, Donohue K. 2011. Environment-dependent inbreeding depression: its ecological and evolutionary significance. *New Phytologist* **189**: 395–407.

Cheptou P-O, Donohue K. 2013. Epigenetics as a new avenue for the role of inbreeding depression in evolutionary ecology. *Heredity* **110**: 205–206.

Crnokrak P, Barrett S. 2002. Perspective: purging the genetic load: a review of the experimental evidence. *Evolution* **56**: 2347–2358.

Crnokrak P, Roff R. 1999. Inbreeding depression in the wild. *Heredity* **83**: 260–270.

Crow JF. 1958. Some possibilities for measuring selection intensities in man. *Human Biology* **30**: 1–13.

Dechamps C, Lefèbvre C, Noret N, Meerts P. 2007. Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. *New Phytologist* **173**: 191–198.

Dechamps C, Noret N, Mozek R, Escarré J, Lefèbvre C, Gruber W, Meerts P. **2008**. Cost of adaptation to a metalliferous environment for *Thlaspi caerulescens*: a field reciprocal transplantation approach. *New Phytologist* **177**: 167–177.

Dubois S. **2005**. Étude d'un réseau de populations métallicoles et non-métallicoles de *Thlaspi caerulescens* (Brassicaceae). Structure génétique, démographie et pressions de sélection.

Dubois S, Cheptou P-O, Petit C, Meerts P, Poncelet M, Vekemans X, Lefèbvre C, Escarré J. **2003**. Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* **157**: 633–641.

Enders L, Nunney L. **2012**. Seasonal stress drives predictable changes in inbreeding depression in field-tested captive populations of *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences* **279**: 3756–3764.

Epinat G, Lenormand T. **2009**. The evolution of assortative mating and selfing with in- and outbreeding depression. *Evolution* **63**: 2047–2060.

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière Y, Delay B. **2000**. Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. *New Phytologist* **145**: 429–437.

Escarré J, Lefèbvre C, Raboyeau S, Dossantos A, Gruber W, Marel J, Frérot H, Noret N, Mahieu S, Collin C, Oort F van. **2011**. Heavy metal concentration survey in soils and plants of the Les Malines mining district (Southern France): implications for soil restoration. *Water, Air, & Soil Pollution* **216**: 485–504.

Fox C, Reed D. **2011**. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution* **65**: 246–258.

Fox C, Scheibly K, Reed D. **2008**. Experimental evolution of the genetic load and its implications for the genetic basis of inbreeding depression. *Evolution* **62**: 2236–2249.

Frankham R. **2005**. Genetics and extinction. *Biological Conservation* **126**: 131–140.

Geyer C, Ridley C, Latta R, Etterson J, Shaw R. **2013**. Local adaptation and genetic effects on fitness: calculations for exponential family models with random effects. *The Annals of Applied Statistics* **7**: 1778–1795.

Geyer C, Wagenius S, Shaw R. **2007**. Aster models for life history analysis. *Biometrika* **94**: 415–426.

Gillespie J. **1973**. Polymorphism in random environments. *Theoretical Population Biology* **4**: 193–195.

Glémin S. **2003**. How are deleterious mutations purged? Drift versus nonrandom mating. *Evolution* **57**: 2678–2687.

Goodwillie C, Kalisz S, Eckert C. 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. *Annual Review of Ecology, Evolution, and Systematics* **36**: 47–79.

Hereford J. 2014. Inbreeding depression does not increase in foreign environments: a field experimental study. *AoB Plants* **6**: plu009.

Husband B, Schemske D. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* **50**: 54–70.

Ingrouille M, Smirnoff N. 1986. *Thlaspi caerulescens* J. & C. Presl. (T. alpestre L.) in Britain. *New Phytologist* **102**: 219–233.

Jiménez-Ambriz G. 2006. Hétérogénéité environementale et polymorphisme chez *Thlaspi caerulescens* (Brassicaceae) : étude conjointe de la diversité génétique neutre et sélectionnée chez une espèce tolérante aux métaux lourds.

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O. 2007. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**: 199–215.

Kärkkäinen K, Kuittinen H, van Treuren R, Vogl C, Oikarinen S, Savolainen O. 1999. Genetic basis of inbreeding depression in *Arabis petraea*. *Evolution* **53**: 1354–1365.

Keller L, Waller D. 2002. Inbreeding effects in wild populations. *Trends in Ecology & Evolution* **17**: 230–241.

Kishony R, Leibler S. 2003. Environmental stresses can alleviate the average deleterious effect of mutations. *Journal of Biology* **2**: 14.

Koch M, Mummenhoff K, Hurka H. 1998. Systematics and evolutionary history of heavy metal tolerant *Thlaspi caerulescens* in Western Europe: evidence from genetic studies based on isozyme analysis. *Biochemical Systematics and Ecology* **26**: 823–838.

Kristensen T, Pedersen K, Vermeulen C, Loeschcke V. 2010. Research on inbreeding in the "omic" era. *Trends in Ecology & Evolution* **25**: 44–52.

Lande R, Porcher E. 2015. Maintenance of quantitative genetic variance under partial self-fertilization, with implications for evolution of selfing. *Genetics* **200**: 891–906.

Lande R, Schemske D. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* **39**: 24–40.

Lande R, Schemske D, Schultz S. 1994. High inbreeding depression, selective interference among loci, and the threshold selfing rate for purging recessive lethal mutations. *Evolution* **48**: 965–978.

Levin D. 2010. Environment-enhanced self-fertilization: implications for niche shifts in adjacent populations. *Journal of Ecology* **98**: 1276–1283.

Liao W, Reed D. **2009**. Inbreeding–environment interactions increase extinction risk. *Animal Conservation* **12**: 54–61.

Lohr J, Haag C. **2015**. Genetic load, inbreeding depression, and hybrid vigor covary with population size: an empirical evaluation of theoretical predictions. *Evolution* **69**: 3109–3122.

Long T, Rowe L, Agrawal A. **2013**. The effects of selective history and environmental heterogeneity on inbreeding depression in experimental populations of *Drosophila melanogaster*. *The American naturalist* **181**: 532–544.

Manna F, Martin G, Lenormand T. **2011**. Fitness landscapes: an alternative theory for the dominance of mutation. *Genetics* **189**: 923–937.

Martin G, Lenormand T. **2006**. The fitness effect of mutations across environments: a survey in light of fitness landscape models. *Evolution* **60**: 2413–2427.

Meerts P, Van Isacker N. **1997**. Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133**: 221–231.

Menzel M, Sletvold N, Ågren J, Hansson B. **2015**. Inbreeding affects gene expression differently in two self-incompatible *Arabidopsis lyrata* populations with similar levels of inbreeding depression. *Molecular Biology and Evolution* **32**: 2036–2047.

Moorad J, Wade M. **2005**. A genetic interpretation of the variation in inbreeding depression. *Genetics* **170**: 1373–1384.

Mousset M, David P, Petit C, Pouzadoux J, Hatt C, Flaven E, Ronce O, Mignot A. **2016**. Lower selfing rates in metallicolous populations than in non-metallicolous populations of the pseudometallophyte *Noccaea caerulescens* (Brassicaceae) in Southern France. *Annals of Botany* **117**: 507–519.

Murren C, Dudash M. **2012**. Variation in inbreeding depression and plasticity across native and non-native field environments. *Annals of Botany* **109**: 621–632.

O'Grady J, Brook B, Reed D, Ballou J, Tonkyn D, Frankham R. **2006**. Realistic levels of inbreeding depression strongly affect extinction risk in wild populations. *Biological Conservation* **133**: 42–51.

Pannell J. **2015**. Evolution of the mating system in colonizing plants. *Molecular Ecology* **24**: 2018–2037.

Peterson M, Kay K. **2015**. Mating system plasticity promotes persistence and adaptation of colonizing populations of hermaphroditic angiosperms. *The American Naturalist* **185**: 28–43.

Plough L. **2012**. Environmental stress increases selection against and dominance of deleterious mutations in inbred families of the Pacific oyster *Crassostrea gigas*. *Molecular Ecology* **21**: 3974–3987.

R Core Team. **2014**. R: A language and environment for statistical computing.

Reed D, Fox C, Enders L, Kristensen T. **2012**. Inbreeding–stress interactions: evolutionary and conservation consequences. *Annals of the New York Academy of Sciences* **1256**: 33–48.

Rileys R. **1956**. The influence of the breeding system on the genecology of *Thlaspi Alpestre* L. *New Phytologist* **55**: 319–330.

Ritland K. **2002**. Extensions of models for the estimation of mating systems using n independent loci. *Heredity* **88**: 221–228.

Ronce O, Shaw F, Rousset F, Shaw R. **2009**. Is inbreeding depression lower in maladapted populations? A quantitative genetics model. *Evolution* **63**: 1807–1819.

Roze D. **2015**. Effects of interference between selected loci on the mutation load, inbreeding depression, and heterosis. *Genetics* **201**: 745–757.

Schemske D, Lande R. **1985**. The evolution of self-fertilization and inbreeding depression in plants. II. empirical observations. *Evolution* **39**: 41–52.

Schmitt J, Gamble S. **1990**. The effect of distance from the parental site on offspring performance and inbreeding depression in *Impatiens capensis*: a test of the local adaptation hypothesis. *Evolution* **44**: 2022–2030.

Schou M, Loeschcke V, Kristensen T. **2015**. Inbreeding depression across a nutritional stress continuum. *Heredity* **115**: 56–62.

Shaw R, Geyer C, Wagenius S, Hangelbroek H, Etterson J. **2008**. Unifying life-history analyses for inference of fitness and population growth. *The American Naturalist* **172**: E35–E47.

Sletvold N, Mousset M, Hagenblad J, Hansson B, Ågren J. **2013**. Strong inbreeding depression in two Scandinavian populations of the self-incompatible perennial herb *Arabidopsis lyrata*. *Evolution* **67**: 2876–2888.

Swindell W, Bouzat J. **2006**. Selection and inbreeding depression: effects of inbreeding rate and inbreeding environment. *Evolution* **60**: 1014–1022.

Vergeer P, Wagemaker N, Ouborg N. **2012**. Evidence for an epigenetic role in inbreeding depression. *Biology Letters* **8**: 798–801.

Waller D, Dole J, Bersch A. **2008**. Effect of stress and phenotypic variation on inbreeding depression in *Brassica rapa*. *Evolution* **62**: 917–931.

Wang A, Sharp N, Agrawal A. **2014**. Sensitivity of the distribution of mutational fitness effects to environment, genetic background, and adaptedness: a case study with *Drosophila*. *Evolution* **68**: 840–853.

Willis J. **1999**. The role of genes of large effect on inbreeding depression in *Mimulus guttatus*. *Evolution* **53**: 1678–1691.

Winn A, Elle E, Kalisz S, Cheptou P-O, Eckert C, Goodwillie C, Johnston M, Moeller D, Ree R, Sargent R, Vallejo-Marín M. **2011**. Analysis of inbreeding depression in mixed-mating plants provides evidence for selective interference and stable mixed mating. *Evolution* **65**: 3339–3359.

Wool D, Sverdlov E. **1976**. Sib-mating populations in an unpredictable environment: effects on components of fitness. *Evolution* **30**: 119–129.

Contributors

Several persons helped me with the planning and the realisation of the two studies described in this manuscript.

- Planning of the studies: A. Mignot, O. Ronce, C. Petit
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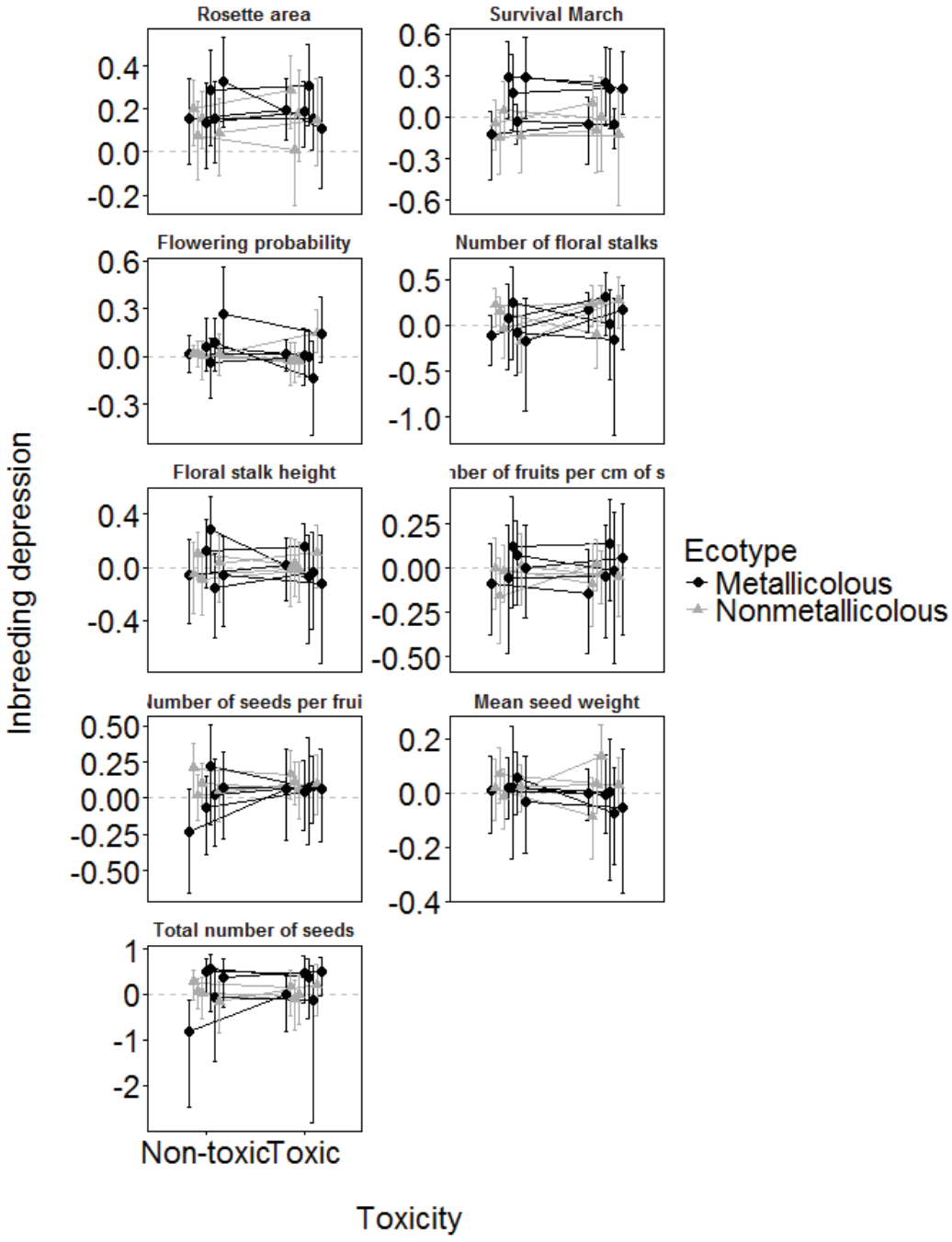
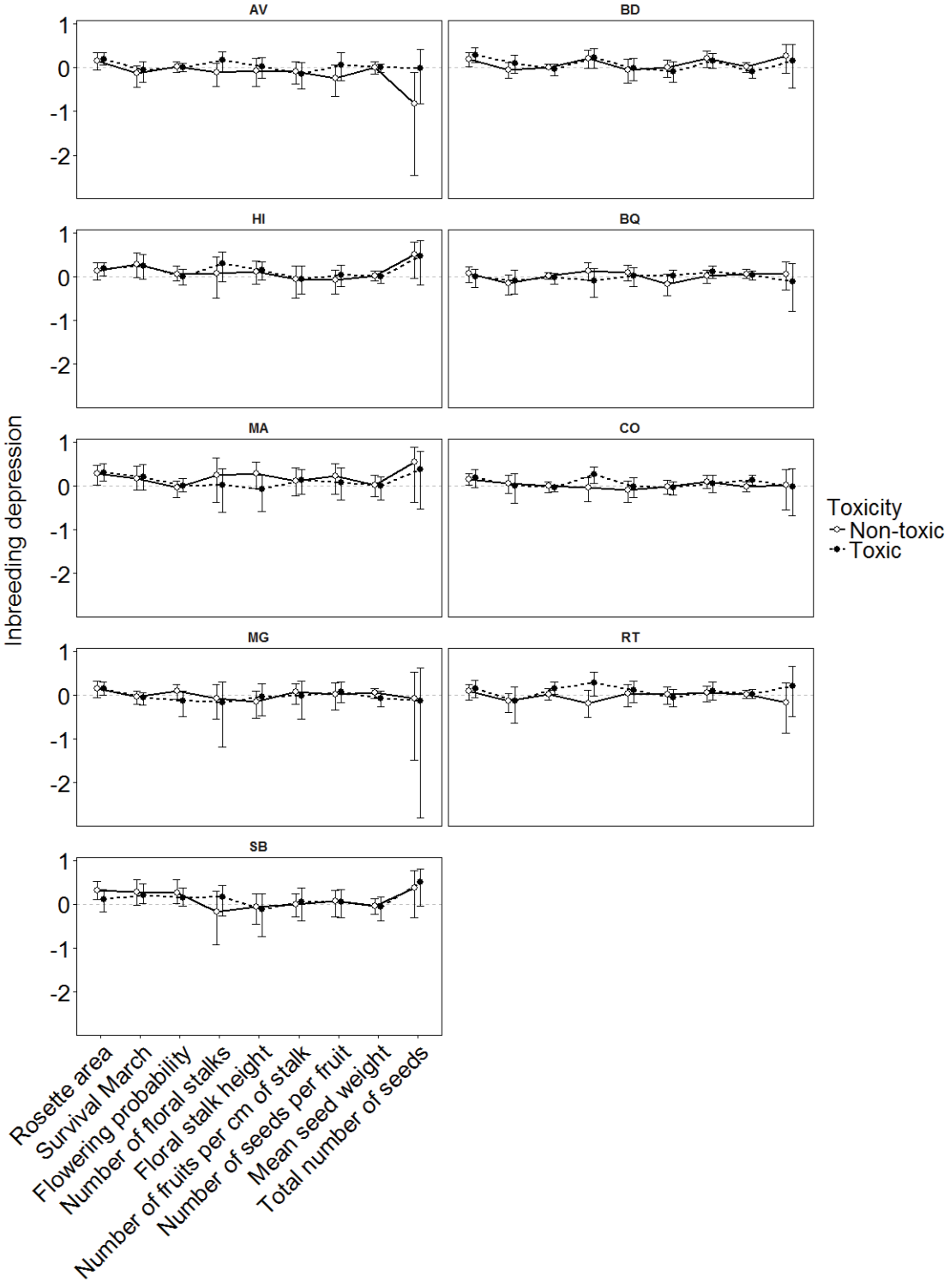
- Genotyping: J. Pouzadoux
- Data analysis: O. Ronce, A. Mignot, M. Segond.

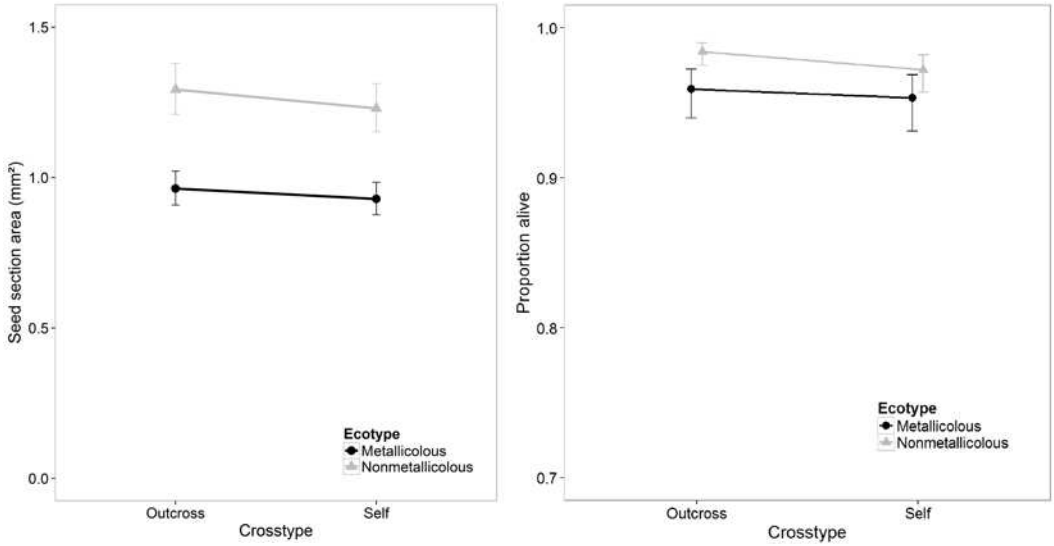
Appendix



Appendix S1. Population inbreeding depression on toxic and non-toxic soil in *N. caerulescens*. Black circles: metallicolous populations. Grey triangles: non-metallicolous populations.



Appendix S2. Population inbreeding depression along the life cycle in *N. caerulescens*. Filled circles: toxic soil. White circles: non-toxic soil.



Appendix S3. Predicted mean seed area and germination rates in both ecotypes and cross types. Error bars represent approximate 95% confidence intervals. Black circles represent the metallicolous ecotype and grey triangles represent the non-metallicolous eco

SYNTHÈSE ET PERSPECTIVES

"It is paradoxical, yet true, to say, that the more we know,
the more ignorant we become in the absolute sense,
for it is only through enlightenment that we become conscious of our limitations.
Precisely one of the most gratifying results of intellectual evolution is
the continuous opening up of new and greater prospects."
Nikola Tesla

To self or not to self?

J'ai tout d'abord confirmé, à l'aide de méthodes précises et peu biaisées, que *N. caerulescens* a un système de reproduction mixte dans les Cévennes et sur le Causse du Larzac, avec des taux d'autofécondation allant de 25% pour l'écotype métallicole à 40% pour l'écotype non métallicole (**Figure 1**). Remarquablement, la variation entre populations d'un même écotype n'était pas significative pour les populations étudiées, ce qui suggère qu'un facteur commun entre ces populations et différent entre les deux écotypes influence le système de reproduction chez cette espèce.

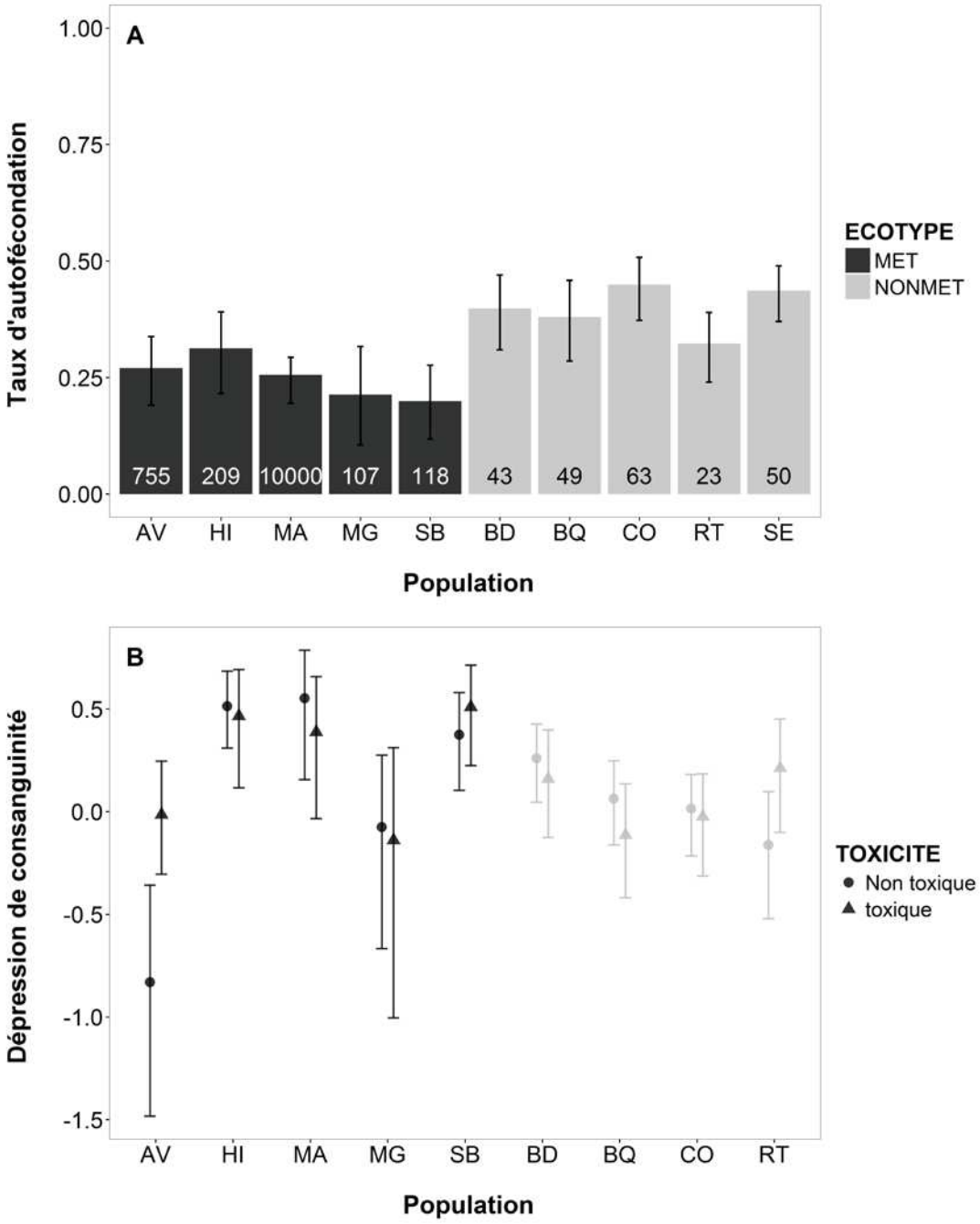


Figure 1. Résultats principaux de la thèse. A) Taux d'autofécondation et taille efficace des dix populations étudiées. Taux d'autofécondation obtenus avec RMES en le contraignant à être le même les deux années; taille efficace obtenue avec MNLE. B) Dépression de consanguinité sur le nombre total estimé de graines. Gris clair : écotype non métallicole, gris foncé : écotype métallicole. Points : sol non toxique, triangles : sol toxique.

Une première question est de savoir si ces résultats peuvent être généralisés à l'ensemble de l'aire de répartition de *N. caerulescens*. Le système de reproduction de l'espèce est potentiellement plus variable à cette échelle qu'au sein de la région étudiée dans cette thèse. En effet, les études génétiques de *N. caerulescens* dans d'autres régions ont trouvé des F_{IS} allant de zéro à un (Besnard et al. 2009; Gonneau 2014). Même si inférer le taux d'autofécondation à partir du coefficient de consanguinité comporte des biais, on peut néanmoins imaginer que cette extrême variation représente, au moins pour certaines populations, une variation réelle du système de reproduction (**Figure 2**). Ceci est d'autant plus probable que les différentes populations sont géographiquement éloignées, à des altitudes et climats différents les unes des autres. L'étude d'un grand nombre de populations pourrait permettre de corréler des paramètres des populations avec le système de reproduction (écotype, toxicité du sol, taille des populations, latitude, marginalité de l'aire, etc.). Un premier de ces facteurs pourrait être la distance entre stigmate et anthères à maturité. D'après la description de Riley (1956), les anthères matures des fleurs de *N. caerulescens* en Angleterre sont proches du stigmate, ce qui, d'après lui, devait rendre l'autofécondation très probable. Dans le Sud de la France, les anthères semblent moins proches du stigmate. Il serait intéressant d'étudier l'herkogamie dans différentes populations de l'aire de répartition. Si elle varie, il serait alors logique d'essayer de la corréler au taux d'autofécondation.

J'ai ensuite testé la densité de plantes en fleurs comme facteur susceptible d'expliquer la variation du taux d'autofécondation en populations naturelles. Ce facteur est connu pour influencer l'attraction et le comportement de butinage des pollinisateurs. En outre, la densité des populations métallicoles semble en général plus forte que la densité des populations non métallicoles (Dubois 2005). Ce facteur semblait donc prometteur pour expliquer les différences de taux d'autofécondation entre écotypes.

La première étude s'est soldée par un résultat négatif : le taux d'autofécondation était totalement indépendant de la densité estimée par la distance aux dix plus proches voisines. Mais la densité atteinte lors de cet épisode reproductif était telle que j'ai souhaité faire une autre étude dans une population où la densité est plus variable.

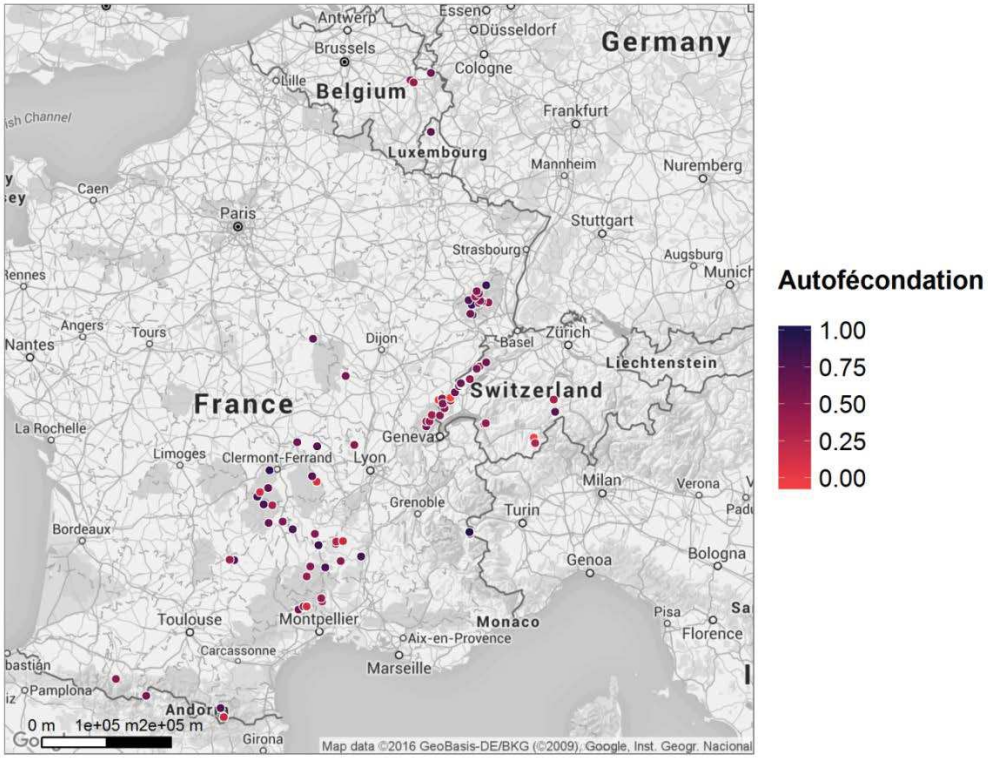


Figure 2. Taux d'autofécondation dans les différentes populations étudiées dans Gonneau (2014) et Besnard et al. (2009). Estimations de l'autofécondation à partir du coefficient de consanguinité. Données de carte ©2016 Geobasis – DE/BKG

Dans la seconde étude, l'effet de la densité n'était toujours pas significatif, malgré de fortes différences de densité entre groupes étudiés. Il est à noter que pour une différence du taux d'autofécondation du même ordre de magnitude que celle observée entre les dix populations des deux écotypes, la puissance statistique de cette analyse est beaucoup plus faible, malgré une taille d'échantillonnage respectable. La différence de taux d'autofécondation entre forte et faible densités, si elle avait été significative, n'aurait dans tous les cas pas expliqué toute la différence observée entre écotypes. Le principal message à retenir est que la densité affecte peu le taux d'autofécondation à l'échelle où nous l'avons mesurée, et/ou ses effets sont noyés par des facteurs confondants. Une manipulation expérimentale de la densité et du nombre d'inflorescences pourrait être utilisée pour essayer de séparer les effets de la géitonogamie et de la densité sur le comportement des pollinisateurs et sur le taux d'autofécondation.

Il semble nécessaire d'étudier plus précisément les mécanismes de l'autofécondation chez *N. caerulescens* et de quantifier leur contribution relative aux taux d'autofécondation estimés.

L'autofécondation peut-elle s'effectuer de manière autonome et si oui, en quelles proportions ? Ou est-elle essentiellement dépendante du comportement des pollinisateurs ? Une expérience est actuellement en cours en conditions contrôlées en manipulant l'accès de différents vecteurs de pollinisation pour répondre à ces questions. Au vu des taux d'allofécondation dans les populations naturelles, il semble dans tous les cas important d'identifier les communautés de pollinisateurs visitant *N. caerulescens* dans différentes populations au cours de la saison. La caractérisation des pollinisateurs est un travail en cours, qui demande un fort investissement étant donné la longue floraison de l'espèce. Il est possible que la présence de métaux dans l'environnement modifie le comportement des pollinisateurs, et l'on pourrait tester si ces derniers ajustent leur comportement en fonction de la présence d'éléments traces dans la plante ou dans l'environnement. Un autre facteur intéressant à tester serait la fragmentation du milieu. En effet, les populations non métallicoles poussent souvent dans des clairières au milieu de buis ou de forêts de chênes verts. La taille des clairières et/ou le nombre de plantes par clairière, plus que leur densité *per se*, pourrait influencer l'autofécondation. En interaction ou non avec le comportement des pollinisateurs, la morphologie des plantes et leur phénologie pourraient expliquer les différences d'autofécondation entre écotypes. Il serait alors intéressant de tester si les différences d'autofécondation mesurées en populations naturelles se maintiennent lorsque les plantes métallicoles et non métallicoles se reproduisent en jardin commun, afin de séparer les effets environnementaux et génétiques affectant la variation du taux d'autofécondation entre écotypes.

Structure génétique

La différenciation entre populations suggère l'existence de faibles flux de gènes entre populations, confirmant les résultats d'études précédentes (Dubois et al. 2003; Jiménez-Ambriz et al. 2007; Gonneau 2014). L'évolution de l'autofécondation dans les populations métallicoles de *N. caerulescens* est donc peu contrainte par la présence de flux de gènes en provenance de populations non métallicoles dans la même région. Il est difficile de conclure quant à la participation de l'autofécondation à ces faibles flux de gènes. En effet, toutes choses égales par ailleurs, 25 à 40% d'autofécondation équivaut à une réduction de 25 à 40% de pollen exporté chez une autogame partielle par rapport à une allogame complète. Mais si les rares flux de gènes

ne sont dus qu'à la dispersion occasionnelle des graines, exporter plus ou moins de pollen ne devrait pas beaucoup affecter la différenciation entre populations.

L'échelle des flux de gènes à l'intérieur des populations est un paramètre très important pour comprendre l'adaptation locale à des pressions de sélection variables à fine échelle, telles que générées par des niveaux de toxicité ou de luminosité différentes. L'étude de la structure intra-mine à Saint Hippolyte révèle que cette population est structurée. Ce résultat vient conforter les résultats de Dubois (2005), qui mettaient en évidence une structure faible mais significative entre les différents sous-milieux (ouvert/talus/sous-bois) de trois mines étudiées. Une analyse de la structure spatiale à fine échelle est en cours à Saint Hippolyte, ce qui nous permettra de mieux comprendre les mouvements de gènes à l'intérieur de la mine. Des échantillons de sol peuvent être prélevés dans les différents quadrats de la mine où nous avons génotypé les plantes. Ces échantillons permettront de comparer les données de différenciation génétique aux différences de toxicité. De forts flux de gènes peuvent empêcher l'adaptation locale (Lenormand 2002 pour revue). Si des différences environnementales existent à faible échelle à l'intérieur des populations alors la réduction des flux de gènes, entre autres via l'autofécondation, peut favoriser l'adaptation aux conditions locales (par exemple Antonovics 1968, 2006; McNeilly et Antonovics 1968).

Taille efficace

L'estimation des tailles efficaces dans les dix populations étudiées suggère que les populations non métallicoles des Causses ont une taille efficace assez faible, et les populations métallicoles des Cévennes des tailles faibles à modérées (**Figure 1**). Ce résultat est cohérent avec les taux d'autofécondation estimés, qui prédisent que la dérive devrait être un peu plus forte chez les populations non métallicoles. Toutefois, il semble que la différence observée de taille efficace soit supérieure à celle prédite uniquement sur la base des différences modérées d'autofécondation entre écotypes. Une structuration spatiale ou temporelle, ou un nombre d'individus reproducteurs différents, pourraient également être impliqués. Ce dernier facteur en particulier est prometteur, dans la mesure où les populations métallicoles semblent comporter plus d'individus. Des comptages pourraient permettre de comparer les deux écotypes plus précisément pour ce facteur.

La faible taille efficace des populations non métallicoles devrait théoriquement compromettre leur survie sur le long terme, même si elle doit également conduire à une plus faible dépression de consanguinité comparée aux populations métallicoles. Les résultats de l'expérience sur la dépression de consanguinité ne vont pas dans le sens d'une valeur sélective systématiquement plus faible dans les populations non métallicoles. Par ailleurs, les résultats obtenus sur la diversité génétique lors de la première partie de cette thèse ne sont pas non plus cohérents avec une plus faible taille efficace des populations non métallicoles. Ces observations pourraient être cohérentes avec des différences récentes de processus démographiques entre écotypes, qui augmenteraient la force de la dérive, mais n'auraient pas encore affecté le fardeau génétique ou la diversité. Mais cela pourrait être dû à des scénarios plus compliqués, impliquant des effets historiques de fondation plus forts chez les métallicoles, qui auraient abaissé la diversité de ces dernières, en conjugaison avec des différences démographiques plus récentes entre les deux écotypes qui expliqueraient les effets de dérive récents.

Dépression de consanguinité

La dépression de consanguinité chez *N. caerulescens* était, en conditions contrôlées, faible à modérée (**Figure 1**), notamment par comparaison avec d'autres plantes avec des taux d'autofécondation équivalents (Winn et al. 2011). En populations naturelles, les forts F_{IS} et taux d'autofécondation estimés sur les adultes suggèrent que la dépression de consanguinité n'est pas non plus extrêmement forte dans ces conditions ou que les taux d'autofécondation réels sont beaucoup plus élevés que ceux mesurés dans notre étude. Cette dernière hypothèse n'est toutefois pas cohérente avec les taux d'autofécondation estimés sur des plantules germées en serre, qui sont du même ordre de grandeur que ceux estimés sur les adultes échantillonnés (voir chapitre 1 et 2). Il est donc peu probable que l'on soit en présence d'un cas d'interférence sélective, où un grand nombre de mutations récessives létales ségrégent dans la population, empêchant virtuellement tous les individus issus de l'autofécondation de survivre, et prévenant donc la purge de ces mutations (Lande et al. 1994; Winn et al. 2011; Roze 2015).

Pour certains traits, nous avons observé une différence de dépression de consanguinité entre les écotypes métallicole et non métallicole, dont le sens est conforme à la différence attendue connaissant les taux d'autogamie et/ou les tailles efficaces. Il est difficile de savoir si

l'autofécondation purge plus la dépression de consanguinité dans l'écotype non métallicole, ou si la plus faible dépression de consanguinité permet d'avoir des taux d'autofécondation plus élevés dans l'écotype non métallicole.

Nous observons une remarquable variation des patrons selon les composantes de valeur sélective utilisées pour calculer la dépression. Il serait intéressant de tester plus rigoureusement les différences de dépression entre traits à l'aide d'une analyse multivariée. La variabilité observée entre les différentes composantes de la valeur sélective n'est pas vraiment expliquée, n'étant clairement reliée ni au caractère précoce ou tardif des traits, ni à leur variation (CV), ni à leur sensibilité au stress. Nous avons donc souhaité utiliser une mesure intégrative de valeur sélective qui combine toutes ces composantes. L'utilisation d'une mesure agrégée de valeur sélective peut être compliquée d'un point de vue statistique, car la distribution obtenue par tirages dans différentes lois des différentes composantes n'est pas classique. L'analyse ASTER répond à ce problème en modélisant explicitement ces tirages conditionnels. Néanmoins, ces analyses ont également leurs limites, en particulier parce que modéliser les effets aléatoires est plus compliqué. Nous n'avons pas détecté de dépression de consanguinité significative sur la mesure intégrative de valeur sélective, alors que nous en avons détecté pour plusieurs traits de vie. Il est possible que des effets faibles et de signes opposés se compensent à l'échelle de la valeur sélective totale, mais il est également possible que notre analyse de valeur sélective totale ne possède pas la puissance statistique pour détecter une faible dépression de consanguinité. Par ailleurs, une difficulté intrinsèque aux analyses fondées sur tout le cycle de vie est que les fardeaux affectant différentes étapes du cycle de vie ne sont pas nécessairement génétiquement indépendants. Les traits de reproduction sont mesurés sur les individus qui ont survécu. Ceux-ci représentent peut-être un échantillon non aléatoire de la diversité génétique initialement présente si une purge des individus consanguins les plus faibles se produit au fur et à mesure de l'expérience.

Nous n'avons quasiment pas trouvé d'effet significatif de la toxicité au sulfate de zinc sur la dépression de consanguinité en conditions contrôlées, malgré une toxicité réelle sur les populations non métallicoles et parfois sur les populations métallicoles, avérée par une mortalité perceptible. Nous n'avons pas non plus détecté d'effet sur la dépression de consanguinité de l'interaction entre histoire de la sélection (écotype) et conditions de test (toxicité au zinc). Il est possible que ce résultat négatif, qui fait suite à plusieurs résultats

équivoques dans la littérature, soit dû à la combinaison de plusieurs effets opposés de la consanguinité sur l'expression des mutations dans différents environnements. Par exemple, on s'attend à ce que la présence de mutations à effets antagonistes ou conditionnellement neutres génère plus de dépression lorsque la population est mal adaptée. Ainsi, la présence de mutations dont la dominance change en fonction de l'environnement pourrait aboutir à plus de dépression de consanguinité lorsque la population est adaptée.

Une dépression de consanguinité aussi faible, si elle est confirmée en conditions naturelles, ne devrait pas représenter une barrière forte à l'évolution de l'autofécondation. On peut alors se demander pourquoi *N. caerulescens* n'est pas plus autogame dans la région d'étude. Une première possibilité est que les taux d'autofécondation que nous avons mesurés ne sont pas à l'équilibre et évoluent lentement vers un système plus autogame. Une autre possibilité est que l'autofécondation est essentiellement influencée - et donc contrainte - par les pollinisateurs, et que le taux d'autofécondation ne peut pas augmenter sans évolution préalable de traits favorisant la géitonogamie ou l'autofécondation autonome. Ce type de contraintes fonctionnelles entre l'autofécondation et l'allofécondation est particulièrement bien illustré par le modèle de Johnston et al. (2009) qui prédit dans de nombreuses combinaisons de paramètres l'évolution de taux d'autofécondation stables du fait de contraintes entre composantes de la reproduction.

En ce qui concerne la dépression de consanguinité, il serait intéressant d'estimer de manière indirecte une partie de la dépression de consanguinité en conditions naturelles dans les populations de *N. caerulescens* en comparant le taux d'autofécondation estimé sur des plantules (aussi tôt que possible) au taux d'autofécondation estimé pendant la floraison de la même génération. Cela nous informerait de la magnitude de la dépression de consanguinité sur la survie jusqu'à la floraison en populations naturelles. Il serait particulièrement intéressant de faire cette mesure dans différentes populations de l'aire de *N. caerulescens* et de la suivre pendant plusieurs années. En revanche, *N. caerulescens* ne semble pas être un très bon modèle pour démêler plus avant les effets potentiels du stress et de l'histoire de la sélection sur la dépression de consanguinité dans différents environnements. En effet, le peu de dépression de consanguinité et son insensibilité à un type de stress au moins sont peu prometteuses, et la lourdeur des expériences qui permettraient d'obtenir plus d'informations rend ce modèle peu attrayant pour continuer.

Conclusion générale

Ce travail a montré que *N. caerulescens* se reproduit selon un système de reproduction mixte, qui dépend de l'histoire d'adaptation des populations, avec un plus fort taux d'autofécondation dans l'écotype non métallicole que dans l'écotype métallicole. La densité locale de plantes en fleur semble n'influencer qu'assez peu les taux d'autofécondation dans ces populations. Pour la première fois, la dépression de consanguinité a été mesurée chez cette espèce modèle, et il apparait qu'elle est modérée en conditions contrôlées, souvent plus forte chez les plantes métallicoles que non métallicoles et dépend peu de la toxicité du sol. Cela signifie que l'évolution de plus forts taux d'autofécondation dans ces populations est sans doute peu contrainte par la dépression de consanguinité.

On s'attend globalement à des différences faibles à modérées entre écotypes métallicole et non métallicole en ce qui concerne un certain nombre de processus évolutifs. Le système mixte de *N. caerulescens* lui confère plusieurs avantages : assurance reproductive potentielle, sélection plus efficace des allèles bénéfiques, purge des allèles délétères. Par ailleurs, le niveau de diversité génétique neutre, et la dépression de consanguinité modérée suggèrent que les désavantages liés à l'autofécondation ne sont pas très forts pour le moment. Dans le modèle de Peterson et Kay (2015), des taux d'autofécondation intermédiaires équivalents à ceux observés chez *N. caerulescens* prédisent une meilleure probabilité d'adaptation à un nouvel environnement suivant un épisode de colonisation, car ils combinent les avantages de l'autofécondation tout en évitant ses conséquences les plus négatives.

Si *N. caerulescens* est capable de s'autoféconder de manière autonome sans être pénalisée et de maintenir ce système mixte, cela a pu encourager la colonisation de nouveaux habitats, dont certains assez extrêmes lors de la période post-glaciaire de l'Europe. Néanmoins, ce n'est pas une espèce invasive, ni extrêmement commune, ce qui suggère que d'autres caractéristiques limitent la colonisation de nouvelles aires géographiques, telles qu'une faible capacité de dispersion des graines, ou de la sensibilité à la compétition.

Bibliographie

Antonovics J. **1968**. Evolution in closely adjacent plant populations V. Evolution of self-fertility. *Heredity* **23**: 219–238.

Antonovics J. **2006**. Evolution in closely adjacent plant populations X: long-term persistence of prereproductive isolation at a mine boundary. *Heredity* **97**: 33–37.

Besnard G, Basic N, Christin P-A, Savova-Bianchi D, Galland N. **2009**. *Thlaspi caerulescens* (Brassicaceae) population genetics in western Switzerland: is the genetic structure affected by natural variation of soil heavy metal concentrations? *New Phytologist* **181**: 974–984.

Dubois S. 2005. *Etude d'un réseau de populations métallicoles et non-métallicoles de* Thlaspi caerulescens *(Brassicaceae). Structure génétique, démographie et pressions de sélection.* Université Montpellier II Sciences et Techniques du Languedoc, Montpellier, France.

Dubois S, Cheptou P-O, Petit C, Meerts P, Poncelet M, Vekemans X, Lefèbvre C, Escarré J. **2003**. Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* **157**: 633–641.

Gonneau C. 2014. *Distribution, écologie et évolution de l'hyperaccumulation des éléments en traces par* Noccaea caerulescens. Université de Lorraine, Nancy, France

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O. **2007**. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**: 199–215.

Johnston M, Porcher E, Cheptou P-O, Eckert C, Elle E, Geber M, Kalisz S, Kelly J, Moeller D, Vallejo-Marín M, Winn A. **2009**. Correlations among fertility components can maintain mixed mating in plants. *The American Naturalist* **173**: 1–11.

Lande R, Schemske D, Schultz S. **1994**. High inbreeding depression, selective interference among loci, and the threshold selfing rate for purging recessive lethal mutations. *Evolution* **48**: 965–978.

Lenormand T. **2002**. Gene flow and the limits to natural selection. *Trends in Ecology & Evolution* **17**: 183–189.

McNeilly T, Antonovics J. **1968**. Evolution in closely adjacent plant populations IV. Barriers to gene flow. *Heredity* **23**: 205–218.

Peterson M, Kay K. **2015**. Mating system plasticity promotes persistence and adaptation of colonizing populations of hermaphroditic angiosperms. *The American Naturalist* **185**: 28–43.

Rileys R. **1956**. The influence of the breeding system on the genecology of *Thlaspi Alpestre* L. *New Phytologist* **55**: 319–330.

Roze D. **2015**. Effects of interference between selected loci on the mutation load, inbreeding depression, and heterosis. *Genetics* **201**: 745–757.

Winn A, Elle E, Kalisz S, Cheptou P-O, Eckert C, Goodwillie C, Johnston M, Moeller D, Ree R, Sargent R, Vallejo-Marín M. **2011**. Analysis of inbreeding depression in mixed-mating plants provides evidence for selective interference and stable mixed mating. *Evolution* **65**: 3339–3359.

ANNEXE

CHARACTERIZATION AND MULTIPLEXING OF 21 MICROSATELLITE MARKERS FOR THE HERB *NOCCAEA CAERULESCENS* (BRASSICACEAE)

Characterization and Multiplexing of 21 Microsatellite Markers for the Herb *Noccaea caerulescens* (Brassicaceae)

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Primer Note

Characterization and multiplexing of 21 microsatellite markers for the herb *Noccaea caerulescens* (Brassicaceae)[1]

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- *Premise of the study:* Multiplexed microsatellite markers were developed for population genetic studies in the pseudometallophyte *Noccaea caerulescens* (Brassicaceae), a model species to investigate metal tolerance and hyperaccumulation in higher plants.
- *Methods and Results:* Microsatellite loci were isolated through pyrosequencing of an enriched DNA library. Three multiplexes combining four previously published and 17 newly designed markers were developed. The new markers were screened in metallicolous and nonmetallicolous populations from southern France. The total number of alleles per locus ranged from five to 18. The observed heterozygosity per locus and per population ranged from 0 to 0.83, and expected heterozygosity ranged from 0 to 0.89.
- *Conclusions:* The investigated loci showed reasonable to high levels of polymorphism at the regional scale. The multiplex set should be helpful in investigating genetic diversity, population structure, and demographic history in *N. caerulescens* at various spatial scales.

Key words: Brassicaceae; heavy metal tolerance; microsatellite; *Noccaea caerulescens*; pseudometallophyte.

The Alpine pennycress, *Noccaea caerulescens* (J. Presl & C. Presl) F. K. Mey. (Brassicaceae), occurs over a large range in Europe. The species is particularly known for its capacity to grow on soils with a high concentration of trace elements such as zinc, cadmium, and nickel. Considering its phylogenetic proximity with *Arabidopsis thaliana*, this characteristic makes *N. caerulescens* a favorite model plant for the study of the genetic bases of metal homeostasis, as well as of the ecological and evolutionary processes involved in local adaptation to extreme environments (Assunção et al., 2003). In order to understand the effect of soil metals on the evolution of *N. caerulescens* populations, it is necessary to study the population genetics of the species. So far, however, the low number of available molecular markers (Basic and Besnard, 2006; Jiménez-Ambriz et al., 2007), low polymorphism, presence of null alleles (Basic and Besnard, 2006; Besnard et al., 2009), and low amplification rate (E. Flaven, personal observation), as well as the absence of protocols for high-throughput genotyping, has not allowed the performance of deep population genetic studies. Here, we introduce 17 new microsatellite markers organized in three multiplexes to reduce genotyping time and costs. The multiplexes also include formerly published markers, thus providing a complete resource in this species.

METHODS AND RESULTS

***Microsatellite library construction*—**Genomic DNA of three individuals from the Baraquette population (Appendix 1) was extracted using a cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990) followed by RNase treatment, and mixed. Development of the microsatellite library was outsourced to Genoscreen (Lille, France). It involved coupling multiplex microsatellite enrichment isolation techniques with 454 GS FLX Titanium pyrosequencing of the enriched DNA, according to the protocol of Malausa et al. (2011). Enrichment was performed using probes containing the following motifs: AG_{10}, AC_{10}, AAC_8, AGG_8, ACG_8, AAG_8, $ACAT_6$, and $ATCT_6$. Sequence data were automatically screened to detect microsatellite motifs, leading to 1852 candidate loci. Primers were designed in silico by Genoscreen, using the QDD pipeline (Meglécz et al., 2010).

***Biological validation*—**Biological validation of a subset of these loci was simultaneously performed at Institut des Sciences de l'Évolution de Montpellier

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TABLE 1. Characterization of 21 microsatellite loci in *Noccaea caerulescens*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Ct (μM)	Multiplex	Fluorescent dye	Post-PCR dilution	Position *A.t.*[a]	Position *N.c.*[a]	Chromosomal blocks[b]	Allele size range (bp)	GenBank accession no.	Publication
Ncpm09	F: TAGACGCTGCGTTTTGAAGA	CT_{18}	58	0.4	NcM1	6-FAM	1/150	1	2	B	96–130	KR065729	
	R: CCTCTGTTGAGTGAATGGTTCTC												
Ncpm13	F: CCAAAACTATGCCGATCTCA	AG_{16}	58	0.2	NcM1	VIC	1/150	NA	NA	NA	158–166	KR065730	
	R: CCACGAGCGAATCTTCTTGT												
Ncpm21	F: GTCACCACTTGCTACGGGAT	CTT_{10}	58	0.2	NcM1	VIC	1/150	3	7	F	240–277	KR065731	
	R: CATTGGATAGCACAGAGGCA												
Ncpm23	F: TTTCATGTCTCGGATCCTCC	TTC_{11}	58	0.4	NcM1	6-FAM	1/150	NA	NA	NA	197–263	KR065732	
	R: GCAGAGCGCAATCTAAGGAC												
Ncpm31	F: GATTATCGAGCTTACTAAAAGCAGC	CTT_{12}	58	0.2	NcM1	NED	1/150	5	4	W	58–101	KR065733	
	R: GTGTTGAAGCGCAATGAAGA												
Tc-up1	F: TGCTCTGTTTCTCTCCACATTC	$(CA)_n(CT)_n$	50	0.2[d]	NcM1	NED	1/160	NA	NA	NA	132–170	AJ746212	Basic and Besnard, 2006
	R: TTCCTTGCTTCTTCTCTTCCA												
Ncpm07	F: TGGAATGGTTCTGTGGACAA	TCA_{18}	58	0.4	NcM2	6-FAM	1/150	NA	NA	NA	109–139	KR065734	
	R: TTCTGGAATTGGCTGCTTCT												
Ncpm14	F: GACCACATCTCGTCTTGCCT	CTT_{15}	58	0.4	NcM2	PET	1/150	NA	NA	NA	108–123	KR065735	
	R: GACCCTAACTACAGGCTGTGAAA												
Ncpm19	F: CCAACAATGGATTGGGAGAG	$GTGTA(TG)_{14}$	58	0.4	NcM2	VIC	1/150	NA	NA	NA	100–124	KR065736	
	R: TCCCCATCACTCCAGCTAAG												
Ncpm29	F: CTCCCTCTTCTCCTACCTACACATA	AC_{17}	58	0.4	NcM2	NED	1/150	NA	NA	NA	78–94	KR065737	
	R: GGTTGAAAGTAGGAGTGAGTCAAGA												
Tc-up4	F: GTTTTGTCCGCTTTGCTTCC	CT_{13}	50	0.2[d]	NcM2	VIC	1/140	NA	NA	NA	253–262	AJ746216.1	Basic and Besnard, 2006
	R: GCCATAGACTTTCTCATTGATTC												
9C7/Thlc3	F: GTCACGAGTTTCACCATT	AG_{13}	58	0.4	NcM2	VIC	1/150	NA	NA	NA	152–191		Jiménez-Ambriz et al., 2007
	R: ATCTTCCACAATTGTGCC												
Tc-up2	F: TGAGAAGAGGAGACACAGGAAC	$(AG)_5(AG)_5(GA)_6$	58	0.2	NcM2	PET	1/150	NA	NA	NA	232–244	AJ746213.1	Basic and Besnard, 2006
	R: CACTTACCAAATCGAAAACTGCTCC												
Nc02	F: GGAGCTGTGGTTTCTGAAGG	AGG_8	68/47[c]	0.06	NcM3	VIC	None	4	3	O	170–191	KR065738	
	R: AGCATCGTATTCCGATCCAG			0.28									
Nc03	F: TGAGCTTCTTCAGTCCCGAT	AC_{12}	68/47[c]	0.06	NcM3	NED	None	5	4	S	188–194	KR065739	
	R: TATGAGCTCGTCGCTCACAG			0.28									
Nc04	F: ACGGTCGCATACCAAAAAGT	AG_{11}	68/47[c]	0.06	NcM3	VIC	None	2	5	J	123–143	KR065740	
	R: AGGATGCACTCCTTGAGACC			0.28									
Nc06b	F: GCGTCTTCTCTCCATCCTCA	AG_{13}	68/47[c]	0.07	NcM3	PET	None	4	6	U	89–153	KR065741	
	R: GGATTTCCAATTCAATCTTCCC			0.37									
Nc07b	F: CCAGTTTCCAACGGCATAGT	AC_{10}	68/47[c]	0.06	NcM3	NED	None	3	7	F	105–112	KR065742	
	R: TTGGTTTGGTTTCTTCTTGTGA			0.28									
Nc19	F: CGGATTGTTGTGAATCCCAT	AGG_{11}	68/47[c]	0.07	NcM3	PET	None	2	5	J	199–235	KR065743	
	R: ACCTCTTCTTTCGCCCTTGT			0.28									
Nc20	F: ACACAACTCCAAAGGCTTCA	AG_{11}	68/47[c]	0.15	NcM3	6-FAM	None	4	3	O	205–234	KR065744	
	R: TTTTGTTCTAACCGTTACTCTTT			0.75									
Nc22	F: TTGCTTTCACATGTCTTGACG	AG_{10}	68/47[c]	0.11	NcM3	6-FAM	None	2	5	K	111–121	KR065745	
	R: GAACAGAACAAGAAGACATGAATGA			0.56									

Note: Ct = final concentration of primers; NA = not available; T_a = annealing temperature.

[a] Relative chromosomal position in *Arabidopsis thaliana* and *Noccaea caerulescens*, respectively (Schranz et al., 2006).

[b] Genome blocks in the "ancestral karyotype" blocks (Schranz et al., 2006).

[c] Following Godé et al. (2012), touchdown PCR was performed. During the first 10 cycles, annealing temperature was decreased from 68°C to 50°C in increments of 2°C. This was followed by 27 cycles with an annealing temperature at 47°C.

[d] Standalone PCR.

(ISEM) and at Laboratoire Évolution Écologie et Paléontologie (Evo-Eco-Paleo), with requirements for levels of genetic polymorphism at different spatial scales. At ISEM, amplification trials were performed on seven individuals from five populations from southern France (Appendix 1). DNA extraction followed a classic CTAB protocol (Doyle and Doyle, 1990). Based on type and number of repeat units, repeat structures, and amplicon size, 32 candidate loci from the library were selected and tested separately. The PCR reactions were carried out in a total volume of 10 µL, containing 1 µL of DNA template, forward and reverse primers (0.2 µM), and 1× QIAGEN Multiplex PCR Master Mix (QIAGEN, Courtaboeuf, France). Cycling conditions were: an initial denaturation step at 95°C for 15 min, then 30 cycles consisting of 30 s at 94°C, 90 s at 58°C, and 1 min at 72°C, followed by a final extension of 30 min at 60°C. PCRs were conducted on Eppendorf Mastercycler pro, Mastercycler nexus gradient (Eppendorf, Hamburg, Germany), and Techne TC-5000 (GMI, Ramsey, Minnesota, USA) machines. Amplification products were visualized with agarose gel (2%) with ethidium bromide stain. Due to inadequate amplification yield, low specificity, or unexpected size, only 15 markers were kept. Forward primers were labeled with one of the FAM, NED, VIC, or PET fluorescent dyes (Applied Biosystems, Waltham, Massachusetts, USA). PCR products were analyzed separately through electrophoresis on an ABI3130 Genetic Analyzer (Applied Biosystems). Nine loci were finally retained based on presence of polymorphism and quality of profiles.

At Evo-Eco-Paleo, 20 primer pairs corresponding to 20 additional loci from the library were selected. They were tested separately on 23 individuals scattered in the European species range (Koch and German, 2013; Appendix 1). Total DNA was extracted using the QIAGEN DNeasy kit (QIAGEN). Extraction and test PCR were performed according to Godé et al. (2012). Each primer pair was tested using FAM labeling. PCR reactions were carried out in a total volume of 10 µL, containing 1 µL of 1/20 diluted DNA template, 2 µM of forward and reverse primers, and 1× QIAGEN Multiplex PCR Master Mix. A final set of eight markers was selected based on the quality of genotyping profiles, compatibility of amplicon sizes for multiplexing, and relative positions on the genome (Table 1). Genomic positions of microsatellite loci were determined by BLASTN searches of microsatellite flanking sequences against the *Arabidopsis thaliana* genome and by using the synteny among chromosomal blocks determined for different Brassicaceae species and the ancestral karyotype (Schranz et al., 2006).

***Screening of the new microsatellite markers*—**All forward primers were labeled with fluorescent dyes, and markers were combined in multiplex PCR based on size compatibility and annealing temperatures (Table 1). Primer dimerization was checked using OligoAnalyzer 1.0.3 (Integrated DNA Technologies, Coralville, Iowa, USA). In addition to newly defined markers, four previously developed markers (Tc-up1, Tc-up2, Tc-up4, Thlc3; Basic and Besnard, 2006; Jiménez-Ambriz et al., 2007) were added to the multiplexes to increase the number of available, multiplexed markers (results not shown, see Table 1 for details). However, due to differing annealing temperatures, Tc-up1 and Tc-up4 were processed in separate PCR and added in the post-PCR steps.

Seventy-four individuals from four populations (Appendix 1, identified as "natural populations screening") were analyzed. DNA extraction followed the protocol from Doyle and Doyle (1990). The PCR reactions were carried out following the protocols described above: ISEM section for the first (NcM1) and second (NcM2) multiplexes, Evo-Eco-Paleo section for the third (NcM3), except that forward and reverse primers were mixed (concentrations in Table 1). Three microliters of diluted PCR product (dilutions in Table 1) were transferred in a mix of 15 µL of Hi-Di Formamide (Applied Biosystems) and 0.15 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Raw data were analyzed using GeneMapper (version 5.0; Applied Biosystems). Automatic analysis and manual check of all the peaks were performed. Detection of the presence of null alleles in populations was performed with FreeNA (Chapuis and Estoup, 2007). Two new loci (Ncpm31 and Ncpm07) harbor null alleles, with frequencies between 10% and 15%. Expected heterozygosity, intrapopulation fixation index, and linkage equilibrium tests were computed using FSTAT (version 2.9.3; Goudet, 1995), and observed heterozygosity was computed in GENETIX (version 4.05; Belkhir et al., 2004). No linkage disequilibrium was detected between loci (with Bonferroni correction). The number of alleles for each locus ranged from five to 18 (Table 2). The observed heterozygosity per locus and per population ranged from 0 to 0.83, and the expected heterozygosity ranged from 0 to 0.89. Hardy–Weinberg equilibrium was tested in GENEPOP (Rousset, 2008), and most of the loci in the four populations were at equilibrium. This result contrasts with previous studies in *N. caerulescens* (Dubois et al., 2003; Basic and Besnard, 2006; Jiménez-Ambriz et al., 2007; Besnard et al., 2009) and may be due to sample size.

TABLE 2. Statistical analysis of the 17 new microsatellite markers in four populations[a] of *Noccaea caerulescens* in southern France.

		All				Avinières (n = 18)				Saint Bresson (n = 22)				Saint Hippolyte (n = 17)				Coulet (n = 17)			
Locus	% amp	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]	A	H_o	H_e	F_{IS}[b]
Ncpm09	100	16	0.563	0.699	0.108	8	0.722	0.794	0.091	5	0.545	0.598	0.089	10	0.823	0.893	0.078	6	0.353	0.46	0.232
Ncpm13	100	5	0.260	0.346	0.130	3	0.500	0.606	0.175	3	0.500	0.530	0.057	1	0.000	0.000	NA	4	0.176	0.224	0.213
Ncpm21	100	9	0.505	0.570	0.136	4	0.500	0.556	0.10	5	0.682	0.639	–0.068	3	0.412	0.588	0.300	3	0.294	0.449	0.344
Ncpm23	100	9	0.444	0.557	0.199	4	0.389	0.562	0.308	3	0.409	0.427	0.043	5	0.412	0.566	0.273	4	0.529	0.638	0.170
Ncpm31	98.6	9	0.493	0.694	0.396	6	0.667	0.771	0.136	4	0.111	0.645	0.828*	5	0.412	0.763	0.460	5	0.529	0.658	0.196
Ncpm07	98.6	8	0.485	0.606	0.210	6	0.833	0.725	–0.149	2	0.364	0.359	–0.012	3	0.062	0.383	0.837*	5	0.412	0.717	0.426
Ncpm14	100	5	0.254	0.405	0.357	3	0.500	0.650	0.231	2	0.091	0.089	–0.024	3	0.235	0.318	0.260	2	0.059	0.346	0.830
Ncpm19	100	11	0.544	0.647	0.130	5	0.722	0.730	0.011	4	0.591	0.639	0.075	2	0.187	0.425	0.559	7	0.706	0.774	0.088
Ncpm29	100	9	0.579	0.733	0.174	6	0.611	0.753	0.189	6	0.773	0.696	–0.110	7	0.529	0.833	0.364	5	0.529	0.746	0.291
Nc02	97.3	6	0.384	0.482	0.081	5	0.750	0.693	–0.042	4	0.273	0.290	0.060	1	0.000	0.000	NA	5	0.562	0.727	0.226
Nc03	98.6	4	0.296	0.467	0.316	3	0.350	0.292	–0.14	4	0.429	0.699	0.387	2	0.059	0.059	0.000	4	0.375	0.7	0.464
Nc04	100	9	0.528	0.618	0.125	6	0.700	0.778	0.143	4	0.318	0.290	–0.097	4	0.647	0.695	0.069	5	0.529	0.732	0.276
Nc06b	100	18	0.582	0.740	0.233	9	0.700	0.815	0.046	7	0.636	0.812	0.216	7	0.437	0.627	0.302	5	0.412	0.717	0.426
Nc07b	98.6	5	0.501	0.592	0.194	4	0.500	0.672	0.255	4	0.571	0.570	–0.002	4	0.471	0.498	0.055	3	0.294	0.57	0.484
Nc19	97.3	8	0.585	0.628	0.073	5	0.684	0.768	0.158	5	0.762	0.698	–0.092	5	0.529	0.686	0.228	4	0.562	0.575	0.022
Nc20	97.3	9	0.269	0.330	0.305	4	0.600	0.544	–0.021	6	0.619	0.664	0.068	1	0.000	0.000	NA	3	0.125	0.235	0.469
Nc22	100	6	0.347	0.522	0.305	2	0.150	0.157	–0.063	5	0.727	0.778	0.065	2	0.235	0.507	0.536	3	0.235	0.605	0.611

Note: % amp = percentage of successful amplification; A = number of alleles; F_{IS} = intrapopulation fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; NA = not available.

[a] Information on geographic locations and vouchers is provided in Appendix 1.

[b] F_{IS} values significantly different from zero after Bonferroni correction ($P < 0.0006$) are indicated with an asterisk.

CONCLUSIONS

Three multiplexes including 17 new and four published microsatellite markers were developed and validated in natural populations. These loci exhibit substantial polymorphism within and between populations. They should provide sufficient power to study population structure and mating system, and to infer demographic history at different spatial scales.

LITERATURE CITED

Assunção, A. G. L., H. Schat, and M. G. M. Aarts. 2003. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* 159: 351–360.

Basic, N., and G. Besnard. 2006. Gene polymorphisms for elucidating the genetic structure of the heavy-metal hyperaccumulating trait in *Thlaspi caerulescens* and their cross-genera amplification in Brassicaceae. *Journal of Plant Research* 119: 479–487.

Belkhir, K., P. Borsa, L. Chikhi, N. Raufaste, and F. Catch. 2004. GENETIX 4.0. 5.2, Software under Windows™ for the genetics of the populations. University of Montpellier, Montpellier, France.

Besnard, G., N. Basic, P.-A. Christin, D. Savova-Bianchi and N. Galland. 2009. *Thlaspi caerulescens* (Brassicaceae) population genetics in western Switzerland: Is the genetic structure affected by natural variation of soil heavy metal concentrations? *New Phytologist* 181: 974–984.

Chapuis, M.-P., and A. Estoup. 2007. Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 24: 621–631.

Doyle, J. J., and J. L. Doyle. 1990. DNA extraction from *Arabidopsis*. *Focus (San Francisco, Calif.)* 12: 13–15.

Dubois, S., P.-O. Cheptou, C. Petit, P. Meerts, M. Poncelet, X. Vekemans, C. Lefèbvre, and J. Escarré. 2003. Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* 157: 633–641.

Godé, C., I. Decombeix, A. Kostecka, P. Wasowicz, M. Pauwels, A. Courseaux, and P. Saumitou-Laprade. 2012. Nuclear microsatellite loci for *Arabidopsis halleri* (Brassicaceae), a model species to study plant adaptation to heavy metals. *American Journal of Botany* 99: e49–e52.

Goudet, J. 1995. FSTAT: A computer program to calculate *F* statistics, version 1.2. *Journal of Heredity* 86: 485–486.

Jiménez-Ambriz, G., C. Petit, I. Bourrié, S. Dubois, I. Olivieri, and O. Ronce. 2007. Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: Role of gene flow, selection and phenotypic plasticity. *New Phytologist* 173: 199–215.

Kiefer, M., R. Schmickl, D. A. German, T. Mandáková, M. A. Lysak, I. A. Al-Shehbaz, A. Franzke, et al. 2014. BrassiBase: Introduction to a novel knowledge database on Brassicaceae evolution. *Plant and Cell Physiology* 55: e3–e3.

Koch, M. A., and D. A. German. 2013. Taxonomy and systematics are key to biological information: *Arabidopsis*, *Eutrema* (Thellungiella), *Noccaea* and *Schrenkiella* (Brassicaceae) as examples. *Frontiers in Plant Science* 4: 267.

Malausa, T., A. Gilles, E. Meglécz, H. Blanquart, S. Duthoy, C. Costedoat, V. Dubut, et al. 2011. High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* 11: 638–644.

Meglécz, E., C. Costedoat, V. Dubut, A. Gilles, T. Malausa, N. Pech, and J.-F. Martin. 2010. QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics (Oxford, England)* 26: 403–404.

Rousset, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

Schranz, M. E., M. A. Lysak, and T. Mitchell-Olds. 2006. The ABC's of comparative genomics in the Brassicaceae: Building blocks of crucifer genomes. *Trends in Plant Science* 11: 535–542.

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Appendix 1. Voucher and location information for *Noccaea caerulescens* populations used in the development and testing of microsatellites.

Population	Type of population	Collection date	Locality	Geographic coordinates	Sample names and storage location[a]	Voucher no.	Use
Angleur	Metallicolous	2009	Angleur	50°36′44.61″N, 5°36′38.74″E	B02.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Anjeau	Nonmetallicolous	October 2007	Saint-Laurent-le-Minier	43°55′3.33″N, 3°37′52.52″E	AN_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Auxelles-Haut	Nonmetallicolous	2009	Auxelles-Haut	47°44′21.52″N, 06°46′35.84″E	F03.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Auxy	Nonmetallicolous	2009	Auxy	46°57′44.13″N, 04°23′47.05″E	F05.01[b,d]	503854	Biological validation of primer pairs (Evo-Eco-Paleo)
Avinières	Metallicolous	March 2013	Saint-Laurent-le-Minier	43°55′55.67″N, 3°39′46.19″E	12_AV_ID_X[c]		Natural populations screening
Baraquette	Nonmetallicolous	October 2007	Saint-Laurent-le-Minier	43°55′6.73″N, 3°36′54.82″E	BQ_Z_X_2007[c]		Library construction
Breinigerberg	Metallicolous	2009	Breining	50°44′12.73″N, 06°14′30.92″E	G01.01[b,d]	501266, 501267	Biological validation of primer pairs (Evo-Eco-Paleo)
Col du Lautaret	Nonmetallicolous	2007	Lautaret	45°02′07″N, 06°24′20″E	F01.01[b,d]	501429	Biological validation of primer pairs (Evo-Eco-Paleo)
Coulet	Nonmetallicolous	September 2013	Saint-Maurice-Navacelles	43°49′44.59″N, 3°33′41.64″E	12_CO_ID_X[c]		Natural populations screening
Coulet	Nonmetallicolous	October 2007	Saint-Maurice-Navacelles	43°49′44.59″N, 3°33′41.64″E	CO_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Fellering	Serpentine	2009	Bergenbach	47°54′22.90″N, 06°57′25.50″E	F04.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Goebelsmühle	Nonmetallicolous	2009	Goebelsmühle	49°55′22.07″N, 06°3′44.08″E	L02.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Husavik	Nonmetallicolous	2006	Husavik	66°01′38.89″N, 17°17′21.71″W	I01.01[b,d]	911812	Biological validation of primer pairs (Evo-Eco-Paleo)
Lichtenau	Metallicolous	2006	Blankenrode	51°32′0.34″N, 08°54′17.02″E	G05.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Lintich	Metallicolous	2010	Bakomi	48°26′05.48″N, 18°55′09.43″E	SK01.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Mostviertiel	Nonmetallicolous	2006	Tormäuer	47°51′08.70″N, 15°17′13.20″E	A01.01[b,d]	406733	Biological validation of primer pairs (Evo-Eco-Paleo)
Moyen Age	Metallicolous	October 2007	Saint-Laurent-le-Minier	43°55′52.16″N, 3°38′26.09″E	MG_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Oslo	Nonmetallicolous	2009	Hovedoya	59°53′39″N, 10°43′44″E	N01.01[b,d]	501275–501278	Biological validation of primer pairs (Evo-Eco-Paleo)
Papeterie	Metallicolous	2006	Ganges	43°56′10.98″N, 03°40′19.88″E	F16.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Prayon	Metallicolous	2009	Prayon	50°35′3.31″N, 5°40′23.69″E	B01.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Ramponenche	Metallicolous	2010	Florac	44°20′18.25″N, 03°40′05.45″E	F02.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Saint Baudille	Nonmetallicolous	October 2007	Montpeyroux	43°44′40.73″N, 3°29′9.96″E	BD_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Saint Bresson	Metallicolous	October 2007	Pommiers	43°56′35.89″N, 3°37′57.02″E	SB_Z_X_2007[c]		Biological validation of primer pairs (ISEM)
Saint Bresson	Metallicolous	September 2013	Pommiers	43°56′35.89″N, 3°37′57.02″E	12_SB_ID_X[c]		Natural populations screening
Saint Hippolyte	Metallicolous	April 2014	Saint-Hippolyte-du-Fort	43°58′17.07″N, 3°49′59.98″E	Zi_Qj_Hi_2014_X[c]		Natural populations screening
Saint Jost	Metallicolous	2009	Virneburg	50°21′08.95″N, 07°06′33.65″E	G02.01[b,d]	501261, 501262	Biological validation of primer pairs (Evo-Eco-Paleo)

Appendix 1. Continued.

Population	Type of population	Collection date	Locality	Geographic coordinates	Sample names and storage location[a]	Voucher no.	Use
Silberberg	Metallicolous	2009	Silberberg	52°12′38.72″N, 07°56′46.13″E	G03.01[b,d]	504547	Biological validation of primer pairs (Evo-Eco-Paleo)
Somerset	Metallicolous	2010	Priddy	51°15′39.06″N, 02°39′02.12″W	UK1.06[b,d]	501343	Biological validation of primer pairs (Evo-Eco-Paleo)
Son	Nonmetallicolous	2000	Esterri d'Aneu	42°35′51.50″N, 01°04′23.82″E	SP01.02[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Špania Dolina	Nonmetallicolous	2010	Bakomi	48°48′29.87″N, 19°08′14.65″E	SK02.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Stauffenbergallee	Nonmetallicolous	2009	Dresden	51°06′04.48″N, 13°47′17.61″E	G04.01[b,d]	280040	Biological validation of primer pairs (Evo-Eco-Paleo)
Štiavnické	Nonmetallicolous	2010	Bakomi	48°26′0.48″N, 18°51′29.86″E	SK03.01[b]		Biological validation of primer pairs (Evo-Eco-Paleo)
Uppsala	Nonmetallicolous	2010	Uppsala	59°50′40.68″N, 17°44′54.18″E	S01.01[b,d]	501430	Biological validation of primer pairs (Evo-Eco-Paleo)

[a] Letters in sample names are defined as: X = number of the plant on which a leaf was collected; Zi = number of the area in the population; Qj = number of the quadrat in the area; Z = plants on which seeds were collected. Several specimens were collected in each population.

[b] Vouchers of leaves were deposited at Laboratoire Évolution Écologie et Paléontologie (Evo-Eco-Paleo), Villeneuve d'Ascq, France.

[c] Vouchers of leaves were deposited at Institut des Sciences de l'Évolution (ISEM), Montpellier, France.

[d] Herbarium specimens of the corresponding population were collected and deposited by M. Koch at the Centre for Organismal Studies (COS), Biodiversity and Plant Systematics, Universität Heidelberg, Heidelberg, Germany. They are available from the BrassiBase database (Kiefer et al., 2014).

Le système de reproduction a une influence majeure sur l'évolution des populations, car il affecte les flux de gènes, la dérive et la sélection. C'est donc un des déterminants à prendre en compte lors de l'étude de l'adaptation à des environnements hétérogènes en présence de flux de gènes. Dans cette thèse, j'ai étudié le système de reproduction d'une Brassicacée tolérante et hyperaccumulatrice de métaux lourds, *Noccaea caerulescens*. Cette espèce vit sur des sols hautement contaminés en éléments traces tels que le plomb, le zinc, ou le cadmium, mais aussi sur des sols non contaminés. Les deux types de populations (écotypes) sont trouvés à quelques kilomètres l'un de l'autre dans la région du Nord de Montpellier. Dans un premier temps, j'ai caractérisé la variation du système de reproduction en populations naturelles. En utilisant des marqueurs microsatellites, j'ai montré que les populations vivant dans les mines avaient un taux d'autofécondation plus faible que les populations vivant sur sol non pollué. En outre, je n'ai pas trouvé de variation temporelle du taux d'autofécondation. Pour essayer de comprendre ce qui peut expliquer cette variation entre écotypes, j'ai ensuite étudié l'effet de la densité sur le taux d'autofécondation, ce facteur étant connu pour influencer le comportement des pollinisateurs et donc le système de reproduction. Je n'ai pas détecté d'effet de la densité sur le taux d'autofécondation à l'intérieur de deux mines, en utilisant deux méthodes différentes, et en variant les échelles spatiales. Enfin, je me suis intéressée à la dépression de consanguinité, autre acteur majeur de l'évolution du taux d'autofécondation. J'ai testé des prédictions sur l'effet de l'histoire d'adaptation à un environnement sur l'expression de la dépression de consanguinité, dans cet environnement ou dans un nouvel environnement (potentiellement stressant). J'ai trouvé que la dépression de consanguinité chez *N. caerulescens* est, en jardin commun, faible à modérée. Les plantes métallicoles étaient mieux adaptées au sol pollué que les plantes non métallicoles, et souffraient parfois d'une plus forte dépression de consanguinité. La toxicité du sol ne semblait que très peu influencer l'expression de la dépression de consanguinité, et ceci de la même manière chez les plantes adaptées ou non à la pollution. *N. caerulescens* a donc un système de reproduction mixte, qui n'est pas contraint par une forte dépression de consanguinité (en conditions expérimentales). Ce système de reproduction devrait faciliter la sélection tout en n'augmentant pas trop la dérive génétique, et donc confère de bonnes capacités d'adaptation à de nouveaux environnements.

Mating system deeply influences the evolution of populations, as it affects gene flow, genetic drift, and selection. It is thus a very important factor to study in the context of adaptation to heterogeneous selection pressures in the presence of gene flow. I studied the mating system of a tolerant Brassicaceae, *Noccaea caerulescens*, which grows on both normal and highly toxic soils, such as former mine wastes, contaminated with Lead, Zinc or Cadmium. One can find the two types of populations (ecotypes) separated by a couple of kilometres in the region north of Montpellier. I first characterized mating system variation in natural conditions in populations of the South of France. I found that self-fertilisation rates were higher in non-metallicolous populations than in metallicolous populations but did not detect temporal variation. To better understand these differences between ecotypes, I investigated the effect of the density of flowering plants on self-fertilisation, as density often influences pollinators behaviour and thus, mating system. I found no effect of density on self-fertilisation rates, in the two studied populations and using to different methods and different spatial scales. Finally, I estimated inbreeding depression, which is a major factor of mating system evolution. I used the two ecotypes of *N. caerulescens* to test whether the history of adaptation to an environment affects the expression of inbreeding depression in this environment and in new, potentially stressing environments. I found weak to moderate inbreeding depression in *N. caerulescens* in experimental conditions. Metallicolous populations were better adapted to soil toxicity than non metallicolous populations, and sometimes had higher inbreeding depression. There was little effect of toxicity on inbreeding depression, similarly for populations adapted or not to toxic soils.*Noccaea caerulescens* has a mixed mating system, which does not seem to be highly constrained by inbreeding depression. This mixed mating system is expected to have beneficial effects on selection, while not increasing genetic drift too much. This may provide good capacities to adapt to new environments.