Biogeographic history and mating system of Rhodendron ferrugineum in the French Pyrenees

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THÈSE

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Histoire biogéographique et système de reproduction de Rhododendron ferrugineum dans les Pyrénées

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ED SEVAB : Écologie, biodiversité et évolution

Unité de recherche :

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Introduction

L'impact des changements globaux actuels tel que l'augmentation des rejets de CO_2, l'intensification de l'agriculture, la fragmentation des habitats ou les dépôts d'azote sont au centre de nombreuses études et débats (Vitousek 1994 ; Walker and Steffen 1997 ; Norby 1998 ; Tilman et al. 2002). Les concentrations atmosphériques en dioxyde de carbone (CO_2) augmentent depuis 1957 (Keeling et al. 1995 ; Fig. 1a) majoritairement en raison de rejets de CO_2 d'origine anthropogénique (Hugues 2000 ; Parmesan et Yohe 2003) et ont de nombreuses conséquences sur les écosystèmes. L'augmentation des émissions de CO_2 et divers gaz à effet de serre (CH_4, N_2O) induit notamment une détérioration de la couche d'ozone. Cette détérioration de 2.2% à 6.7% dans l'hémisphère nord entraine une augmentation des radiations d'UV-B de 3 à 11% (Feldheim and Conner 1996). Une autre conséquence de l'augmentation des rejets de gaz à effet de serre est l'augmentation de la température moyenne annuelle sur Terre d'environ 0.6°C sur les 100 dernières années, de 0.95 °C en France et de 1.1°C dans les Pyrénées par exemple (Jones et al. 2001 ; Walther et al. 2002). La prédiction d'une augmentation plus ou moins drastique des températures par les différents modèles climatiques (Fig. 1b) pourrait induire la fonte des glaciers de montagnes et des calottes glaciaires, provoquant ainsi une élévation du niveau de la mer de 5 cm d'ici la fin du 21ème siècle (Raper and Braithwaite 2006). Les prédictions actuelles suggèrent que les effets des changements climatiques seront les plus faibles au niveau des tropiques et les plus forts aux latitudes et altitudes élevées (Körner 1999).

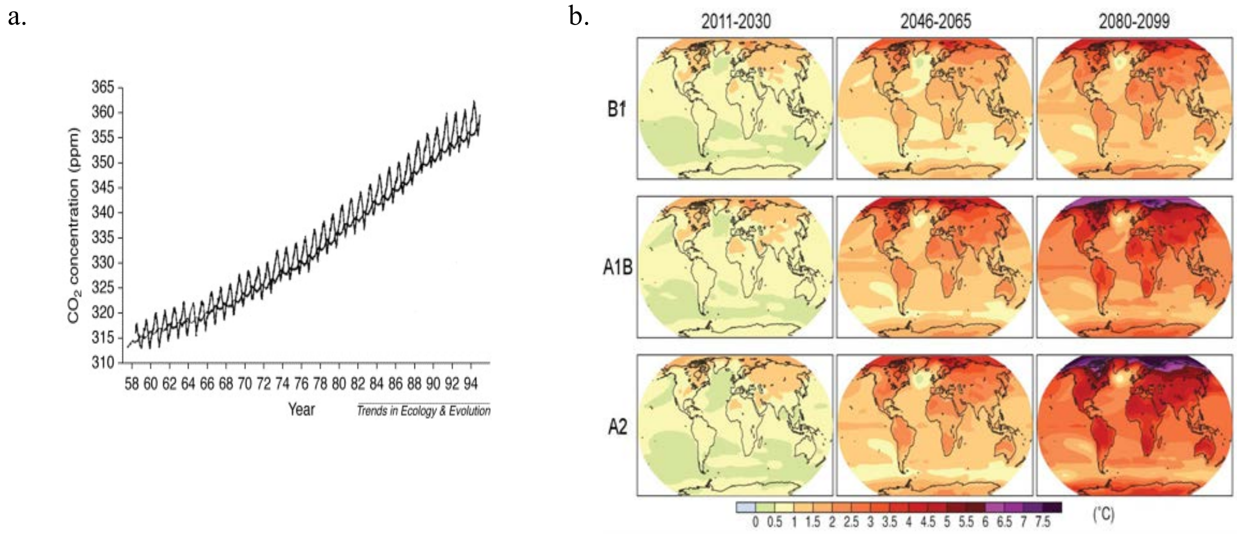

Figure 1. a. Concentration en CO_2 mesurée à l'observatoire de Mauna Loa, Hawaii (20°N, 156°W) et au Pôle sud de 1958 à 1994. Les données de Mauna Loa sont caractérisées par un cycle saisonnier prononcé (Keeling et al. 1995). b. Projections de la hausse moyenne des températures de surface selon différents scénarios d'émissions de gaz à effet de serre (B1 scénario « optimiste », A1B « modéré » et A2 « pessimiste ») pour les prochaines décennies (IPCC 2007).

Les précipitations moyennes à la surface de la Terre pourraient elles aussi augmenter de 1 à 3% (Allen et Ingram 2002 ; Held et Soden 2006). Cependant les prédictions de répartition des précipitations semblent peu homogènes : en effet elles pourraient augmenter de 10% à 20% d'ici à la fin du XXIe siècle en Europe du Nord, surtout en hiver, et baisser dans les mêmes proportions autour du bassin méditerranéen. Par ailleurs, les modifications d'usage des terres dues aux pressions anthropiques (urbanisation, déforestation, défrichage, intensification agricole) entrainent une restructuration des paysages et par conséquent une modification des écosystèmes (Fig. 2) avec notamment une fragmentation des habitats. Ces pressions anthropiques et plus particulièrement l'intensification de l'agriculture s'accompagnent également de rejet de polluants et de molécules telle que l'azote. L'azote est un facteur déterminant dans la composition des communautés végétales et la constante augmentation de son utilisation (Galloway 1995) entraine des modifications de la structuration des communautés.

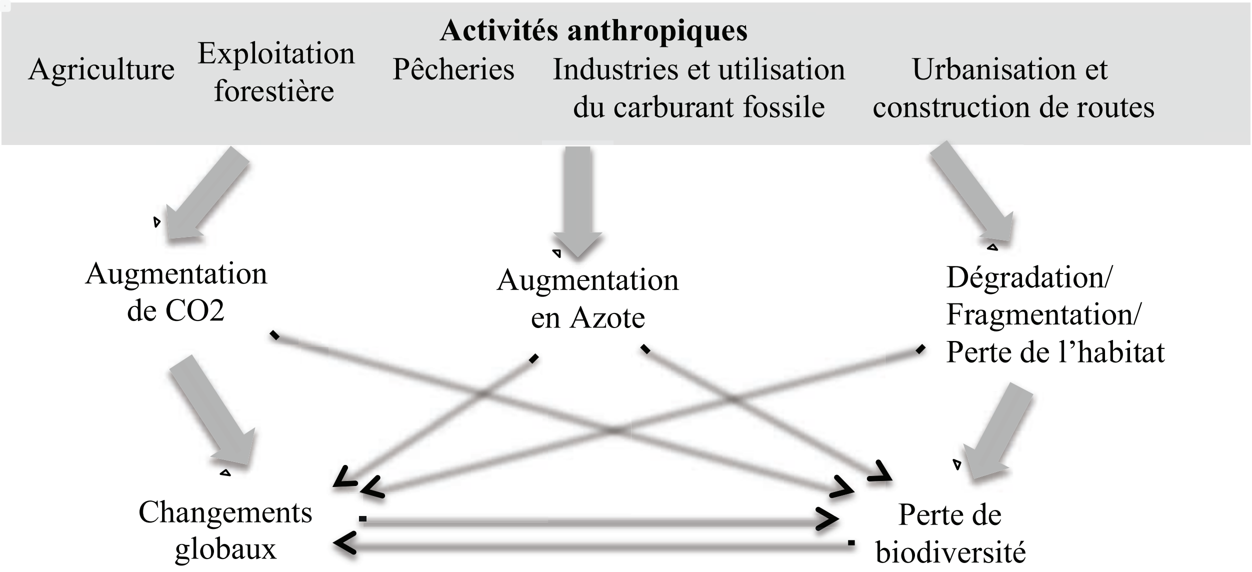

Figure 2. Principaux paramètres des changements globaux selon Vitousek (1994) et Groom et al. (2006). L'épaisseur des flèches représente l'importance des effets.

Les changements globaux récents affectent la physiologie (Hugues 2000), la distribution (Parmesan and Yohe 2003; Root et al. 2005; Walther et al. 2005; Lavergne et al. 2006) et la phénologie des espèces (Menzel and Fabian 1999; Visser and Holleman 2001; White et al. 2003; Zavaleta et al. 2003), ainsi que la dynamique des populations et les interactions entre espèces (Walther et al. 2002). Plusieurs études ont modélisé la distribution future des espèces. Que ce soit à une échelle régionale (Huntley et al. 1995 ; Peterson et al. 2002 ; Erasmus et al. 2002 ; Thuller 2004) ou locale (Gottfried and al. 1995 ; Guisan and Theurillat 2000) elles sont alarmistes quant aux risques d'extinction de certaines d'entre elles durant le prochain siècle (Thomas et al. 2004).

Les espèces peuvent faire face aux changements globaux en modifiant leur aire de répartition ou par le biais d'adaptations génétiques pouvant entrainer des modifications phénologiques ou physiologiques. Des études montrent un décalage dans la période de reproduction d'espèces animales et dans la phénologie de floraison ou de reproduction chez les espèces végétales (Archaux 2003 ; Cleland et al. 2007). Par ailleurs, des preuves paléo-écologiques (Huntley 1991; Prentice et al. 2000; Davis and Shaw 2001) et diverses observations du changement d'aire de répartition des espèces (Walther et al. 2005; Parmesan, 2006) montrent que la migration (*i.e.* un changement directionnel de l'aire de répartition) est l'une des réponses les plus probables des espèces face aux changements globaux. Les réponses des espèces dépendent de leur structure génétique, un niveau diversité génétique élevée peut permettre une adaptation rapide aux changements globaux. Chez les espèces végétales, cette structure génétique est déterminée par les facteurs abiotiques tels que climat agissant à une large échelle spatio-temporelle ou localement (microclimat), les interactions biotiques et les traits d'histoire de vie de l'espèce (*i.e.* mode de reproduction). Des relations entre variabilité génétique et variables environnementales ont été montré maintes fois (Hedrick et al. 1976; Aitken et al. 2008; Balkenhol et al. 2009). Les effets combinés des facteurs biotiques et abiotiques peuvent induire une adaptation locale des espèces et une forte différentiation génétique entre les populations (Li et al. 1999 ; 2000 ; 2001) et par conséquent déterminer leurs capacités de réponses aux changements globaux.

L'objectif principal de ce travail est d'étudier les effets de variables environnementales sur le système de reproduction d'une espèce végétale entomogame à l'échelle du massif Pyrénéen. Dans la première partie de l'introduction, je m'intéresserai aux mouvements des espèces végétales en réponse à des variations climatiques passées et actuelles et à l'impact sur la structure génétique de ces espèces. Dans une seconde partie, j'étudierai plus particulièrement comment les menaces sur les relations plantes-pollinisateurs vont affecter la reproduction des espèces végétales. Les changements globaux induisent de nombreux changements dans les interactions plantes-pollinisateurs de par le déclin des pollinisateurs, la fragmentation des habitats, les décalages de phénologie ou les remplacements de communautés. En réponse à ces changements, différentes stratégies de reproduction peuvent être observées et permettent aux espèces végétales de survivre dans des conditions très diverses. Le système de reproduction d'une espèce détermine ses opportunités d'évolution en impactant les paramètres génétiques des populations tels que leur taille effective, les flux de gènes ou la répartition de la diversité génétique intra et inter-populationnelle. Les changements globaux agissent à la fois sur les communautés de pollinisateurs et sur les communautés de plantes : il faut donc étudier les réponses de ces différents acteurs aux changements mais également les évolutions de leurs interactions. Enfin, dans une troisième et dernière partie, je verrai quelles sont les conséquences des changements globaux sur les milieux alpins, points chauds de biodiversité et qui semblent particulièrement sensibles aux changements globaux.

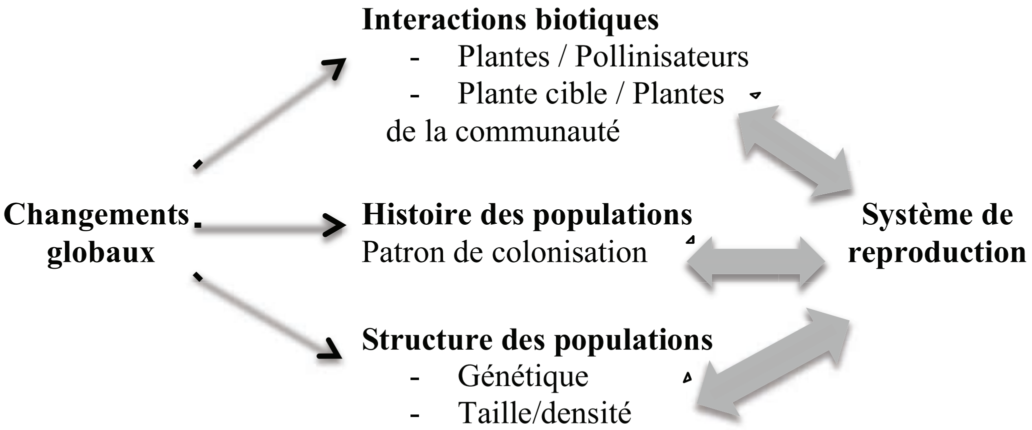

Figure 3. Contexte de l'étude : Impacts des changements globaux sur les espèces végétales.

Histoire biogéographique : inférer le futur à partir du passé

Le maintien d'une diversité génétique élevée au sein d'une espèce est indispensable pour permettre son adaptation à des conditions environnementales changeantes (Frankel et al. 1995 ; Freeman and Heron 2007 ; Pauls et al. 2013). Par conséquent, il semble indispensable d'étudier l'impact des changements climatiques passés sur la structure génétique actuelle d'une espèce (Hahn et al. 2012) afin d'en inférer les effets des changements futurs.

La biogéographie est considérée comme une science étudiant la répartition géographique présente et passé des organismes (Lomolino et al. 2005). L'étude biogéographique d'une espèce est l'étude spatio-temporelle de sa diversité biologique, son origine, son évolution et sa régulation dans des espaces hétérogènes et changeants (Blondel 1986). La phylogéographie quant à elle se définit comme l'étude de la distribution biogéographique des lignées généalogiques et des processus qui y ont abouti, elle s'intéresse particulièrement à la variabilité génétique intra-espèce ou entre espèces apparentées (Avise 2000). Les modifications constantes de l'environnement poussent les espèces à se déplacer afin de trouver le milieu le plus propice à leur survie (Pease et al. 1989 ; Hewitt 1996). Ces migrations, de par les événements répétés de goulots d'étranglement le long des routes de colonisation, peuvent entrainer une différentiation inter-populationnelle et peut dans certains cas conduire à l'apparition de nouvelles espèces, de sous espèces ou de lignées génétiques (Mayr 1963). Les séparations entre les populations laissent des marques dans la séquence d'ADN des espèces et peuvent être analysées par des techniques de biologie moléculaire (Encadré 1). La détermination des lignées génétiques et leur localisation permet de mieux appréhender les niveaux de diversité génétique présents chez une espèce et par exemple de déterminer les zones-refuges utilisées durant les dernières glaciations.

Encadré 1 : Marqueurs moléculaires

Des informations sur la structuration génétique des populations peuvent être obtenues grâce à l'utilisation de marqueurs moléculaires tels que :

- les allozymes,
- les RAPD (Random Amplified Polymorphic DNA),
- les ISSR (Inter Simple Sequence Repeat),
- les RFLP (Restriction Fragment Lenght Polymorphisms)
- les AFLP (Amplified Fragment Length Polymorphism),
- les microsatellites,
- les SNP (Single Nucleotide Polymorphism)
- ou des techniques de séquençage haut débit (NGS ; pyroséquençage, technique Illumina, séquençage par ligation).

Les microsatellites font partis des marqueurs les plus utilisés en génétique des populations. Ils permettent d'évaluer la structuration inter-populationnelle, de quantifier les migrations entre ces populations, de comprendre l'organisation fine au sein des populations, mais aussi d'estimer les relations génétiques entre individus et de réaliser des analyses de paternité (Schlötterer and Pemberton 1998 ; Neff and Gross 2001). Actuellement, de très nombreuses études utilisent les marqueurs microsatellites pour résoudre la phylogénie de plusieurs genres (Scariot et al. 2007), retracer des routes de colonisation (Guillemaud et al. 2010), des phénomènes de remises en contact de populations isolées (Estoup et al. 2000), estimer l'impact d'événements climatiques anciens, tels que des glaciations, sur la structure génétique et la distribution des espèces (Hanfling et al. 2002 ; Hewitt 2004 ; Schönswetter et al. 2005), ou encore estimer la différenciation et/ou l'isolement entre plusieurs populations d'une même espèce. Ces marqueurs neutres et co-dominants sont constitués de répétitions en tandem de 2 à 6 nucléotides (Fig. 4) un nombre variable de fois (par exemple AC reproduit *n* fois) et dont le taux de mutation est de 10^{-6} à 10^{-2} par génération selon les espèces (Schlötterer 2000).

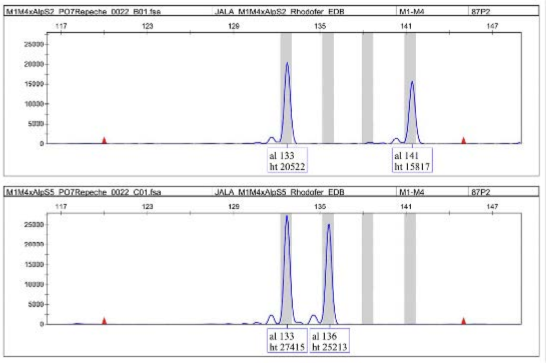

Fig. 4. Exemple de profils microsatellites de deux individus de *R. ferrugineum* obtenus avec Genemapper.

De Candolle (1820) a été le premier à proposer que la distribution actuelle des organismes vivants dépend à la fois de processus écologiques contemporains et historiques. De nombreux évènements historiques ont eu un fort impact sur la répartition des espèces, c'est notamment le cas de la dérive des continents ou des épisodes de glaciations. Durant le Pléistocène (de 2.6 millions d'années à 12000 ans), les variations climatiques ont induits des changements dans les aires de répartition des espèces. La calotte glaciaire de l'hémisphère nord a commencé à se développer il y a environ 2.5 millions d'années et des oscillions climatiques majeures se sont déroulées les 700 derniers milliers d'années avec un cycle de 100 mille ans (Webb and Bartlein 1992). Il a été récemment montré que de fortes amplitudes climatiques ont eu lieu à la fin du Pléistocène, avec des périodes glaciaires et interglaciaires marquées (Roy et al. 1996). Les glaciations ont conduit à des expansions/contractions de l'aire de répartition des espèces entrainant dans certains cas un isolement géographique et génétique des populations suivis par des événements de fondations lors de la recolonisation des aires libérées des glaces, induisant une perte de diversité génétique de l'espèce (Hewitt 2000). En Europe, durant les périodes glaciaires, le bassin Méditerranéen a limité les déplacements des espèces vers le sud, les Alpes et les Pyrénées de par leur orientation est-ouest ont également été des barrières aux migrations nord-sud. Les refuges européens les plus connus sont les péninsules ibérique, italienne et balkanique (Taberlet et al. 1998 ; Hewitt 2000). Ils sont classiquement caractérisés par une diversité génétique élevée. Petit et al. (2003) ont étudié la diversité génétique de 22 espèces ligneuses échantillonnées dans 25 forêts européennes et ont pu estimer la diversité génétique moyenne de chaque forêt. Les diversités génétiques les plus fortes ont été trouvées en Corse, en Italie et dans les Balkans (Fig. 5).

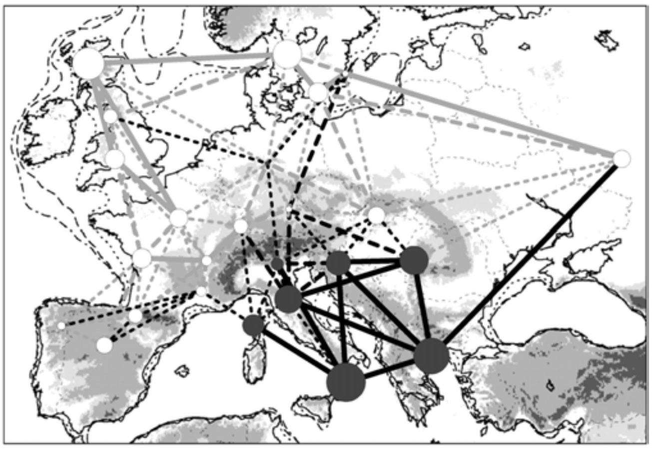

Fig. 5. Etude des divergences génétiques dans 25 forêts européennes. Les cercles noirs représentent les valeurs supérieures et les cercles blancs les valeurs inférieures à la moyenne. Pour chacune des forêts le niveau de divergence avec les 5 forêts les plus proches est indiqué par les lignes, les lignes noires en continue représentent une forte divergence, les lignes en pointillées une divergence intermédiaire et les lignes continues grises une faible divergence (Petit et al. 2003).

Une étude menée par Taberlet et al. (1998) comparant l'histoire phylogéographiques de 10 espèces européennes dont 4 espèces végétales (*Abies alba* ; *Picea abies* ; *Fagus sylvatica* ; *Quercus spp*) a montré une considérable dissimilarité entre les patrons phylogéographiques.

Bien que des congruences spatiales aient été observées dans des zones plus restreintes (Bermingham and Avise 1986 ; Avise 1992 ; Joseph et al. 1995) les résultats obtenus en Europe et en Amérique du Nord (Zink 1996) montrent qu'une adéquation des patrons phylogéographiques à l'échelle d'un continent est peu probable car chaque espèce répond différemment aux pressions environnementales. Si le sapin pectiné (*Abies alba*) et le chêne (*Quercus spp*) se sont déplacés durant les périodes glaciaires vers les refuges connus, l'Epicéa commun (*Picea abies*) a quant à lui survécu dans les Alpes dinariques, les Carpates et dans la région actuelle de Moscou (Huntley and Birks 1983 ; Lagercrantz and Ryman 1990). Le hêtre commun (*Fagus sylvatica*), un arbre originaire des climats tempérés a lui trouvé refuge au sud de l'Europe comme attendu mais il semblerait qu'il n'ait pas survécu dans le refuge ibérique et par conséquent toute la partie ouest de sa zone de distribution a été colonisé à partir du refuge des Balkans (Demesure et al. 1996). En accord avec les modèles de contractions/expansions des aires de répartition, une diversité intra-spécifique plus importante a été détectée dans les zones du sud que dans celles plus au nord récemment colonisées (Demesure et al. 1996 ; Dumolin-Lapègue et al. 1997).

Plus récemment des études ont porté sur les mouvements des espèces en réponse aux changements climatiques actuels (Walther et al. 2002; Parmesan and Yohe 2003; Walther 2003). Lenoir et al. (2008) ont étudié les mouvements altitudinaux de 171 espèces végétales sur une large échelle (de 0 à 2600 m d'altitude sur 6 chaines de montagnes en Europe de l'ouest : les Alpes de l'ouest, le nord des Pyrénées, le Massif Central, l'ouest du Jura, les Vosges et la Corse) durant le 20$^{\text{ième}}$ siècle (1905 - 2005). Dans la période de 1986 à 2005, la plupart des espèces ont une altitude optimale plus élevée que durant la période de 1905 à 1985 (Fig. 6). La tendance à monter en altitude de cet assemblage de 171 espèces dans les massifs montagneux de l'Europe de l'ouest concorde avec d'autres études sur les milieux arctiques et alpins (Grabherr et al. 1994 ; Keller et al. 2000 ; Sturm et al. 2001 ; Lloyd and Fastie 2003 ; Lesica and McCune 2004 ; Walther et al. 2005). Les changements climatiques n'affectent pas seulement les espèces à leur limite d'aire de répartition mais l'ensemble de leur aire. Lenoir et al. (2008) ont également montré que l'augmentation d'altitude était plus prononcée pour les espèces alpines que pour les espèces ubiquistes. Ceci est en accord avec la théorie prédisant que les espèces des milieux alpins sont plus sensibles aux changements climatiques que les espèces vivants dans d'autres milieux (Grabherr et al. 1994 ; Keller et al. 2000 ; Pauli et al. 2007). Le changement d'altitude concerne également plus fortement les espèces herbacées, les mousses et les fougères qui ont des cycles de vie courts, une maturation rapide et/ou une taille réduite que les arbres et les arbustes. La variabilité dans la vitesse de montée en altitude des espèces va entrainer des perturbations dans les interactions biotiques des communautés.

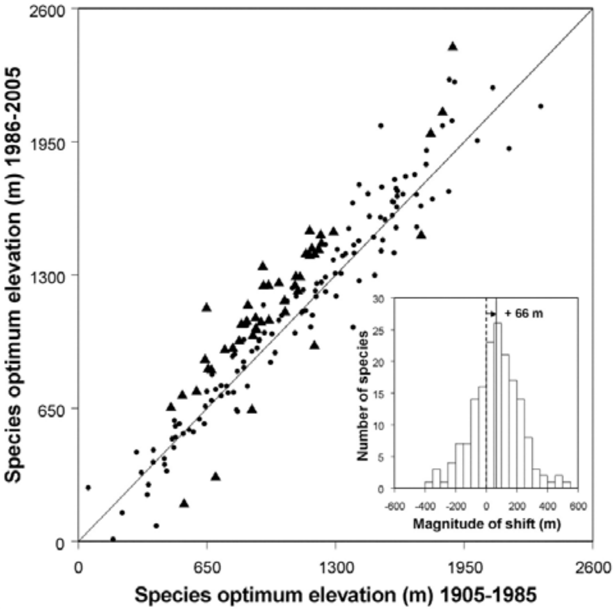

Fig. 6. Altitude optimale pour 171 espèces végétales sur les périodes de 1905-1985 et 1986-2005. Les triangles représentent les espèces (n = 46) ne montrant pas de recouvrement entre leurs optimums altitudinaux de 1905-1985 et de 1986-2005 (IC 95%) et les cercles (n = 125) représentent les espèces montrant un recouvrement entre leurs optimums altitudinaux de 1905-1985 et de 1986-2005 (IC 95%).

Les espèces végétales ont subi de fortes contraintes dans le passé qui ont impacté leur structure génétique (Encadré 2). Suite aux changements globaux actuels elles subissent de nouvelles pressions biotiques et abiotiques. Si des changements d'aire de répartition des espèces sont fréquents suite à ces pressions, de nombreuses autres réponses peuvent être observées.

Encadré 2 : Paramètres génétique mesurés

Une population végétale est généralement définie comme un ensemble d'individus de la même espèce occupant le même habitat et susceptibles de se reproduire entre eux. Chaque individu se distingue par un génotype qui lui est propre et chaque population est caractérisée par une diversité génétique et des fréquences alléliques spécifiques. Les loci sont des portions physiques d'ADN pouvant présenter des variations, lorsqu'il existe des variations pour un allèle ce dernier est dit polymorphe. Sous l'effet des pressions évolutives (mutation, sélection, dérive et migration), les fréquences alléliques sont susceptibles de varier au cours du temps. De nombreux paramètres peuvent être estimés afin d'étudier la structure génétique des populations notamment :

- Richesse allélique : le nombre moyen d'allèles par locus (A) est défini par n_i allèles au locus i et pour L loci tel que $A = \frac{1}{L} \sum_{i=1}^{L} ni$

- Hétérozygotie observée (H_O) : le nombre observé d'individus hétérozygotes dans une population est calculé à partir de la fréquence mesurée des hétérozygotes (nombre d'individus hétérozygotes divisé par le nombre d'individus total de l'échantillon)

- Hétérozygotie attendue (H_E) : le nombre d'hétérozygotes attendus pour une population supposée à l'équilibre de Hardy-Weinberg est calculé à partir des fréquences alléliques $he = 1 - \sum_{n=1}^{n} f2n$. Si plusieurs loci sont considérés l'hétérozygotie moyenne (H_E), représentant la moyenne du taux d'individus hétérozygotes par population, sera la moyenne arithmétique de toutes les valeurs de he.

- Coefficient de consanguinité (F_{IS}) : cet indice mesure la différentiation des individus à l'intérieur d'une population tel que FIS $= 1 - Ho/HE$. Lorsque $F_{IS} > 0$, un déficit en hétérozygotes est détecté (si $F_{IS} = 1$ la fixation est complète, c'est en théorie le cas des populations se reproduisant uniquement par autofécondation), lorsque $F_{IS} = 0$ la population est à l'équilibre de Hardy-Weinberg et lorsque $F_{IS} < 0$ un excès en hétérozygotes est détecté.

- Indice de fixation (F_{IT}) : cet indice mesure la différentiation des individus d'une population par rapport au total des individus $FIT = 1 - Ho/HE$

- Indice de fixation (F_{ST}) : cet indice mesure la différentiation entre les populations $FST = 1 - \frac{1-FIT}{1-FIS}$

Les trois indices sont liés par : $(1- F_{IT}) = (1- F_{IS})(1-F_{ST})$. Si toutes les populations sont à l'équilibre de Hardy-Weinberg, $F_{IS} = 0$ et $F_{IT} = F_{ST}$. Si toutes les populations sont à l'équilibre de Hardy-Weinberg et ont les mêmes fréquences alléliques, les indices ont une valeur nulle.

Interactions plantes-pollinisateurs et systèmes de reproduction

L'impact des changements globaux sur les espèces végétales semble très variable (Walther 2010 ; Doak and Morris 2010 ; Maclean and Wilson 2011). En effet, si des changements importants dans la phénologie de floraison, la production de graines, la reproduction végétative et la distribution des populations ont été observés (Wilson and Nilsson 2009 ; Fridley et al. 2011), dans certains cas les effets de ces changements peuvent avoir été atténués par des réponses du système de reproduction qui permet de maintenir une performance démographique malgré les changements globaux (Eckert et al. 2010).

Une grande majorité des angiospermes est pollinisée par l'intermédiaire d'animaux pour la reproduction sexuée (Kearns et al. 1998) dont une large proportion d'espèces végétales d'intérêt agronomique (près de 90% des espèces natives et 75% des espèces agricoles ; Ollerton et al. 2006; Klein et al. 2007). Les changements globaux ayant un fort impact sur les communautés végétales et animales, des perturbations des relations plantes-pollinisateurs sont attendues.

A. Relations plantes-pollinisateurs

Les études scientifiques sur la pollinisation ont débuté il y a plus de 250 ans (Baker 1983), et l'évolution des relations plantes-pollinisateurs est traditionnellement décrite comme des processus de co-adaptation dans lesquelles les traits morphologiques des fleurs évoluent pour attirer les pollinisateurs et ceux des pollinisateurs évoluent afin d'exploiter au mieux les ressources florales (Stebbins 1970 ; Faegri and van der Pijl 1979). De ce fait, les interactions plantes-pollinisateurs évolueraient vers un fort degré de spécialisation. Cependant, dès la fin des années 90, des études ont montré un scénario relativement différent dans lequel les plantes sont visitées et pollinisées par une large diversité de visiteurs (Ollerton 1996 ; Waser et al. 1996 ; Herrera 1996 ; Waser 1998). Les interactions plantes-pollinisateurs semblent donc s'étendre sur un large gradient d'un système généraliste à un système spécialiste (Herrera 1996 ; Waser et al. 1996).

Les interactions plantes-pollinisateurs sont en partie régulées par la qualité et la quantité de ces interactions (Herrera 1989), *i.e.* fréquence des visites des pollinisateurs et impact sur la reproduction de l'espèce végétale. Afin de comparer l'efficacité relative des pollinisateurs, les chercheurs ont essayé de standardiser les mesures d'efficacité (Thomson and Thompson 1992 ; Inouye et al. 1994). Idéalement, ces mesures devraient prendre en compte la fréquence de visite des pollinisateurs, les charges polliniques stigmatiques et des pollinisateurs, les comportements lors des visites des pollinisateurs (nombre de fleurs visitées, durée des interactions) et la contribution à la production de graines (Theiss et al. 2007). Des pollinisateurs effectuant peu de visites ou transportant de forte charge pollinique mais déposant peu de pollen peuvent être moins efficace que des pollinisateurs ayant des taux de visites moyens mais un transfert de pollen efficace (Thomson and Thompson 1992). Si l'abondance des pollinisateurs les plus efficaces d'une espèce végétale tend à diminuer il est possible qu'un fort impact soit visible sur la reproduction des individus.

✓ *Déclin des pollinisateurs*

Actuellement, un déclin de la diversité et de l'abondance des pollinisateurs a été observé et est au centre de nombreuses discussions (Thomas et al. 2004 ; Hooper et al. 2005 ; Balvanera et al. 2006 ; Grixti et al. 2009; Potts et al. 2010 ; Winfree et al. 2011). Ce déclin résulte des changements globaux tels que l'agriculture intensive, l'invasion d'espèces, la fragmentation et la perte d'habitats, l'utilisation de produits chimiques ou les réchauffements climatiques (Biesmeijer et al. 2006; Diekotter et al. 2007 ; Gonzalez-Varo et al. 2013). Les abeilles et les bourdons, par exemple, montrent un déclin mondial de leur richesse spécifique (Fig. 7; Biesmeijer et al. 2006; Gritxi et al. 2009) et de leur densité (Rasmont et al. 1993 ; Cameron et al. 2011), et ce phénomène s'accentue depuis les années 1950 (Kosior et al. 2007). L'altération des communautés de pollinisateurs entraine des effets en cascade sur la dynamique et la persistance des populations de plantes. La réduction de densité de populations de *Rhabdothamnus solandri* (Gesneriaceae), un arbuste endémique de Nouvelle Zélande, est liée à la baisse d'effectif de ses pollinisateurs les plus efficaces (trois espèces d'oiseaux dans ce cas : *Prosthemadera novaeseelandiae*, Meliphagidae ; *Anthornis melanura*, Meliphagidae ; *Notiomystis cincta*, Notiomystidae; Anderson et al. 2011).

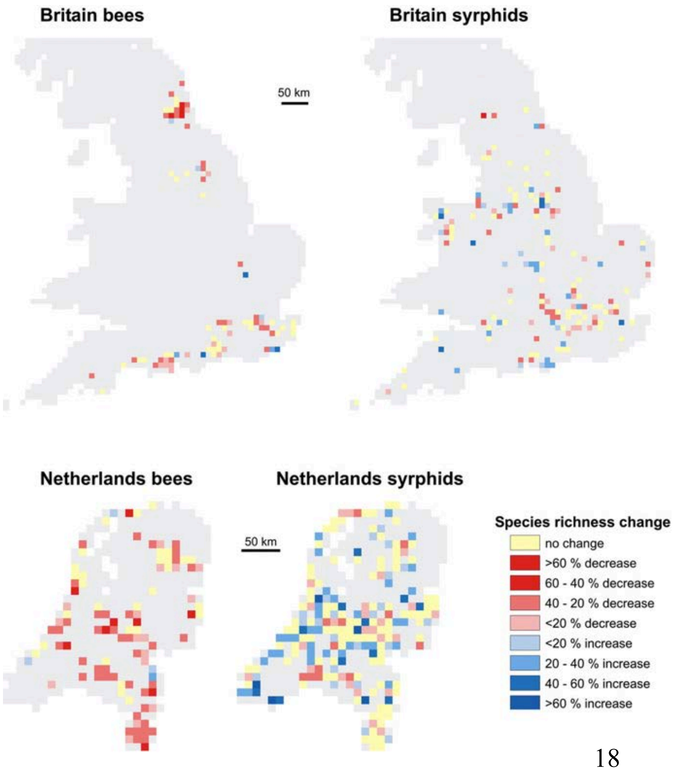

Fig. 7. Changements dans la richesse spécifique de bourdons, d'abeilles et de syrphes dans des quadrats de 10*10km en Angleterre et aux Pays-Bas d'après Biesmeijer et al. 2006.

Plusieurs mécanismes ont été proposés pour expliquer la corrélation positive entre la diversité en pollinisateurs et la qualité du service de pollinisation (Klein 2009). Une grande diversité d'espèces de visiteurs augmente la probabilité pour une plante d'avoir des pollinisateurs efficaces. L'efficacité peut varier entre les groupes de pollinisateurs en fonction de leurs taux de visites mais également de la qualité et quantité de pollen transportée qui dépend de paramètres morphologiques et comportementaux (Sahli et Conner 2007). Des études récentes ont montré que les systèmes de pollinisation pourraient présenter une spécialisation fonctionnelle, c'est à dire qu'une espèce végétale pourrait être préférentiellement visitée par un groupe fonctionnel d'insectes (Ollerton et al. 2007). Ce concept implique que les pollinisateurs peuvent être regroupés en groupes fonctionnels à partir de mesures de traits morphologiques (*i.e.* taille de l'insecte, taille des parties buccales ; Violle et al. 2007) qui se comportent de la même façon et par conséquent exercent une pression de sélection similaire sur les végétaux (Fenster et al. 2004). La complémentarité fonctionnelle entre les espèces peut permettre d'augmenter le service de pollinisation (Fontaine et al. 2006 ; Hoehn et al. 2008 ; Bluthgen and Klein 2011). La redondance des espèces contribuant aux mêmes fonctions est une des théories du fonctionnement des écosystèmes et de l'hypothèse de l'assurance biologique (Lawton and Brown 1993; Naeem 1998). Appliquée aux interactions plantes-pollinisateurs, cette hypothèse suggère que l'extinction d'une espèce pollinisatrice aurait des conséquences limitées sur la reproduction de l'espèce végétale cible grâce à la redondance entre espèces pollinisatrices.

Le déclin des pollinisateurs va donc avoir des conséquences variables sur les espèces végétales en fonction des assemblages de pollinisateurs. Il paraît donc important d'étudier la composition des communautés de pollinisateurs afin de déterminer quels seront les impacts du déclin de ces derniers sur la reproduction des végétaux.

✓ *Décalage phénologiques*

De nombreuses études ont montré que, sur les dernières décennies, la phénologie (*i.e.* période temporelle d'une activité telle que la période de floraison ou de croissance) de nombreuses espèces végétales a été modifiée suite aux changement globaux (Hughes 2000 ; Walther et al. 2002 ; Parmesan and Yohe 2003 ; Root et al. 2003). Ces changements phénologiques montrent que les végétaux s'adaptent et apportent une preuve de l'impact des changements globaux sur les êtres vivants (Parmesan and Yohe 2003).

Les végétaux étant impliqués dans de nombreuses relations avec d'autres espèces et notamment avec des insectes pour leur reproduction (zoogamie), il est nécessaire que les décalages phénologiques chez les espèces mutualistes s'effectuent de manière synchrone afin de ne pas diminuer les capacités de reproduction des espèces. Les mécanismes impliqués dans la phénologie des végétaux n'étant pas les même pour les insectes ou les vertébrés, il est probable que ces changements de phénologies ne soient pas toujours synchrones (Visser et al. 2004). Si les changements phénologiques de deux espèces mutualistes ne se font pas à la même vitesse cela peut entrainer des diminutions de la reproduction des espèces partenaires (Visser et al. 2004).

Les simulations de Memmott et al. (2007) indiquent que les décalages phénologiques dus à des changements climatiques vont entrainer des décalages temporels entre des pollinisateurs et leurs ressources florales. Les prédictions montrent que les pollinisateurs les plus spécialisés seront particulièrement touchés par ces perturbations. En effet, si un pollinisateur se nourrit uniquement d'une espèce végétale et que cette dernière subit une modification dans sa période de floraison sans qu'il n'y ait de décalage dans le cycle de vie du pollinisateurs, ce dernier n'aura que peu ou plus de ressource alimentaire. Les études disponibles sur les traits d'histoire de vie des pollinisateurs en rapport à la disponibilité en nourriture indiquent que la longévité et/ou la fécondité des insectes a diminué avec la diminution de disponibilité en nourriture (Murphy et al. 1983 ; Boggs and Ross 1993) créant sur un plus long terme un risque d'extinction.

✓ *Fragmentation et isolement des populations*

La fragmentation des communautés végétales est un des changements les plus répandus dans les écosystèmes terrestres. La fragmentation des habitats inclut trois composantes (Andren 1994) : (1) la perte d'habitat, (2) la réduction des tailles de populations et (3) l'augmentation de l'isolation des populations. La perte d'habitat peut conduire directement à l'extinction des espèces tandis que les deux autres composantes ont des effets plus complexes. Les espèces deviennent particulièrement vulnérables à l'extinction en dessous d'une certaine taille de population, même si la qualité de l'habitat dans lequel elles résident est optimale (Shaffer 1981 ; Lande 1988). Les probabilités d'extinction tendent à augmenter lorsque ces petites populations sont isolées les unes des autres car le nombre de partenaires sexuels diminue et qu'il y a par conséquent peu de possibilité pour les individus d'échanger des gènes (Brown and Kodrick-Brown 1977). Il est important de noter que certains environnements sont naturellement hétérogènes, créant une fragmentation naturelle des habitats. Cependant, ces milieux naturellement fragmentés diffèrent des fragments induits par les changements globaux de par leur échelle spatiale et temporelle et les degrés de connectivité entre les fragments (Haila 1999).

Des études récentes (Aguilar et al. 2006) ont montré que la fragmentation des habitats entrainant une diminution de la taille des populations végétales et une augmentation de leur isolement induisent une diminution de leur reproduction. Cette diminution est due à la réduction en disponibilité des pollinisateurs (Aizen and Feinsinger 2003) et des partenaires sexuels (Cunningham 2000 ; Duncan et al. 2004 ; Aizen and Harder 2007). En effet, des petites populations isolées, disposant donc de moins de ressources florales, sont moins attractives pour les pollinisateurs (Sih and Baltus 1987 ; Jennersten 1988 ; Aizen and Feinsinger 1994 ; Groom 1998 ; Dauber et al. 2010) entrainant une réduction de la quantité et qualité des services de pollinisation (Wilcock and Neiland 2002). Les changements globaux, et notamment l'augmentation de $CO_{2,}$ entrainent également des modifications dans la composition (qualité et quantité) du nectar (Davis 2003 ; Erhardt et al. 2005) qui aura aussi un impact sur l'attraction des pollinisateurs par les populations végétales.

✓ *Remplacements des communautés végétales*

Les changements globaux peuvent entrainer un déplacement de l'aire de distribution des espèces. Les vitesses de migration de chaque espèce étant différentes, un remplacement progressif des espèces actuelles des communautés végétales sera probablement observé (Pacala and Hurtt 1992; Walther et al. 2002; Walther 2003). Plusieurs changements ont déjà été observés, des études dans les Alpes ont montré un remplacement des espèces de la communauté végétale par des espèces plus thermophiles (Grabherr et al. 1994). Dans certains cas, les espèces végétales dominantes sont peu à peu remplacées par des espèces pionnières qui ont des capacités d'adaptation plus élevées (Halpin 1994). Il est également possible que des espèces peu dominantes « rares » remplacent les espèces dominantes par exclusion compétitive (Street and Semenov 1990). Ces scénarios se basent sur l'hypothèse que les facteurs limitant tels que le type de sol ou l'humidité seront peu affectés par les changements globaux. Des espèces végétales non natives et de zones adjacentes vont probablement devenir de nouveaux éléments des écosystèmes (Fig. 8 ; Walther et al. 2002). Walther et al. (2002) ont en effet montré une forte augmentation d'espèces exotiques dans les communautés végétales du sud de la Suisse. Tous les écosystèmes ne répondront pas de la même manière aux changements globaux, certains ne seront pas sensibles aux changements (Körner 1998 ; Bugman and Pfister 2000) alors que d'autres répondront sur un très long terme (Davis 1989).

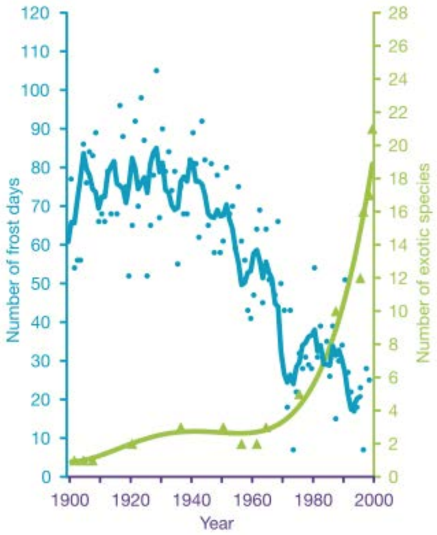

Fig. 8. Changement de végétation au sud de la Suisse. Augmentation du nombre d'espèces invasives au cours du temps suite à une diminution du nombre de jour de gel (d'après Walther et al. 2002).

Les changements des communautés végétales risquent d'avoir une influence sur le transfert de pollen par les pollinisateurs puisque les proportions de pollen conspécifique porté par un pollinisateur dépendent notamment des plantes de la communauté environnante (Duncan et al. 2004 ; Lazaro et al. 2009 ; Sargent et al. 2011). Les plantes de la communauté peuvent avoir des effets de facilitation sur la fréquence de visite des pollinisateurs. En effet, une forte diversité végétale est plus attractive pour les visiteurs et entraine généralement une augmentation de l'abondance, la diversité et la richesse des pollinisateurs (Moeller 2004 ; Ghazoul 2006). Cependant, dans une communauté végétale diversifiée, il est probable que les visiteurs généralistes transportent une quantité élevée de pollen hétérospécifique ce qui va entrainer une limitation en pollen conspécifique (Morales and Traveset 2008 ; Mitchell et al. 2009).

L'assemblage des espèces dans une communauté reflète les interactions entre organismes ainsi que les interactions entre les organismes et l'environnement abiotique. Les changements globaux risquent d'altérer la composition des communautés. Ces menaces sur les relations plantes-pollinisateurs peuvent entrainer une limitation en pollen diminuant ainsi la production de graines et auront de multiples effets sur la persistance de populations végétales ou sur la production agricole (Thomann et al. 2013).

✓ *Limitation en pollen*

Le service en pollinisateurs est défini par son efficacité (quantité et qualité de pollen transporté et déposé sur les stigmates, taux de visites, comportement lors des visites et contribution à la production de graines). Les changements globaux perturbant les interactions plantes-pollinisateurs, ils peuvent, par conséquent, entrainer une limitation en pollen. Les faibles quantités ou qualités de pollen déposées sur les stigmates peuvent résulter d'une limitation en visites (faible nombre de visites ou de pollinisateurs), d'une faible disponibilité en pollen ou d'un transfert inefficace de pollen (perte de pollen durant le transport, transport non spécifique de pollen). De fortes variations dans la limitation en pollen ont été observées en fonction de variations spatio-temporelles de l'environnement (Burd 1994).

Le déclin des pollinisateurs et les changements d'espèces dans les communautés ont potentiellement un fort impact sur le système de reproduction des espèces végétales entomogames. Des études sur la pollinisation et la production de graines le long d'un gradient de fragmentation ont montré que le déclin des pollinisateurs augmente la limitation en pollen dans des populations naturelles (Ashman et al. 2004 ; Aguilar et al. 2006). De plus, une diminution de la densité et de la richesse en pollinisateurs, couplée à une diminution en visite et par conséquent en pollen reçu par les plantes et en graines produites sont fréquemment observées avec une augmentation de la fragmentation des paysages (Steffan-Dewenter and Tscharntke 1999).

La limitation en pollen est un facteur écologique majeur impliqué dans l'évolution du système de reproduction des plantes (Lloyd 1979). En réponse au déclin des pollinisateurs, les espèces entomogames peuvent développer deux stratégies pour maintenir leur succès reproducteur: (i) une augmentation de l'autofécondation, attendue principalement chez les espèces végétales spécialisées envers leurs pollinisateurs. Par exemple, chez *Centaurium erythraea,* des études ont montré une augmentation des taux d'autofécondation spontanée suite au déclin de pollinisateurs (Brys et al. 2011 ; Brys and Jacquemyn 2011). (ii) Un renforcement des interactions avec les pollinisateurs (Ashman et al. 2004 ; Eckert et al. 2010), en augmentant par exemple les quantités de pollen ou de nectar disponibles pour les pollinisateurs. Dans ce deuxième scénario, le renforcement des interactions avec diverses espèces de pollinisateurs pourrait compenser le déclin des espèces les plus efficaces et permettrait le maintien de l'allofécondation via une augmentation des taux de visites et de l'efficacité de transport de pollen des pollinisateurs secondaires. Un plus grand investissement dans l'attraction des pollinisateurs peut être mis en place (taille des fleurs, couleur, qualité du nectar) et notamment par les organes mâles car l'export de pollen augmente avec l'augmentation du nombre de visites (Bell 1985 ; Vamosi and Otto 2002). L'ajustement de la phénologie de floraison avec les périodes de disponibilité des pollinisateurs contribue également à l'augmentation des taux de visites (Elzinga et al. 2007 ; Devaux et Lande 2010). Ce deuxième scénario est surtout attendu chez les espèces végétales généralistes pouvant maintenir leur production de graines grâce aux visites de différentes espèces de pollinisateurs ou pouvant attirer de nouveaux pollinisateurs.

Après près de 30 ans de multiples théories sur le système de reproduction (Lloyd 1979 ; Lande and Schemske 1985 ; Barrett and Harder 1996), le déclin des pollinisateurs permet de tester les prédictions théoriques à large échelle et dans des populations naturelles. Jusqu'à maintenant, très peu d'études empiriques et expérimentales ont étudié les conséquences des changements globaux d'un point de vue évolutif (Bodbyl Roels and Kelly 2011 ; Brys and Jacquemyn 2012) et aucune évidence directe de l'évolution de la reproduction des plantes n'a été montrée *in natura* (Thomann et al. 2013).

B. Systèmes de reproduction des végétaux

La reproduction chez les végétaux est caractérisée par une grande variété de systèmes incluant des processus sexués et asexués. La multiplication asexuée implique la production de nouvelles branches, rejets, spores ou graines génétiquement identiques au sporophyte qui les a produit à l'exception de quelques mutations somatiques (Whithaml'z and Slobodchikoff 1981). La multiplication végétative est extrêmement commune chez les espèces pérennes mais la plupart des espèces combinent reproduction sexuée et asexuée. Elle conduit à une descendance nombreuse, adaptée à un environnement local et qui va coloniser efficacement les milieux. Je m'intéresserai par la suite uniquement à la reproduction sexuée (Encadré 3).

D'après Schemske et Lande (1985) le système de reproduction des espèces végétales se réparti de manière bimodale avec une tendance à l'allogamie ou à l'autogamie. Néanmoins, il a depuis été observé que 42% des espèces ont un système de reproduction mixte où 20 à 80 % des graines sont issus d'autofécondation (Vogler and Kalisz 2001 ; Goodwillie et al. 2005). L'évolution et le maintien d'un système mixte de reproduction sont au cœur de nombreuses discussions (Goodwillie et al. 2005 ; Cheptou and Schoen 2007). L'évolution de l'autofécondation est généralement expliquée par l'opposition de forces génétiques : l'avantage de transmission de l'autofécondation (Fisher 1941) *vs* la dépression de consanguinité.

Encadré 3 : La reproduction sexuée chez les plantes

La **reproduction sexuée** implique la fusion de gamètes mâles et femelles engendrant une descendance. Chez les Angiospermes, les structures produisant les ovules et le pollen peuvent être à l'intérieur d'une même fleur (70% des espèces possèdent des **fleurs hermaphrodites** ; Richards 1986) ou dans des structures séparées soit portées par un même individu (espèces **monoïques**), soit sur des individus différents (espèces **dioïques** ; Holsinger 2000). Les espèces **polygames** portent à la fois des fleurs hermaphrodites, des fleurs mâles et des fleurs femelles sur le même pied.

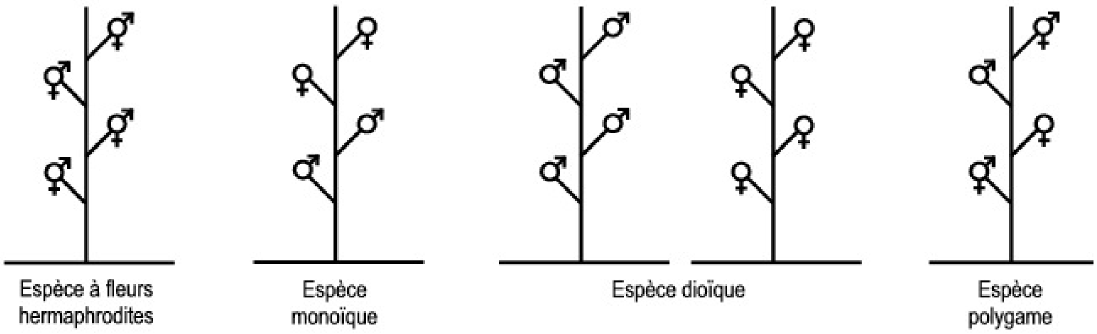

Figure 9. Schémas d'organisation des structures mâles et femelles chez les Angiospermes.

Dans le cas des espèces monoïques et hermaphrodites, la reproduction sexuée sera dite **autogame** (**autofécondation**) lorsque les gamètes mâles et femelles proviennent d'un même individu et **allogame** (**allofécondation**) lorsqu'ils proviennent d'individus distincts. L'autofécondation est une forme de reproduction sexuée dans laquelle les deux gamètes proviennent d'un seul et même individu et dont la descendance donne un individu qui n'est pas nécessairement identique à son parent. Chez les espèces hermaphrodites autocompatibles l'autofécondation peut se produire au sein d'une fleur ou au sein d'un même individu qui est aussi appelé **géïtonogamie**. Beaucoup d'espèces ont mis en place des systèmes pour éviter l'autofécondation telle que :

- la **dichogamie** : décalages temporelles dans la maturation des organes mâles et femelles d'un même individu (McCarty and Quinn 1990).
- l'**herkogamie** : éloignement physique des organes mâles et femelles.
- l'**auto-incompatibilité** : les grains de pollen génétiquement proches des cellules du stigmate ne pourront pas germer (Byers and Waller 1999 ; Lande and Schemske 1985).
- l'**hétérostylie** : les morphes différent par la longueur des styles et des étamines favorisant la fécondation croisée.

- ✓ *Assurance de reproduction*

Darwin (1877) a été le premier à proposer l'hypothèse d'assurance reproductive comme moteur de l'évolution de l'autofécondation. En effet, quand les partenaires sexuels ou les vecteurs de pollinisation sont rares dans un environnement donné, l'autofécondation donne un avantage aux espèces autogames (Darwin 1877; Lloyd 1979). Une faible abondance en pollinisateurs durant les périodes de floraison favorise le passage de l'allofécondation à l'autofécondation spontanée grâce à l'assurance reproductive (Darwin 1877 ; Baker 1955 ; Lloyd 1979 ; Takebayashi and Morel 2001).

Près de 20% des Angiospermes se reproduisent quasiment essentiellement par autofécondation (Barrett 2002). L'autofécondation est obligatoire dans les fleurs dites cléistogames dont la morphologie ne permet pas l'apport de gamètes extérieurs (Oakley and Winn 2008), c'est le cas par exemple chez *Lobelia dortmanna* et *Epipactis phyllanthes.* Les individus issus d'autofécondation ont généralement moins de loci polymorphes et moins d'allèles par loci polymorphes que des individus apparentés allo-fécondés (Brown 1979 ; Gottlieb 1981). Les espèces se reproduisant par autofécondation ont généralement des populations avec une taille efficace plus petite (Schoen and Brown 1991). Par ailleurs, les espèces autogames ou clonales présentent généralement une plus faible diversité génétique et un plus fort degré de différentiation inter-populationnelles que les espèces allogames (Hamrick and Godt 1996).

Plusieurs types d'autofécondation peuvent être observés : le « *primary selfing* » qui intervient avant toute possibilité d'allofécondation, le « *competing selfing* » lorsque l'autopollen est en compétition avec l'allopollen, le « *delayed selfing* » qui intervient en fin de saison de pollinisation (il y a donc dans un premier temps possibilité d'allofécondation). En plus de l'assurance reproductive, le « *primary selfing* » peut être favorisé car il réduit les coûts du maintien du temps de floraison nécessaire à l'allopollinisation. Le « *delayed selfing* » est favorisé quand le service en pollinisateurs est imprédictible (Schoen and Brown 1991 ; Yahara 1992 ; Schoen et al. 1996) ou quand les densités florales sont très importantes (Husband and Barrett 1996). L'autofécondation a cependant un coût car le pollen impliqué dans l'autopollinisation n'entrera pas en jeu dans l'allopollinisation (sauf dans le cas du « *delayed selfing* » où l'autopollen intervient après possibilité d'allopollinisation) : ce concept est connu sous le nom de « *pollen discounting* » (Harder et Wilson 1998). De plus, la dépression de consanguinité s'oppose à l'avantage somatique de l'autofécondation (Herlihy and Eckert 2002).

- ✓ *Dépression de consanguinité*

La dépression de consanguinité est définie comme la réduction de la valeur sélective de la descendance autogame due à l'expression d'allèles récessifs délétères chez les homozygotes par rapport à la descendance allogame (Lande et Schemsk 1985 ; Frankham et al. 2002). Une faible valeur sélective de la descendance autogame pourrait diminuer le bénéfice des individus autogames issus de l'assurance de reproduction et par conséquent la survie des populations principalement autogames (Hedrick and Kalinowski 2000). La dépression de consanguinité est un facteur essentiel dans l'évolution du système de reproduction des plantes (Darwin 1877 ; Charlesworth and Charlesworth 1987). Les études se sont portées sur deux modèles : le modèle de « surdominance » et le modèle de mutations délétères (Crow 1993). Dans le premier modèle, la dépression de consanguinité dépend de la supériorité des hétérozygotes. Etant donné que la descendance issue d'autofécondation a moins de chance d'être hétérozygote elle aura une valeur sélective réduite. Le deuxième modèle suppose que la dépression de consanguinité est causée par des mutations délétères rares pour la plupart récessives. Ces allèles délétères sont maintenus par des mutations récurrentes. La plupart des travaux semblent montrer que la dépression de consanguinité est essentiellement due aux mutations délétères (Charlesworth and Charlesworth 1987 ; Carr and Dudash 2003).

- ✓ *Relations entre autofécondation, assurance reproductive et dépression de consanguinité*

Les relations entre autofécondation, assurance reproductive et dépression de consanguinité sont encore mal connues. Un objectif important est d'expliquer la variabilité des taux de dépression de consanguinité entre espèces, entre populations ou entre familles (*i.e.* plantules provenant du même individu maternel) d'une population. A chacun de ces niveaux les chercheurs ont tenté de déterminer si la dépression de consanguinité est associée à l'autofécondation. En théorie, les grandes populations principalement allogames devraient avoir un taux de dépression de consanguinité relativement fort tandis que les populations principalement autogames ou ayant expérimentées des goulots d'étranglement devraient avoir un plus faible taux de dépression de consanguinité grâce à la sélection (*i.e.* purge de la charge génétique ; Ohta and Cockerham 1974 ; Charlesworth et Charlesworth 1987 ; Karron 1989 ; Barett and Charlesworth 1991). En milieu naturel, les observations ne sont pas toujours consistantes avec ces prédictions. Conformément aux théories, certaines études détectent une corrélation négative entre dépression de consanguinité et autofécondation (Husband and Schemsk 1996 ; Winn et al. 2011) tandis que d'autres études ne détectent aucune corrélation (Byers and Waller 1999). Dans certains cas une forte dépression de consanguinité peut être associée à un taux d'autofécondation élevé (Vaughton et al. 2008; Winn et al. 2011 ; Ruan and Teixeira da Silva 2012).

Des facteurs biotiques et abiotiques peuvent influencer l'évolution du système de reproduction et le degré d'autofécondation effectué par un individu (Darwin 1877 ; Lloyd 1979 ; Holsinger 1991 ; Uyenoyama et al. 1993 ; Barrett and Harder 1996). Des études sur des populations naturelles soulignent l'importance du contexte écologique dans l'expression du système de reproduction d'une espèce (Dudash and Fenster 1997 ; Fishman and Wyatt 1999 ; Eckert 2000; Goodwillie 2001). Des études récentes sur divers taxa ont révélé que les stress environnementaux (Armbruster and Reed 2005; Sedlacek et al. 2013), la compétition (Cheptou et al. 2000) ou une taille réduite des populations augmentent la dépression de consanguinité (Michaels et al. 2008 ; Hirayama et al. 2007). Une augmentation de l'assurance reproductive et de la dépression de consanguinité dues au stress produit par les changements globaux sont donc attendues (Beaumont et al. 2011).

Pouvoir prédire la variation de l'assurance reproductive et la dépression de consanguinité lors des changements globaux semble être essentiel dans la compréhension de l'adaptation des espèces végétales aux contraintes biotiques et abiotiques. Certains paramètres abiotiques pouvant influencer le système de reproduction d'une espèce il semble intéressant de travailler à une large échelle afin de prendre en compte la diversité des milieux dans lesquels les populations d'une même espèce se développent. Le challenge actuel est de savoir dans quelles mesures les caractéristiques de la population (taille, densité, isolement), de la communauté (densité, diversité), des assemblages de pollinisateurs (diversité, densité, efficacité, redondance) déterminent la pollinisation et le succès reproducteur d'une espèce et dans quelles mesures les changements globaux impactent ces différent paramètres.

Les milieux alpins face aux changements climatiques

Les milieux alpins, représentants environ 25% de la surface émergée de la Terre (Kapos et al. 2000). Ils sont considérés comme des points chauds de biodiversité et accueillent près d'un tiers de la flore terrestre (Barthlott et al. 1996 ; Price 2000) dont de très nombreuses espèces endémiques. Dans les massifs montagneux, il est possible de distinguer plusieurs étages de végétations en fonction de l'altitude. L'étage alpin, situé au dessus de la limite des forêts, est dépourvu de ligneux de grande taille (Kudo 1993). La limite inférieure de cet étage est souvent graduelle jusqu'à l'étage subalpin qui est une transition entre les pelouses d'altitude et les forêts. La fourchette altitudinale de cet étage est très variable, elle va de 300 mètres dans les régions subpolaires à plus de 3500 m dans les zones tropicales (Körner 1995). Dans les Pyrénées par exemple il s'étend d'environ 1600 à 2300 m (Fig 10 ; Dupias 1985).

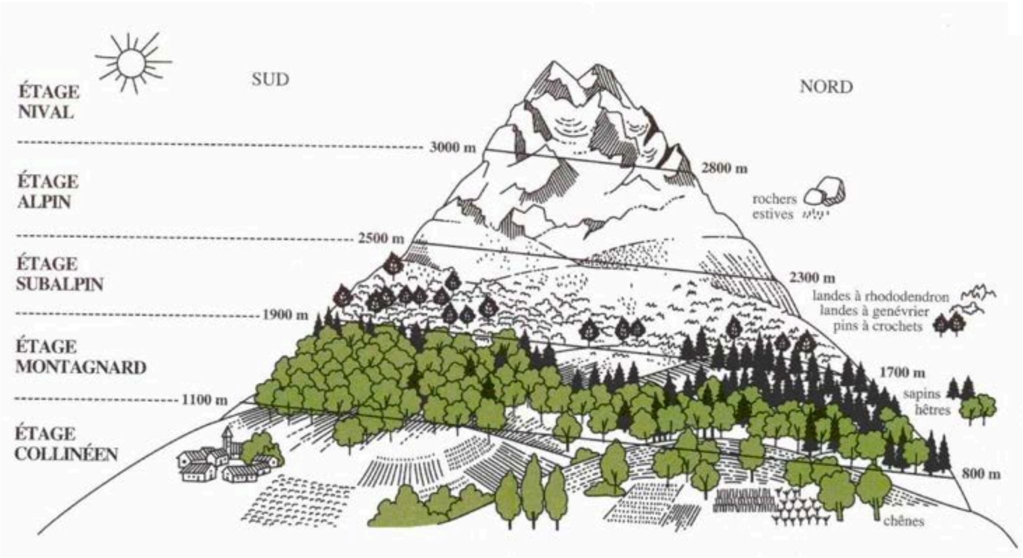

Fig. 10 Schéma des différents étages Pyrénéens de P. Mayoux (2004).

Quatre changements atmosphériques majeurs sont associés à l'altitude (Körner 2007): (i) une diminution de la pression atmosphérique (diminution de 11% tous les 1000 m de dénivelé qui entraine des changements dans les échanges de gaz chez les végétaux), (ii) une réduction des températures atmosphériques (diminution de 5.5 C° tous les 1000 m de dénivelé ; Körner 1995), (iii) une augmentation des radiations solaires, (iv) une augmentation de la fraction d'UV-B. Les précipitations sont un paramètre complexe de l'altitude, les gradients de précipitation sont très variables en fonction des études (Khurshid 1973 ; Lauscher 1976 ; Körner 2003). Par ailleurs, les milieux alpins font partis des zones les moins venteuses sur Terre, à l'exception des crêtes exposées et des sommets (Lauscher 1976 ; Barry 1981). Les changements abiotiques (topographie, température, enneigement, humidité ou composition du sol) le long de gradients altitudinaux et longitudinaux peuvent influencer les processus biologiques chez les plantes. Cela peut notamment induire des patrons d'adaptations génétiques complexes à une échelle locale. Ces forts gradients environnementaux induisant de forts changements de végétation sur de courtes distances (Whiteman 2000) font des écosystèmes alpins des systèmes modèles pour tester des phénomènes physiologiques ou écologiques (Körner and Spehn 2002) et sont largement étudiés depuis 1800 (Humbold and Bonpland 1807 ; Bonnier et Flahaut 1876 ; Larcher and Siegwolf 1985 ; Körner 2003).

La disponibilité en habitat est un facteur majeur dans la diversification et l'évolution des organismes (MacArthur and Wilson 1967 ; Rosenzweig 2003). Lorsque l'on monte en altitude la surface disponible diminue (Fig. 11) et par conséquent les habitats sont réduits (Rabbek 1995 ; Körner 2000). Au-dessus de la limite des arbres, la disponibilité en habitat est en moyenne divisée par deux tous les 167 m de dénivelé (Körner 2007). Les habitats de montagne sont caractérisés par une fragmentation naturelle élevée crée par une topographie hétérogène (Körner 1999, 2001). Par conséquent, les populations de plantes alpines sont généralement isolées spatialement les unes des autres.

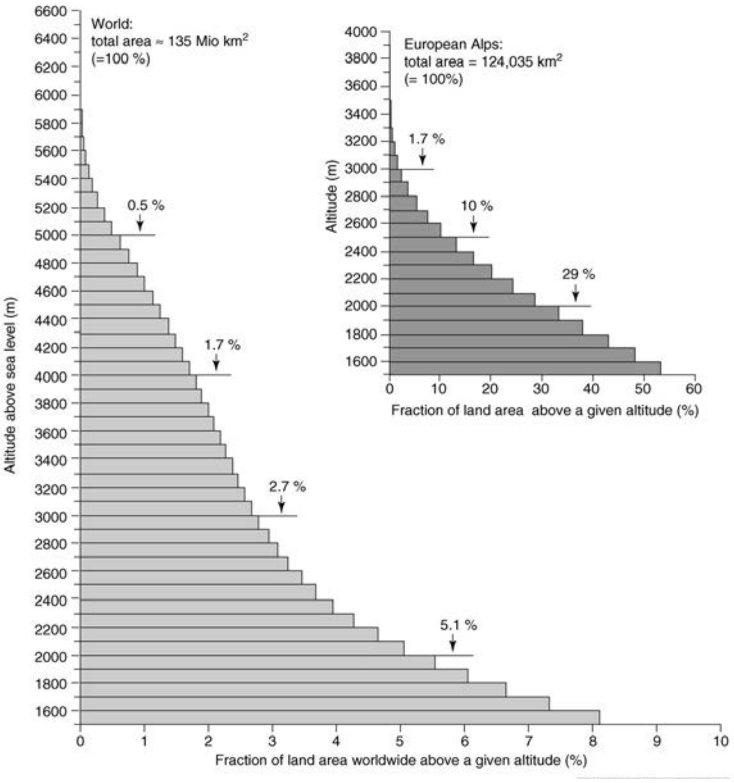

Figure 11. Espace mondial disponible à partir de 1500 m d'altitude (surface totale disponible au-dessus de 1500 m d'altitude = 10.94 million km^2). Le schéma de droite prend l'exemple des Alpes (surface totale disponible au-dessus de 1500 m d'altitude total = 66 000 km^2).

Une des premières réponses des espèces face aux changements climatiques va être une montée en altitude (*i.e.* « thermophilisation » des communautés) afin de trouver des conditions climatiques optimales (MacArthur 1972 ; Peters and Darling 1985). Comme les surfaces habitables en altitude sont de plus petites tailles qu'à basse altitude, les aires de distribution des espèces vont être de plus en plus étroites entrainant une réduction des tailles de populations et les rendant plus vulnérables aux pressions génétiques et environnementales (Peters and Darling 1985 ; Hansen-Bristow et al. 1988 ; Bortenschlager 1993). De plus, la thermophilisation en altitude des espèces va entrainer l'invasion par les forêts des prairies alpines connues pour abriter de très nombreuses espèces et notamment des insectes (Iserbyt 2009).

Les espèces alpines ont déjà subi des changements climatiques drastiques au cours du temps ce qui a eu pour effet de promouvoir des espèces hautement spécialisées avec une forte adaptation pour les opportunités limitées de croissance et de survie (Körner 1999). Le développement des espèces végétales en altitude est contraint par les effets directs et indirects des faibles températures, radiations ou de la faible disponibilité en eau (Körner and Larcher 1988). Les opportunités de croissance sont limitées au printemps et en été et la floraison est limitée à une très courte période de l'année (Kudo 1993 ; Bergman et al. 1996). Les végétaux répondent à ces influences climatiques grâce à des ajustements morphologiques et physiologiques tels que le nanisme, des formes en coussin ou en rosette ou une modification des stratégies de reproduction. Bien que 90% des plantes alpines se reproduisent végétativement (Johnson 1969 ; Bliss 1971 ; Kudo 1993 ; Körner 1999), tels que *Vaccinium vitis-idaea* L., *Vaccinium ovalifolium* J.E. Smithvar. *alpinum* ou *Rhododendron aureum* Georgi., la reproduction sexuée est importante car la production de graines est nécessaire pour la dispersion et la colonisation de nouveaux sites (Kevan 1989). Cependant, les écosystèmes alpins subissent des variations spatio-temporelles en termes de service de pollinisation (Hirao et al. 2006). En effet, les conditions météorologiques difficiles (faibles températures, humidité) peuvent limiter l'abondance et l'activité des pollinisateurs et par conséquent limiter les opportunités de fécondation croisée (Kudo 1993 ; Callaghan and Jonasson 1995). De nombreuses études ont montré que les Diptères et les Hyménoptères sont les pollinisateurs principaux des espèces végétales entomophiles dans les milieux alpins. Dans certains cas, les activités des Hyménoptères peuvent être limitées et les Diptères prédominants (Arroyo et al. 1982; Levesque et Burger 1982; Mc Call and Primack 1992; Totland 1993).

La montée en altitude des espèces, les phénomènes de fragmentation des habitats, les perturbations des relations plantes-pollinisateurs et l'impact des changements globaux sur le système de reproduction font des populations alpines des sujets particulièrement sensibles aux changements globaux. Etant donné que ces milieux sont des points chauds de biodiversité il est important d'étudier les effets des changements globaux sur ces milieux.

Objectifs de la thèse

Le premier objectif de cette thèse a été de déterminer l'histoire biogéographique de *Rhododendron ferrugineum* dans les Pyrénées. Les patrons génétiques et l'histoire phylogéographique de plusieurs espèces (Stehlik et al. 2002 ; Byars et al. 2009) ont été étudiés dans diverses régions de montagnes, mais jusqu'à maintenant peu d'études ont porté sur les Pyrénées (Segarra-Moragues et al. 2007 ; Lauga et al. 2009 ; Escaravage et al. 2011). Le but de cette étude est de comprendre l'impact de l'histoire phylogéographique de cette espèce sur sa structure génétique actuelle. Pour cela, nous avons étudié la structure génétique de 33 populations (29 populations Pyrénéennes, 3 populations Alpiennes et une population du Jura). L'origine asiatique du genre *Rhododendron* (Goetsch et al. 2005) implique une migration est-ouest de l'Asie vers l'Europe. En France, les populations Alpiennes auraient migré vers les Pyrénées. Trois principales hypothèses peuvent être avancées quant à leurs conditions de survie dans les Pyrénées durant les différents épisodes glaciaires: (1) l'espèce peut s'être réfugiée dans l'un des deux principaux refuges à l'est et au sud-ouest de la chaine des Pyrénées, (2) l'espèce peut avoir survécu dans des nunataks, zones refuges de haute altitude réparties le long de la chaine Pyrénéenne, (3) l'espèce peut avoir survécu dans de grands refuges de basse altitude.

Dans un second temps, nous avons étudié la variabilité du système de reproduction de *R. ferrugineum* dans 17 populations des Pyrénées le long de gradients environnementaux. Nous nous sommes intéressés aux relations entre autofécondation, assurance reproductive et dépression de consanguinité le long de gradients environnementaux afin de déterminer le rôle de contraintes environnementales et de la taille des populations sur le système de reproduction de cette espèce. Nous nous attendions à trouver les plus forts taux d'autofécondation et d'assurance reproductive dans les populations les moins susceptibles d'attirer des pollinisateurs (haute altitude, petite taille). Ces populations devraient également être sujettes à un fort taux de dépression de consanguinité. Par conséquent, nous nous attendions à trouver une relation négative entre les taux d'autofécondation et de dépression de consanguinité (Winn et al. 2011). Les taux de dépression de consanguinité devraient également être plus élevés dans des environnements stressants ou dans les populations de grande taille en cas de compétition.

Dans une troisième partie, nous avons déterminé les cortèges de pollinisateurs de 17 populations Pyrénéennes de *R. ferrugineum* sur deux années consécutives. Dans chaque population nous avons regardé s'il y avait une redondance dans les traits morphologiques et dans les capacités de visites des différents groupes de visiteurs présents et nous avons estimé l'efficacité de chacun des cortèges de pollinisateurs. Nous avons également étudié les liens entre efficacité du cortège de pollinisateurs, production de graines de *R. ferrugineum* et taux d'autofécondation.

Plusieurs hypothèses ont pu être proposées : (1) quel que soit le contexte environnemental *R. ferrugineum* a toujours le même assemblage de pollinisateurs. De part cette spécialisation, *R. ferrugineum* serait en danger en cas de changements de cortèges de pollinisateurs suite aux changements globaux (Roth et al. 2014). (2) *R. ferrugineum* est visité par les pollinisateurs présents localement, la composition et la diversité de l'assemblage dépend donc du contexte environnemental. Cela montrerait que cette espèce est capable de s'adapter à divers cortèges de pollinisateurs. Si une redondance en terme d'efficacité existe entre les différents pollinisateurs, la variabilité des cortèges de pollinisateurs n'impactera pas le système de reproduction de *R. ferrugineum*. (3) *R. ferrugineum* utilise les assemblages locaux de pollinisateurs mais la variabilité d'efficacité entre les différents cortège entrainent une variabilité du système de reproduction de l'espèce.

Cadre de l'étude

A. Espèce étudiée: *Rhododendron ferrugineum* L. (Ericaceae)

Le genre Rhododendron est particulièrement diversifié avec 1025 espèces présentes en Asie, en Europe et en Amérique du Nord, des limites du climat tropical jusqu'au climat polaire (Leach 1961). La plupart des espèces se développent en régions montagneuses comme le Caucase, les Carpates, l'Himalaya, les Alpes ou les Pyrénées. Certaines espèces sont également présentes dans les régions forestières d'Inde et de Birmanie. Ce genre est totalement absent d'Amérique latine et d'Afrique. Les formes sont très diverses allant d'arbuste de moins d'un mètre jusqu'à des arbres pouvant atteindre 15 m de haut.

Rhododendron ferrugineum L. (Ericaceae ; Fig. 12) est un arbuste nain sempervirent pouvant atteindre 80 cm de haut. Cette espèce forme des landes, classées d'intérêt communautaire (COM 1992 ; programme Natura 2000 : code 4060-4 ; CORINNE biotope : code 31.42), localement très denses à l'étage subalpin (1400 à 2200 m) dans les Alpes et dans les Pyrénées, sur les faces nord à nord-ouest. Cette espèce est également retrouvée dans le nord des Apennins et dans le Jura. Dans les Pyrénées, de petites populations isolées sont présentes entre 900 à 1500 m d'altitude. *Rhododendron ferrugineum* est une espèce à floraison massive fleurissant de fin mai à fin juillet (en fonction de l'altitude et des conditions climatiques) et pouvant produire plus de 3000 fleurs rouges nectarifères par m^2. La croissance et la floraison débutent simultanément environ 15 jours après la fonte des neiges et dure environ un mois (Delmas et al. 2014). Des études antérieures ont montré que, dans les Alpes, les fleurs pentamères sont protandres (grains de pollen matures avant le stigmate). Les fleurs sont constituées de 10 étamines réparties sur 2 verticilles et de 5 carpelles contenant environ 500 ovules (Escaravage et al. 2001). Les graines, de forme oblongue de 1 à 2 mm, sont matures 4 à 5 semaines après fécondation et sont disséminées par le vent ou l'eau. La production d'un grand nombre de graines est un caractère commun au genre Rhododendron (Sprecht, 1996). *Rhododendron ferrugineum* possède un système mixte de reproduction sexuée et peut également se multiplier végétativement par marcottage (Pornon et al. 1997 ; Escaravage et al. 1998 ; Pornon et al. 2000). Cette espèce entomophile est principalement pollinisée par des Hyménoptères du genre *Apis* et *Bombus* (Escaravage et al. 1997, Escaravage et Wagner 2004 ; Delmas et al. 2013). D'autres pollinisateurs tels que les Diptères et les Coléoptères sont des pollinisateurs secondaires.

Fig. 12. a et b. Populations Pyrénéennes de *R. ferrugineum*. c. Floraison massive de *R. ferrugineum*. d. Bourgeon inflorenscentiel. e. Inflorescence épanouie. f. Capsules en cours de maturation. g. Capsules déhiscentes. h. Emasculation des bourgeons. i. Ensachage des inflorescences. j. Mesure des taux de visites dans un quadrat. K. *Bombus lucorum*. L. *Apis mellifera*. M. Lepidoptère. N. Syrphidae.

B. Site d'étude : le massif Pyrénéen

Situées au sud-ouest de l'Europe, les Pyrénées s'étendent sur près de 430 km le long d'un axe est-ouest entre la Méditerranée et l'Océan Atlantique.

Avec 129 sommets culminants à plus de 3000 m d'altitude, les Pyrénées forment une barrière géographique aux migrations nord/sud de nombreuses espèces animales et végétales. Des études ont suggéré la présence de deux refuges principaux de basse altitude, l'un au sud-ouest et l'autre à l'est des Pyrénées, respectivement sous influences climatiques océaniques et méditerranéennes (Jeannel 1946 ; Médail and Diadema 2009 ; Dzialuck et al. 2009). Par ailleurs, des données biologiques et géologiques démontrent que durant les périodes glaciaires, la calotte glaciaire ne couvrait pas complètement les hauts sommets et descendait rarement en dessous de 1000 m d'altitude (Jalut et al. 1992). Il est donc possible que des nunataks aient été des refuges pour certaines espèces de haute altitude (Segarra-Moragues et al. 2007).

Les vallées Pyrénéenne généralement orientées nord-sud abritent de nombreuses espèces animales et végétales. La flore Pyrénéenne est constituée d'environ 4500 espèces dont 160 endémiques des Pyrénées, telles que la Ramonde des Pyrénées (*Ramonda pyrenaica* ; Gesneriaceae*),* le Vélar des Pyrénées (*Erysimum pyrenaicum* ; Brassicaceae), l'Androsace ciliée (*Androsace ciliata ; Primulaceae)*, l'Hélianthème des Pyrénées (*Hellanthemum pyrenaicum ;* Cistaceae) ou la vesce argentée (*Vicia argentea ;* Fabaceae), (Fig. 13). Des études sur les bourdons des Pyrénées-Orientales ont montré la présence dans la vallée d'Eyne de 33 des 46 espèces de bourdons trouvés en France (Iserbyt et al. 2008).

Fig. 13. Exemple d'espèces végétales endémiques des Pyrénées. a. Androsace ciliée (*Androsace ciliata ;* Primulaceae*)*. b. Chardon bleu des Pyrénées (*Eryngium bourgatii ;* Apiaceae*)*. c. Ramonde des Pyrénées (*Ramonda pyrenaica* ; Gesneriaceae*)*. d. Saxifrage des Pyrénées (*Saxifraga longifolia ;* Saxifragaceae*)*. e. Hélianthème des Pyrénées (*Hellanthemum pyrenaicum ;* Cistaceae). f. Vesce argentée (*Vicia argentea ;* Fabaceae). g. Vélar des Pyrénées (*Erysimum pyrenaicum* ; Brassicaceae). h. Ancolie des Pyrénées (*Aquilegia pyrenaica ;* Ranunculaceae*)*.

Cette étude a été menée durant les saisons de floraison 2011 et 2012 de *R. ferrugineum*. 17 populations ont été étudiées le long de gradients altitudinaux et longitudinaux le long du massif Pyrénéens (Fig. 14). Les populations sont réparties dans les Hautes-Pyrénées (3 populations de haute et 3 populations de basse altitude), en Ariège (3 populations de haute et 3 populations de basse altitude) et dans les Pyrénées-Orientales (3 populations de haute et 2 populations de basse altitude), de 1220 à 2080 m d'altitude (Tableau 1). La distance géographique entre les populations varie de 3 à 250 km avec une moyenne de 150 km. Les paramètres biotiques et abiotiques tels que la taille des populations, la densité de fleurs de *R. ferrugineum*, l'assemblage d'espèces co-florissantes et des cortèges de pollinisateurs ou la température ont été déterminé pour chacune des populations. Nous avons également effectué des tests de pollinisation sur 15 individus de chaque population.

Les études génétiques ont été menées sur 33 populations, 17 décrites précédemment plus 3 populations dans les Alpes (dont une en Autriche), une dans le Jura et 12 supplémentaires dans les Pyrénées (dont 3 en Espagne ; Fig. 14).

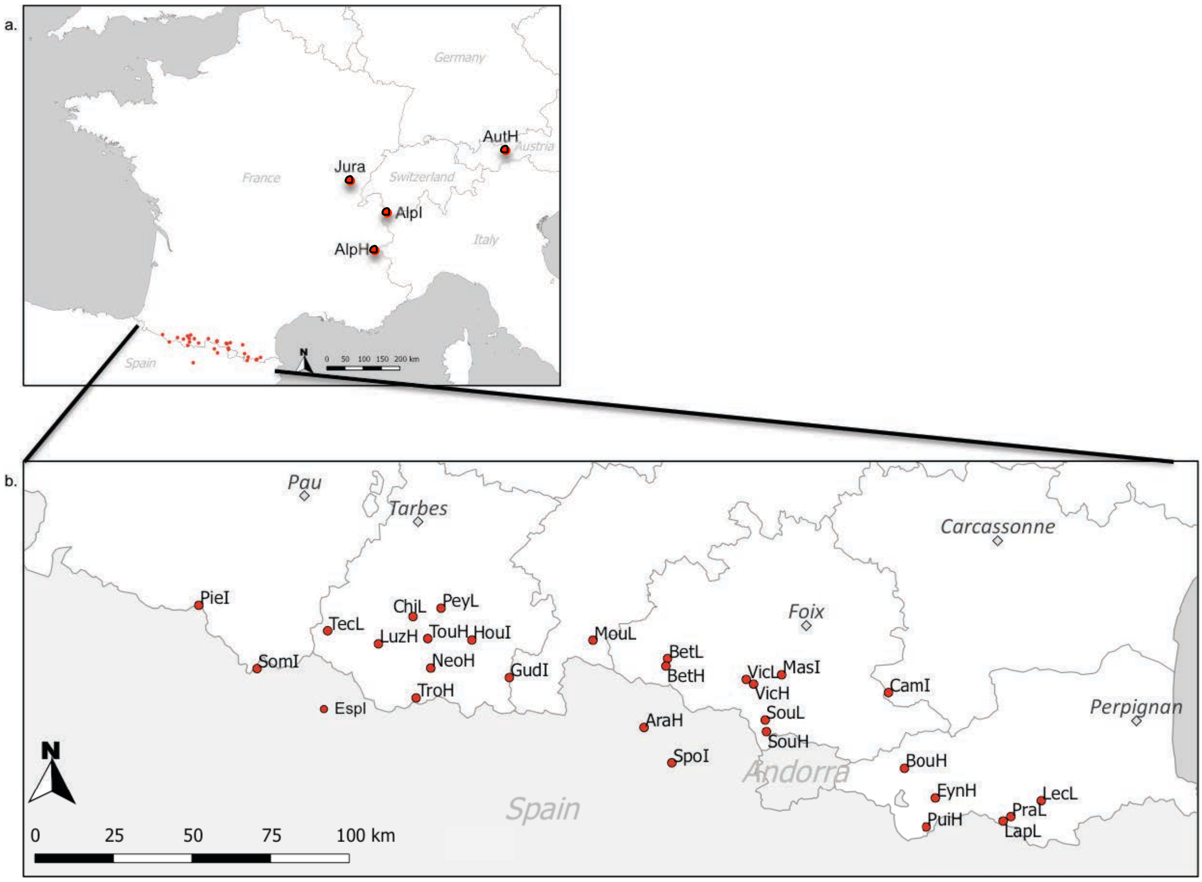

Fig. 14. Localisation des 33 populations, étudiées dans les Alpes (n=3), le Jura (n=1) et les Pyrénées (n=29).

Tableau 1. Informations géographiques et taille des populations de *R. ferrugineum* étudiées

Massif	**Sites**	**Code des populations**	**Localisation**	**Taille des populations (ha)**
Alpes	La Vormaine	AlpI	45°02′07″N 06°24′19″E	>100
	Col du Lautaret	AlpH	46°00'08"N 06°57'18"E	5-10
	Parscherkofel	AutH	47°12'32"N 11°27'18"E	50-100
Jura		JuraL	42°57'N 05°44'E	<1
Est des Pyrénées	Léca	LecL	42°28'07"N 02°31'40"E	<1
	Prats de Mollo	PraL	42°25'21"N 02°24'38"E	<1
	Lapreste	LapL	42°24'34"N 02°22'51"E	<1
	Eyne	EynH	42°28'27"N 02°07'05"E	5-10
	Puigmal	PuiH	42°23'26"N 2°05'0"E	5-10
	Lac des Bouillouses	BouH	42°33'31"N 01°59'42"E	50-100
Pyrénées centrales	Camurac	CamI	42°46'30"N 01°55'45"E	10-20
	Massat	MasI	42°49'18"N 01°30'75"E	<1
	Soulcem	SouH	42°39'28"N 01°27'27"E	50-100
	Soulcem	SouL	42°41'28"N 01°27'07"E	<1
	Etang de Lhers	VicH	42°47'36"N 01°24'16"E	20-50
	Etang de Lhers	VicL	42°48'23"N 01°22'31"E	1-5
	Espot	SpoI	42°33'47"N 01°05'36"E	<1
	Bethmale	BetL	42°51'42"N 01°04'02"E	1-5
	Bethmale	BetH	42°50'24"N 01°03'42"E	50-100
	Val d'Aran	AraH	42°39'45"N 00°58'56"E	<1
	Le Mourtis	MouL	42°54'32"N 00°46'31"E	5-10
	Peyragudes	GudI	42°47'42"N 00°27'14"E	5-10
	Hourquette d'ancizan	HouI	42°53'57"N 00°18'13"E	5-10
	Néouvielle	NeoH	42°48'N 00°09'E	50-100
	Le Peyras	PeyL	42°59'15"N 00°10'46"E	5-10
	Col du Tourmalet	TouH	42°53'57"N 00°07'54"E	5-10
	Cirque de Troumouse	TroH	42°43'41"N 00°05'40"E	5-10
	Chiroulet	ChiL	42°57'38"N 00°04'17"E	1-5
	Luz-Ardiden	LuzH	42°52'45"N 00°03'34"W	10-20
	Huesca	EspI	42°45'08"N 00°14'18"W	>100
	Lac du Tech	TecL	42°54'40"N 00°15'32"W	<1
	Col du Somport	SomI	42°47'41"N 00°31'38"W	<1
Pyrénées de l'ouest	Col de la Pierre Saint Martin	PieI	42°58'07"N 00°45'52"W	<1

CHAPITRE 1

- **Development of 18 microsatellite markers in *Rhododendron ferrugineum* (Ericaceae) for investigating genetic structure at margins.**

- **Microsatellite marker analysis reveals the complex phylogeographic history of *Rhododendron ferrugineum* (Ericaceae) in the Pyrenees.**

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TECHNICAL NOTE

Development of 18 microsatellite markers in *Rhododendron ferrugineum* (Ericaceae) for investigating genetic structure at margins

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Abstract Eighteen novel microsatellite markers were developed in *Rhododendron ferrugineum* using pyrosequencing technology. They were tested on populations in the Alps, Jura and Pyrenees to study genetic diversity at the species range margins. Protection of populations at margins is important for conserving the genetic diversity of species. We found 2–7 alleles per locus. The expected and observed heterozygosity ranged from 0.07 to 0.79 and from 0.05 to 0.85, respectively. These loci were successfully amplified in 22 other *Rhododendron* species and four Ericaceae species. These markers will be useful for investigating the genetic structure of *R. ferrugineum* and related species at their distribution margins.

Keywords Ericaceae · Microsatellite markers · Population genetics · Pyrosequencing · *Rhododendron*

Climate change has been suggested to modify geographic distribution and abundance of plant and animal species. In mountain areas, climate change will lead to changes in species distribution along altitudinal gradients, a fragmentation of populations and therefore a modification of species range margins (Lenoir et al. 2008). Peripheral populations tend to occur in less suitable and isolated environments making them sensitive to stochastic events, potentially resulting in localized population extinctions (Lesica and Allendorf 1995). Furthermore, according to the "rear edge population hypothesis", marginal populations commonly harbor the bulk of species' genetic diversity and could be relictual populations (Hampe and Petit 2005). Therefore, studying population dynamics at the edge of a species' range is crucial to understanding its response to environmental changes and its susceptibility to extinction (Bruni et al. 2012).

Rhododendron ferrugineum is an alpine shrub distributed from 1,000 to 2,600 m in the Alps, Jura and Pyrenees. This shrub, having an ecological interest (Natura 2000 program), is patchily distributed with large central populations and small marginal populations. Building on the work of Delmas et al. (2011), we isolated and developed eighteen new microsatellite markers to examine the genetic diversity of *R. ferrugineum* and determine which populations are more suitable to protect.

Production and pyrosequencing of enriched DNA libraries was carried out by Genoscreen (Lille, France) using a 454-FLX Titanium (Roche Applied Science, Meylan, France) as described in Malausa et al. (2011). For this purpose, 20 mg of lyophilized leaf tissue was ground with tungsten beads at 30 Hz for 90 s using TissueLyser II and genomic DNA was extracted using the DNeasy-Plant Mini Kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. The resulting sequences were subsequently sorted and selected using the open access program QDD (Meglecz et al. 2010). A first set of 200 concatenated sequences longer than 80 bp and containing microsatellite motifs with at least six repeats and tandem-repetition-free flanking regions was tested in separate PCRs on four individuals from different parts of the Pyrenees. Amplifications were carried out in 10 µl mix containing 10 ng template DNA, 0.7× Qiagen Multiplex PCR Master Mix, 10 µM of

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each primer and RNase free water. Cycling conditions were 15 min at 95 °C, 30× (30 s at 95 °C, 90 s at 56 °C, 45 s at 72 °C) and 30 min at 60 °C. Among the 200 primer pairs, 145 produced amplicons of the expected size on agarose gels. A set of 48 primers pairs with a large range of PCR product sizes was chosen and amplified with fluorescent-labeled forward primers (Eurofins MWG Operon, Courtaboeuf, France) following the same PRC conditions as used in the previous amplification. To maximize the chance to isolate polymorphic microsatellite loci, these 48 primers pairs were tested on seven individuals collected in three different areas in the French Pyrenees. Among the 48 tested markers, 18 were polymorphic (Table 1). Four multiplex PCRs were optimized using Multiplex manager 1.1 (Holleley and Geerts 2009) and were tested on 70 individuals from four populations—one from the Alps (45°2′N–6°24′E), one from Jura (46°45′N–5°40′E) and two from the French Pyrenees (Léca 42°28′N–2°31′E, Luz-Ardiden 42°52′N–0°03′W). PCR

Table 1 Characteristics of 18 polymorphic microsatellite markers in *Rhododendron ferrugineum*

Locus	GenBank accession number	Repeat motif	Allele size range (bp)	Primer sequences (5′–3′)	Multiplex markers set	Fluorolabel
RF96P2	KC008581	$(TCT)_9$	73–79	F: AGGCCTTCTACTACAACAACTCA R: TCCACCCTTTCCTTCTCTTTC	1	Yakima Yellow
RF85	KC008582	$(CAA)_5$	148–159	F: TGGTGCTGTGACAAAGGGTA R: TCCGAGGAAATCAATGAAGG	1	Atto550
RF153	KC008583	$(GA)_7$	207–235	F: CCACACGCTAGGGAACTTTT R: TCAGCGTCGAAGAATCTCAA	1	Fam
RF140	KC008584	$(AC)_{13}$	196–208	F: ATGGCTAGCTTTGTGCTGCT R: TGCACATGAGTTCCTCAACA	1	Yakima Yellow
RF175	KC008585	$(TG)_{10}$	282–318	F: TTCTGAACCCTGCACTTCCT R: CCAGCGGAGACAGTATGGAT	1	Atto565
RF213	KC008586	$(AC)_9$	87–113	F: CGTCTAAGAAAGTCGCACCA R: GAAGGGTTGAAGGATGACGA	2	Atto565
RF163P1	KC008587	$(GA)_{12}$	179–191	F: TGAATGGTTCAAGCACCAAA R: GCTGTTCTTGCTGACATGGA	2	Fam
RF122P2	KC008588	$(TTC)_7$	209–238	F: TTCTCAGTTCCTTCTTCGGC R: GTAAGAGAAAAGGACGCGCA	2	Yakima Yellow
RF113P2	KC008589	$(GTT)_8$	312–327	F: TCGTCAAATGCAGCCAAAC R: CAACGAACAATCATGCTCAAA	2	Atto550
RF202	KC008590	$(TG)_{15}$	86–104	F: CATTTTCCAGCACAAAACTAAATG R: TGTGACGGTGTATCGGAAGA	3	Atto565
RF128	KC008591	$(GA)_{11}$	146–156	F: ATAAACGGCTCTCAAATGCG R: GATGTGTTTCCGGCGTAGTT	3	Fam
RF157P1	KC008592	$(TC)_{12}$	200–216	F: CTTCCTCTCCCATGCCATAA R: CCTTTCCGAAGCACCAATAA	3	Atto550
RF105P2	KC008593	$(GAA)_6$	460–466	F: GACTTGCTCACGGGAACCTA R: ATTGGATCATGGGTCAAAGC	3	Yakima Yellow
RF87P2	KC008594	$(CAC)_7$	133–142	F: CCTTTCCTCGCAATGAAGAA R: AGGAAGGTGATGAGGAGGGT	4	Fam
RF182	KC008595	$(CT)_{12}$	150–164	F: TCTGTCCGACCGAATCTGTA R: CAGCAGCCATTAGACAGAAAAA	4	Yakima Yellow
RF146P1	KC008596	$(TC)_{10}$	211–261	F: CCCCTTAAATGGGAGGCTTA R: AAACTCCAAGGGCATGTTTG	4	Atto550
RF114P3	KC008597	$(CTT)_7$	385–394	F: TGCACTAACCCAGAAATCATGT R: TCATAATCGGGTTTCAGCTA	4	Atto565
RF126P1	KC008598	$(TG)_9$	438–450	F: AATTAACCATCAACCGTAGGGTAA R: TCAAACAACTTCATCCCTTCC	4	Atto550

Table 2 Initial primer screening in four populations of *Rhododendron ferrugineum*

	Alps ($n = 20$)			Luz/Pyrenees ($n = 20$)			Jura ($n = 10$)			Léca/Pyrenees ($n = 20$)		
	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E
RF113P2	4	0.70	0.64	3	0.60	0.57	4	0.53	0.49*	3	0.49	0.45
RF122P2	1	0.00	0.00	2	0.20	0.18	2	0.07	0.07	1	0.00	0.00
RF163P1	4	0.60	0.66	3	0.60	0.60	3	0.07	0.18*	3	0.34	0.40
RF213	5	0.55	0.55	2	0.05	0.05	3	0.47	0.44	3	0.48	0.55
RF105P2	2	0.50	0.50	2	0.30	0.26	2	0.40	0.29	2	0.38	0.40
RF128	3	0.35	0.30	3	0.20	0.19	2	0.33	0.26	3	0.48	0.25*
RF157P1	6	0.75	0.79	4	0.65	0.65	4	0.33	0.42	3	0.14	0.15
RF202	4	0.50	0.58	3	0.70	0.62	3	0.53	0.38	3	0.53	0.65
RF85	2	0.15	0.21	3	0.15	0.14	3	0.40	0.41	3	0.57	0.65
RF87P2	3	0.65	0.61	3	0.45	0.37	3	0.40	0.45	3	0.14	0.15
RF96P2	2	0.15	0.12	2	0.45	0.31	2	0.67	0.35*	2	0.51	1.00*
RF114P3	2	0.25	0.30	2	0.20	0.26	2	0.27	0.22	2	0.38	0.50
RF126P1	2	0.10	0.10	2	0.20	0.26	2	0.33	0.32	1	0.00	0.00
RF140	3	0.35	0.41	3	0.40	0.60	2	0.13	0.34	3	0.23	0.25
RF146P1	7	0.65	0.65	3	0.35	0.37	2	0.07	0.18	2	0.33	0.30
RF153	5	0.50	0.68	2	0.50	0.47	4	0.47	0.45	3	0.49	0.45
RF175	5	0.65	0.58	3	0.60	0.67	5	0.40	0.40	2	0.33	0.30
RF182	3	0.80	0.61	3	0.55	0.48	2	0.13	0.13	1	0.00	0.00

Number of alleles observed (N_a), observed (H_o) and expected (H_e) heterozygosity, significant deviation from HWE (*) after correction for multiple tests Bonferroni procedure; $p < 0.01$)

amplifications were carried out as above. The 18 loci were coamplified and pooled before electrophoresis. Fragment lengths were read on a ABI3730 sequencer (Applied Biosystems, Courtaboeuf, France) and scored with GENEMAPPER version 4.0 software with the GeneScan^{TM}–600LIZ$^{®}$ (Applied Biosystems, Courtaboeuf, France) as an internal size standard.

In order to avoid sampling clones within populations, leaves were sampled from at least 5 m spaced individuals and GenAlex 6.5 was used to detect potential clones among samples (Peakall and Smouse 2006). None of the collected individuals appeared to be clonal. GENEPOP 4.0 (Raymond and Rousset 1995) was used to estimate observed (H_O) and expected heterozygosity (H_E), to determine departures from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD).

Mean allelic diversity varied from 2 to 7. The observed and expected heterozygosity ranged from 0.07 to 0.79 and from 0.05 to 0.85, respectively (Table 2). One locus (RF122P2) was monomorphic in the Alps population and three loci (RF122P2, RF126P1 and RF182) were monomorphic in Léca (Pyrenees). Four loci (RF113P2, RF163P1, RF128 and RF96P2) deviated significantly from HWE in maximum two populations. Significant LD was discovered in one loci pair (RF157P1-RF140).

Cross-species amplification was successful for 22 other *Rhododendron* species (Appendix). The 18 microsatellite markers were also tested in four Ericaceae species: *Vaccinum myrtillus, Arctostaphylos uva-ursi, Arbutus unedo* and *Calluna vulgaris*. Five markers amplified 1–3 of these species.

The microsatellite markers described here will be useful for investigating the importance of genetic structure in *Rhododendron* species. Such markers will provide valuable information concerning the genetic diversity at margins but also the genetic structure of this and related species, facilitating development of conservation measures in *Rhododendron* species.

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Appendix

See Table 3.

Table 3 Cross species amplification tests for 18 *Rhododendron ferrugineum* microsatellite loci in 22 *Rhododendron* species and 4 species from the Ericaceae family

	RF96P2	RF85	RF153	RF140	RF175	RF213	RF163P1	RF122P2	RF113P2	RF202	RF128	RF157P1	RF105P2	RF87P2	RF182	RF146P1	RF114P3	RF126P1
R. brachycarpum	−	−	−	−	+	−	+	+	+	+	−	−	−	+	+	−	−	+
R. camtchaticum	−	−	−	−	−	+	+	−	+	+	−	+	−	−	−	−	+	−
R. canadense	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+
R. commonae	+	−	−	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+
R. edgeworthii	−	+	−	+	+	−	+	+	+	+	+	+	−	+	+	−	−	+
R. ericoides	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
R. herzogii	+	+	−	−	+	−	+	+	+	+	−	+	−	+	−	−	−	+
R. hirsutum	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
R. hyperythrum	+	+	−	−	−	+	+	+	+	+	−	+	−	−	−	+	+	+
R. lagunculicarpum	+	+	−	+	+	−	+	+	+	+	+	+	−	+	−	−	−	+
R. luteum	+	+	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	+
R. macrophyllum	+	+	−	−	+	−	+	+	+	+	−	−	−	+	−	−	+	+
R. mariesii	−	+	−	−	+	+	+	+	+	+	+	−	−	+	−	−	−	+
R. molle	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+
R. occidentale	+	+	−	−	+	+	+	+	+	−	+	−	−	+	−	+	+	+
R. ponticum beaticum	+	+	−	−	+	+	+	+	+	+	−	−	−	+	+	−	+	+
R. quadrasianum	+	+	−	+	−	+	+	+	+	+	+	+	−	+	+	−	−	+
R. rarum	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+
R. saxifragoides	+	+	−	+	+	−	−	−	−	+	+	+	−	+	+	−	+	+
R. schlippenbachii	−	+	−	+	+	+	+	−	+	+	+	−	−	−	−	−	−	+
R. vaseyi	+	+	−	−	−	+	+	−	+	+	+	−	−	+	−	−	−	+
R. wardii	−	−	−	−	+	+	+	−	+	+	−	−	−	+	−	−	+	+
Vaccinum myrtillus	−	−	−	−	+	−	−	−	+	−	−	−	+	−	−	+	−	−
Arctostaphylos uva-ursi	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Arbutus unedo	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
Calluna vulgaris	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−

Amplification success is indicated by + and failure by −

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Microsatellite Marker Analysis Reveals the Complex Phylogeographic History of *Rhododendron ferrugineum* (Ericaceae) in the Pyrenees

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Abstract

Genetic variation within plant species is determined by a number of factors such as reproductive mode, breeding system, life history traits and climatic events. In alpine regions, plants experience heterogenic abiotic conditions that influence the population's genetic structure. The aim of this study was to investigate the genetic structure and phylogeographic history of the subalpine shrub Rhododendron ferrugineum across the Pyrenees and the links between the populations in the Pyrenees, the Alps and Jura Mountains. We used 27 microsatellite markers to genotype 645 samples from 29 Pyrenean populations, three from the Alps and one from the Jura Mountains. These data were used to estimate population genetics statistics such as allelic richness, observed heterozygosity, expected heterozygosity, fixation index, inbreeding coefficient and number of migrants. Genetic diversity was found to be higher in the Alps than in the Pyrenees suggesting colonization waves from the Alps to the Pyrenees. Two separate genetic lineages were found in both the Alps and Pyrenees, with a substructure of five genetic clusters in the Pyrenees where a loss of genetic diversity was noted. The strong differentiation among clusters is maintained by low gene flow across populations. Moreover, some populations showed higher genetic diversity than others and presented rare alleles that may indicate the presence of alpine refugia. Two lineages of R. ferrugineum have colonized the Pyrenees from the Alps. Then, during glaciation events R. ferrugineum survived in the Pyrenees in different refugia such as lowland refugia at the eastern part of the chain and nunataks at high elevations leading to a clustered genetic pattern.

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Introduction

The maintenance of genetic diversity within populations is essential for the conservation of species since it can enable adaptation to future environmental changes [1], [2]. With high genetic diversity, it is more likely that some individuals will possess alleles that could allow the population to adapt to a new habitat [3]. High genetic diversity may be maintained via gene flow among individuals and among populations. In recent years, many studies have shown strong impacts of global change on species' genetic diversity [4]. Because climate warming is greater at high than at low elevations [5] alpine biodiversity hotspots that are home to many endemic and rare species may be particularly threatened by climate change [6]. Thus, to predict how these biodiversity hotspots will deal with climate changes, it is important to understand the genetic patterns of the species and their changes over time [7].

Genetic structure and genetic divergence within and among plant populations result from a number of contemporary and historical factors acting at various temporal and spatial scales. Alpine habitats are typically characterized by small spatial changes in environmental variables such as topography, temperature, snow cover, soil moisture or bedrock [8] favorable to phenotypic differentiation and local genetic variability over short geographical distances. In addition to abiotic factors, life history traits can also influence genetic features of populations. For instance, selfing perennials and clonal species generally exhibit lower levels of genetic diversity and higher levels of population differentiation than outcrossing perennials [9]. In contrast, mixed mating systems maintain a high level of genetic diversity and may be advantageous for plants in variable alpine environments [6]. The combined effects of both life history traits and small-scale abiotic heterogeneity may result in local genetic adaptations and high genetic differentiation of populations, notably where gene exchanges between populations are restricted as is often the case in mountain habitats [10]. Therefore, plant-community fragmentation pattern, steep climatic gradients leading to asynchronous flowering phenology or disjoint pollinator communities [7], and topographical barriers may hamper gene flow (through seed and pollen movements) and may increase differentiation between populations.

At large scales, species genetic patterns are often shaped by past climate-driven range dynamics [11]. In temperate regions, species ranges have changed dramatically over the Pleistocene with its successions of glaciations and warming periods. In mountains, periodic advances and retreats of glaciers have, on several occasions, forced species to descend to lowland and peripheral refugia and/or to survive in isolated ice-free areas above glaciers (nunataks). Genetic isolation, bottlenecks and potential allopatric evolution of populations in refugia have been followed by repeated founding events during population expansions, which have led to loss of genetic diversity and heterozygosity [11]. Additionally, populations located at eroding margins of species distribution ranges following climate warming could have persisted in suitable isolated lowland habitats [12]. It has been suggested [13–14] that such small populations isolated for so long could display low within-population genetic diversity (as a result of genetic drift) but have disproportionally high levels of between-population differentiation and regional genetic diversity and distinctiveness [15].

The genetic pattern and phylogeography of many species [16], [17] have been investigated in various mountainous regions, but to date, only a very limited number of studies have focused on the Pyrenees [18–20]. The Pyrenees are a mountain range stretching across the isthmus that lies between the Iberian Peninsula and the rest of the European continent. Acting as a biogeographical barrier during postglacial expansions, they are a limit for numerous Iberian and northern species. However, lower lands at the eastern and western extremities of the mountain range have been corridors for migration of several plant and animal species [21,22]. Due to oceanic influences to the west and Mediterranean influences to the east of the chain, the climate varies greatly along a longitudinal gradient in the Pyrenees (Météo France data). This bioclimatic pattern potentially allowed some species to survive glaciations in various refugia along the chain. Studies have suggested that species have survived glaciations [23–25] in two main refugia - one in south central and another in the eastern Pyrenees - thanks to mild Mediterranean and oceanic climatic influences. However both geological and biological data [26] demonstrate that during glacial periods the ice sheet did not completely cover the highlands and rarely descended below 1000 m a.s.l except for big glaciers pushed down into large valleys and to the plains [23]. These nunataks and unglaciated peripheral area could have provided refugia for some species [18] and determined several recolonizing routes sculpturing a specific and complex genetic structure in the Pyrenees.

Here we investigate the genetic structure and phylogeographical history of the alpine shrub *Rhododendron ferrugineum* (Ericaceae) in the Pyrenees. This species is insect pollinated and reproduces both sexually (mixed mating system) and vegetatively through layering. It is widely distributed at high elevation, on non-calcareous bedrock. The aim of the present study is to understand to what extent the phylogeographical history of the species has shaped its present genetic patterns. The Asian origin of the genus *Rhododendron* [27] implies an east/west migration from Asia to Europe. In France, this would lead to the Alps being colonized by *R. ferrugineum* before the Pyrenees, with the Pyrenean populations most likely derived from populations from the Alps. After the migration to the Pyrenees, the species had to survive successive glaciation events. Different hypotheses of glacial refugia and postglacial colonization and their consequences on the genetic patterns of *R. ferrugineum* may be proposed: 1. The species could have survived glaciation episodes in one or both of the two main refugia at the eastern and south central part of the Pyrenees (HYP 1; [24]). Thus, the highest genetic diversity should be found in one or both of these areas and the genetic pattern would be shaped by founder effects i.e. gradual reduction of genomic variability along the colonizing routes from main refugia. This would have occurred because colonization usually involves only a fraction of the genetic diversity present in refugial areas [28]. 2. The species could also have survived in nunataks along the mountain chain (HYP 2). Hypothetically, high elevation nunatak populations would have experienced long periods of isolation, inbreeding and genetic drift [18]. This pattern would lead to a patchy distribution of genetic clusters with low levels of genetic diversity and high population differentiation; 3. A pattern with less differentiated clusters than in the previous hypothesis may be observed if the species had survived in large lowland refugia in which extensive gene flow had occurred. The recolonization of highland areas from lowland refugia could have left small, isolated and marginal rear-edge populations like those currently observed at low altitude along the chain (HYP 3). Indeed, the strong abiotic mountain heterogeneity may have allowed remnant populations to match suitable ecological conditions at intermediate or low altitudes. Moreover, the long life span of the clones [29,30] could have permitted their survival even with low seedling recruitment. Such rear-edge populations should exhibit more rare alleles and higher among-population genetic diversity than populations recently established from high altitude source populations [15]. These three hypotheses are not exclusive and it is conceivable that the genetic pattern observed may result from the different mechanisms at work successively or simultaneously within the bioclimatic regions of the Pyrenees inducing diverse phylogenetic patterns along the chain.

Materials and Methods

Species studied

Sampling of the species of interest was conducted in accordance to national and international guidelines (authorization from the ONF for the populations BetL, BetH, LecL, PraL, PuiH, from the Parc National des Pyrénées for the population TroH, from the Conseil général des Pyrénées-Orientales for population BouH, and no permissions were needed for the other sites).

Rhododendron ferrugineum L. (Ericaceae) is an evergreen shrub with a mean height of 70 cm that dominates subalpine landscapes in the Alps and Pyrenees between 1500 to 2200 m a.s.l, on north-to-west facing slopes, sometimes reaching 90–100% of the vegetation cover. It is also found in the Jura Mountains, in the Northern Apennines and in the Apuan Alps [31]. In the Pyrenees, infrequent smaller and isolated populations can be found lower down, between 900–1500 m a.s.l. A single population of some scattered individuals remains at low altitude (1500 m a.s.l.) in the French Jura Mountains. *Rhododendron ferrugineum* is a clonal, self-compatible [32] mass-flowering species (producing until 3000 flowers per m^2; [33]) pollinated by honeybees and bumblebees [32,34].

Study sites and sample collection

We sampled 29 populations across the Pyrenees, the population in the Jura Mountains and three populations in the Alps (Figure 1, Table 1) ranging from 1070 m to 2200 m a.s.l. The populations were divided into three groups according to their elevation: low altitude (hereafter L; from 1000 to 1450 m a.s.l), intermediate altitude (hereafter I; from 1450 to 1900 m a.s.l.), high altitude (hereafter H; from 1900 to 2200 m a.s.l.). In the Pyrenees, populations were sampled along a longitudinal transect through three regions: Languedoc-Roussillon (eastern part), Midi-Pyrénées (central part) and Aquitaine (western part). Due to calcareous bedrock, populations in the western part of the Pyrenees are rare and we only sampled one. An estimation of population size is given in Table 1 [<1 ha, 1–10 ha, 10–20 ha, 20–50 ha, 50–100 ha, >100 ha). The mean geographical distance between populations from Pyrenees *vs.* Alps, Pyrenees *vs* Jura Mountains and Alps *vs.* Jura Mountains was 900 km (ranging from 515 to 1430 km), 695 km (from 590 to 830 km) and 335 km (from 165 to 640 km) respectively. The distances between populations across the

Pyrenees ranged from 3 to 370 km with an average of 126 km. In each of the 33 populations (except for the very small JuraL and LapL populations), young leaves of twenty individuals were sampled (a total of 645 individuals sampled), separated by at least 5 m in order to avoid sampling clonal individuals, were dried in silica gel in the field and then stored at −20°C.

DNA extraction and microsatellite markers

For each individual sampled, 20 mg of leaf tissue was ground with two tungsten beads (diameter 3 mm) in a sterile 2 ml Eppendorf tube at 30 Hz for 90 s using TissueLyser II (Qiagen). Then, the quality and amount of DNA obtained from the DNeasy-Plant Mini Kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions was verified using a Nanodrop ND 1000 spectrophotometer (Peqlab, Erlangen, Germany).

We used nuclear microsatellites as suitable molecular markers to reconstruct regional phylogeographical patterns [18]. Chloroplasts (personal observation) or other types of genetic markers such as AFLP proved to be little informative in this species [35]. The 27 polymorphic microsatellite markers (Table S1) were previously developed [36,37] using pyrosequencing technologies (454 FLX Titanium, Roche Applied Science, Meylan, France). Forward primers were labeled with fluorochromes (Eurofins MGW Operon, Courtaboeuf, France) and were used in six multiplexed PCRs, optimized using Multiplex Manager 1.1 [38].

Amplifications were carried out in a 10 µl mix containing 10 ng of template DNA, 0.7 × Qiagen Multiplex PCR Master Mix, 10 µM of each primer and RNase free water. Cycling conditions were 15 min at 95°C, 30 × (30 s at 95°C, 90 s at 56°C, 45 s at 72°C) and 30 min at 60°C. Each set of reactions included a negative (water) and a positive (known genotype) control. Fragment lengths were read on an ABI 3730 sequencer (Applied Biosystems, Courtaboeuf, France) and scored with Genemapper version 4.0 software with the GeneScan – 600 LIZ (Applied Biosystems, Courtaboeuf, France) as internal size standard.

Data analysis

Genetic diversity. All 645 individuals were tested for clonality with GenAlEx 6.5 software [39]. The presence of null alleles at each locus was examined using Micro-Checker software [40], and tests for departures from Hardy-Weinberg equilibrium (HWE) were tested by Fisher's exact tests using Genepop 3.4 [41]. We used GenAlEx 6.5 software to calculate for each locus and population the number of alleles, the allelic richness (Ar), the observed (H_O) and expected heterozygosity (H_E) and the inbreeding coefficient (F_{IS}).

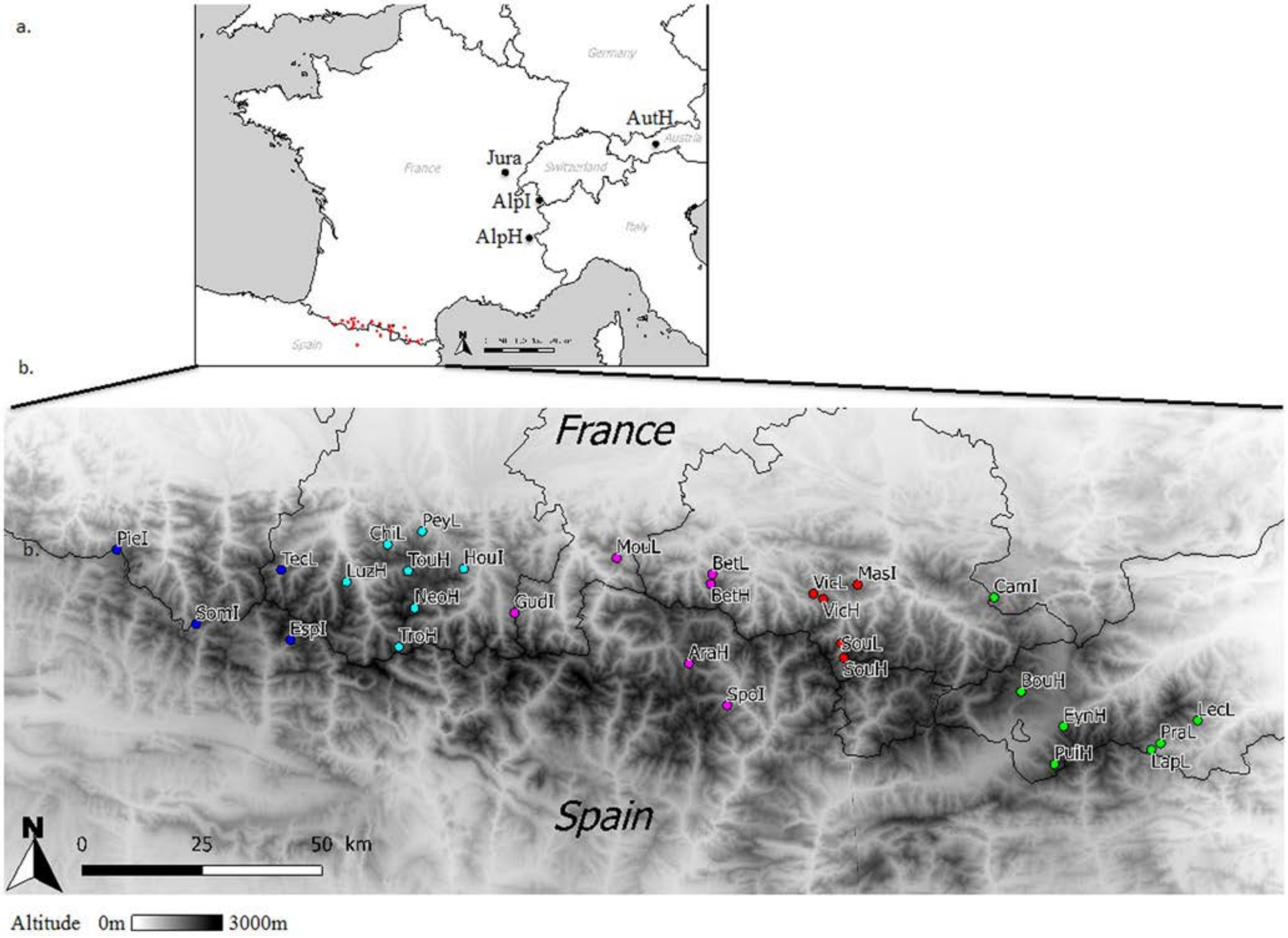

Figure 1. Location of the sampled populations. a. Location of the sampled *Rhododendron ferrugineum* populations in the Alps, Jura Mountains and Pyrenees, b. detailed map of the Pyrenean populations (the colors correspond to the five genetic clusters).
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Table 1. Geographical information and genetic diversity of *Rhododendron ferrugineum* populations.

Moutain range	Sites	Code population	Location	Population size (ha)	Altitude (m)	n	Ar	Private allele	H_O	H_E	F_{IS}
Alps	La Vormaine	AlpI	45°02′07″N 06°24′19″E	>100	1560	20	3.70	1	0.49	0.49	**0.04**
	Col du Lautaret	AlpH	46°00′08″N 06°57′18″E	5–10	2100	20	4.30	10	0.59	0.57	**−0.01**
	Parscherkofel	AutH	47°12′32″N 11°27′18″E	50–100	2000	20	2.89	2	0.41	0.39	**−0.03**
Jura		JuraL	42°57′N 05°44′E	<1	1300	10	2.67	0	0.48	0.44	**−0.06**
Eastern Pyrenees	Léca	LecL	42°28′07″N 02°31′40″E	<1	1250	20	2.37	1	0.34	0.30	−0.11
	Prats de Mollo	PraL	42°25′21″N 02°24′38″E	<1	1450	20	2.44	1	0.31	0.32	**0.02**
	Lapreste	LapL	42°24′34″N 02°22′51″E	<1	1230	15	2.44	0	0.42	0.34	−0.21
	Eyne	EynH	42°28′27″N 02°07′05″E	5–10	1915	20	2.78	0	0.38	0.35	**−0.06**
	Puigmal	PuiH	42°23′26″N 2°05′0″E	5–10	2045	20	2.74	0	0.39	0.34	−0.10
	Lac des Bouillouses	BouH	42°33′31″N 01°59′42″E	50–100	2005	20	2.56	0	0.37	0.34	**−0.05**
Central Pyrenees	Camurac	CamI	42°46′30″N 01°55′45″E	10–20	1600	20	2.92	0	0.42	0.38	−0.09
	Massat	MasI	42°49′18″N 01°30′75″E	<1	1500	20	2.59	0	0.41	0.40	**0.0007**
	Soulcem	SouH	42°39′28″N 01°27′27″E	50–100	2035	20	2.63	0	0.41	0.39	**−0.01**
	Soulcem	SouL	42°41′28″N 01°27′07″E	<1	1240	20	2.74	0	0.37	0.39	0.08
	Etang de Lhers	VicH	42°47′36″N 01°24′16″E	20–50	1940	20	2.74	0	0.38	0.35	−0.07
	Etang de Lhers	VicL	42°48′23″N 01°22′31″E	1–5	1275	20	2.44	0	0.36	0.32	−0.09
	Espot	SpoI	42°33′47″N 01°05′36″E	<1	1515	20	4.18	0	0.35	0.43	0.10
	Bethmale	BetL	42°51′42″N 01°04′02″E	1–5	1385	20	2.67	0	0.40	0.37	**−0.06**
	Bethmale	BetH	42°50′24″N 01°03′42″E	50–100	1920	20	2.92	0	0.40	0.37	**−0.03**
	Val d'Aran	AraH	42°39′45″N 00°58′56″E	<1	2075	20	2.26	0	0.29	0.29	**0.05**
	Le Mourtis	MouL	42°54′32″N 00°46′31″E	5–10	1440	20	2.59	0	0.33	0.33	**0.04**
	Peyragudes	GudI	42°47′42″N 00°27′14″E	5–10	1650	20	2.67	0	0.39	0.37	**−0.03**
	Hourquette d'ancizan	HouI	42°53′57″N 00°18′13″E	5–10	1565	20	2.74	0	0.42	0.40	**−0.04**
	Néouvielle	NeoH	42°48′N 00°09′E	50–100	2200	20	2.56	0	0.47	0.44	**−0.03**
	Le Peyras	PeyL	42°59′15″N 00°10′46″E	5–10	1220	20	2.33	0	0.36	0.36	**0.03**
	Col du Tourmalet	TouH	42°53′57″N 00°07′54″E	5–10	1950	20	2.81	1	0.42	0.39	**−0.04**
	Cirque de Troumouse	TroH	42°43′41″N 00°05′40″E	5–10	2080	20	2.44	0	0.39	0.38	**0.02**
	Chiroulet	ChiL	42°57′38″N 00°04′17″E	1–5	1240	20	2.70	0	0.38	0.37	**0.02**
	Luz-Ardiden	LuzH	42°52′45″N 00°03′34″W	10–20	1905	20	2.63	0	0.37	0.37	**0.02**
	Huesca	EspI	42°45′08″N 00°14′18″W	>100	1640	20	2.27	0	0.20	0.30	0.32
	Lac du Tech	TecL	42°54′40″N 00°15′32″W	<1	1240	20	2.41	0	0.39	0.34	−0.11
	Col du Somport	SomI	42°47′41″N 00°31′38″W	<1	1645	20	2.26	0	0.33	0.37	0.16
Western Pyrenees	Col de la Pierre Saint Martin	PieI	42°58′07″N 00°45′52″W	<1	1830	20	2.00	0	0.28	0.29	**0.09**

Altitudinal classes are given in the code population (L: low, I: intermediate, H: high altitude).
n = sample size, Ar = allelic richness, H_O = observed heterozigosity, H_E = expected heterozygosity, F_{IS} = within population coefficient of inbreeding (non-significant values are in bold, $P<0.01$).
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Population structure and differentiation. The pairwise F_{ST} (level of genetic differentiation; [42]) among populations were calculated with Genodive v2.0 [43]. Then, the statistical significance of the pairwise F_{ST} values was assessed by 1000 random permutations of individuals across populations with Genetix 4.05 [44].

To analyze the genetic structure among populations, we used Structure software (ver. 2.3.3; [45]) in order to determine the most likely number of homogenous clusters in the sample (K). The admixture model was applied with 50 runs for each K value from 1 to "number of populations +1", each run comprising a burn-in period of 20 000 iterations followed by 100 000 iterations. The optimal value of *K* was evaluated by considering the highest mean likelihood value of *K*, *i.e.*, L (*K*), as well as the ΔK method [46]. These results were computed and visualized in the online interface Structure Harvester [47].

To evaluate the distribution of genetic variance within and among populations, and among groups when populations were nested into genetic clusters, altitude (low, intermediate and high altitude) or population size (according to the six population size groups in Table 1) we performed analyses of molecular variance (AMOVA; [48]) using Genodive v2.0. The significance of the results was tested using 999 random permutations of the data.

Mantel tests [49] were performed (''ncf'' package implemented in R, R Development Core Team, 2008) to test for isolation-by-distance (IBD; divergence due to drift and mutation proportionally increasing with geographical distance), investigating the correlation between matrices of genetic (F_{ST}) and geographical (km) distances. Mantel test were also used to determine wheter elevational separation was related to genetic differentiation. Calculation of the gene flow on the basis of rare alleles was conducted [50] and the number of migrants (N_m) was corrected for the number of samples.

Results

A total of 153 alleles at 27 microsatellite loci were detected across 645 individuals at all sites (Table S2). No clones were identified among the individuals sampled as revealed by the test of clonality.

Significant departures from HWE across loci were detected in 12 out of 33 (or 36%) sites. The RF87P2 marker was the only locus that showed no departures from HWE. Most of the loci showed significant departures from HWE in two to seven populations, except RF14P3, RF47P1 (11 populations each), RF56P1 (13 populations) and RF96P2 (31 populations). These instances likely reflect occasional departures from random mating rather than the presence of null alleles. Indeed, the probability of null alleles was low for all loci, according to Oosterhout's method [40], mean estimation of null alleles was −0.031 (ranging from −0.3675 to 0.1352). No differences in the results were observed when including the microsatellite marker RF96P2 or not, so we decided to keep it in the analyses.

Genetic diversity and allelic richness among populations

The number of alleles ranged from 2 to 15 (mean 6.25) alleles per locus. A total of 16 private alleles (restricted to a single population; Table 1) was detected (10.45%), mostly in AlpH (10), AutH (2), AlpI (1) and also in some Pyrenean populations (TouH, PraL, LapL had one private allele each). Allelic richness (Ar) varied across sites (2–4.30). The highest Ar were observed in the Alps (AlpI = 3.70 and AlpH = 4.30) and in one of the southernmost populations of the central Pyrenees (SpoI = 4.18), while the lowest Ar were observed at the western end of the Pyrenees (PieI = 2.00, SomI = 2.26, EspI = 2.27) and in the central Pyrenees (AraH = 2.26). Ar, H_O and H_E were 4.6, 0.37 and 0.35 respectively in the Pyrenees, and 5.2, 0.5 and 0.45 in the Alps. Expected (H_E) and observed heterozygosity (H_O) ranged from 0.20 to 0.59 and from 0.29 to 0.56 respectively for all sites. H_E was high in the Alps and Jura Mountains (ranging from 0.39 to 0.57), and in some Pyrenean populations (SpoI and NeoH, 0.43 and 0.44 respectively) and the lowest in the easternmost (LecL, PraL, AraH) and westernmost (EspI, PieI) Pyrenean populations. These latter populations were small and isolated (<1 ha) except the large and central population EspI (>100 ha). Significant heterozygosity excesses (F_{IS} coefficients ranging from −0.07 to −0.21) were detected in three eastern (LecL, LapL, PuiH) and four central populations of the Pyrenees (CamI, VicH, VicL, TecL). In contrast, significant heterozygosity deficiency was found in four populations with particularly high values of F_{IS} coefficients in SomI (0.16) and EspI (0.31). Because the frequencies of null alleles did not appear to be significant, the highest F_{IS} values could be due to inbreeding.

Genetic differentiation and structuration among populations

The F_{ST} values (Table S3) were significantly different from zero for all population pairs, except for three pairwise comparisons: BetH/BetL and SouH/SouL that were geographically really close (less than 3 km) and CamI/EynH (35 km apart). The mean F_{ST} value was relatively low (0.267) and most of the highest values (>0.33) were found in the Pyrenees between eastern and western populations, reaching 0.45 for LecL/AraH. The population from the Jura Mountains showed a highly variable level of F_{ST} (from 0.25 to 0.42).

To infer the relationship between populations we used the clustering algorithm implemented in the program Structure. Comparing all 33 populations (K: 1 to 34), ΔK as a function of K reached a peak at K = 2 and then reached a second peak at K = 6 (Figure 2a). At K = 2, populations from the Alps, Jura and the eastern Pyrenees were assigned to the same cluster while populations from western and central Pyrenees were clearly grouped apart. Individuals from AlpI showed a similar proportion of membership to both genetic clusters. At K = 6, populations from the Alps and Jura were assigned to one cluster (CL1) and those of the Pyrenees were grouped in 5 clusters (CL2 to CL6) along a longitudinal gradient. Except between CL5 and CL4 or CL6, there was low admixture between the other Pyrenean clusters.

The analysis of molecular variance (AMOVA; Table 2) based on F_{ST} showed that as expected for polymorphic loci such as microsatellites, the within-population variance component was rather high (>66%, P = 0.001). The among variation component (F_{ST}) ranged from 9% to 31% according to the grouping and was statistically significant in all combinations tested (P = 0.001). At a superior hierarchical level, the genetic clusters could explain a significant part of the total genetic variance (24.4%, P = 0.001) whereas it was not significant when grouping populations into altitudinal groups (P = 0.956) or population size (P = 0.222).

Allelic richness (Ar) was highest in CL1 (Alps and Jura, 5.2) and lowest in CL6 (western Pyrenees, 2.9) and ranged from 3.2 to 3.8 in the other clusters (Table 3). F_{ST} values were all significantly different among clusters (Table 4). The highest F_{ST} values were found between CL2 (eastern Pyrenees) and CL5 or CL6 (western Pyrenees, 0.29 and 0.31 respectively). The F_{ST} values between CL1 and the Pyrenean clusters were quite low (ranging from 0.16 to 0.20).

There was a positive relationship (Mantel test) between genetic distance and geographical distance matrices across populations located in the Pyrenees (R = 0.784, P = 0.001). The increase of isolation with geographical distance was consistent with the previous results (see Structure analyses). When including populations from the Alps and Jura, the Mantel test was still positive (R = 0.26) but not significant (P = 0.383), confirming the lack of strong differentiation among the populations from the Alps and Pyrenees. At the Pyrenean scale, the correlation among elevational distances and FST was slightly positive but not significant (R = 0.092, P = 0.06).

Gene flow among populations

Gene flow among all studied populations (Alps, Pyrenees and Jura) was low and reached a mean value of Nm = 0.72 migrants per generation. The values reached 2.88 for all Pyrenean populations but 2.10 in the eastern lineage (CL2) and 5.9 in the western lineage (CL3 to CL6).

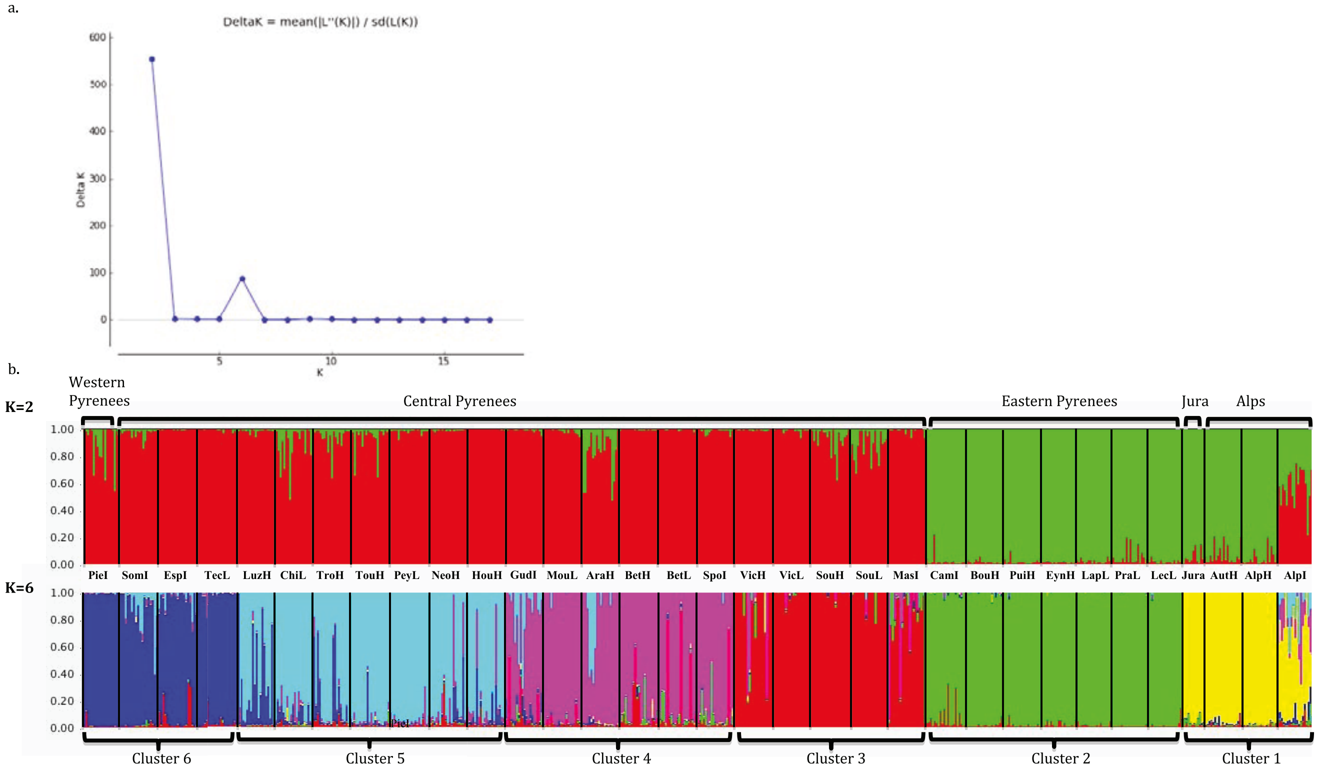

Figure 2. Genetic structure of the 33 populations. a. Plot of Delta K according to K. b. STRUCTURE clustering results obtained at K = 2 and K = 6. Each individual is represented by a thin bar corresponding to the sum of assignment probabilities to the K cluster. Black bars separate populations.
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Discussion

Genetic diversity in R. ferrugineum populations

Microsatellite analysis revealed a relatively high level of genetic variability in *R. ferrugineum* populations in the Pyrenees (Ar: 2–4.18, H_E: 0.29–0.44) but lower than in the Alps and the Apennine populations (Ar: 2.71–5.57, H_E: 0.37–0.68; [31]) or in other long-lived species found in the Pyrenees (*Pinus uncinata*: H_E: 0.83–0.99, (25); *Quercus petraea*: H_E: 0.76–0.84, [51]). Although clonality and selfing are known to decrease genetic variability [52], the long life span of individuals [29,30] and the strong life-time inbreeding depression eliminating most selfed individuals (personal data) may contribute to maintaining relatively high genetic diversity even in small *R. ferrugineum* populations. Accordingly, most of the populations studied showed a F_{IS} close to zero and a great proportion of the genetic variation was found within populations as observed in other *R. ferrugineum* populations [31] and other alpine species [19], [53], [18]. However, selfing has probably contributed to individual recruitment in populations harboring higher heterozygote deficiency (SomI, PieI, EspI, SpoI). Such high heterozygote deficiency has also been reported in the Apennines and the Alps [31]. Surprisingly, significant heterozygote excess

Table 2. Partitioning of the observed microsatellite variation (AMOVA) based on F_{ST} of *Rhododendron ferrugineum* populations.

Source of Variation	SSD	d.f.	MS	Var-comp	% Variation	P-value
Within Population	4355.120	548	7.947	7.947	0.662	0.001
Among Population	732.618	24	30.526	1.135	0.095	0.001
Among Genetic cluster	1461.242	4	365.310	2.929	0.244	0.001
Within Population	4354.038	548	7.945	7.945	0.690	0.001
Among Population	2103.864	26	80.918	3.659	0.310	0.001
Among Altitude	93.988	2	46.994	−0.167	0.00	0.956
Within Population	4366.874	548	7.894	7.894	0.685	0.001
Among Population	2079.120	23	83.837	3.372	0.293	0.001
Among Population size	106.877	5	56.922	0.258	0.022	0.222

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Table 3. Genetic diversity of *Rhododendron ferrugineum* per genetic cluster.

	Ar	H_O	H_E	F_{IS}
Cluster 1	5.630	0.498	0.584	0.148
Cluster 2	3.630	0.375	0.364	−0.030
Cluster 3	3.185	0.386	0.406	0.048
Cluster 4	3.778	0.360	0.390	0.078
Cluster 5	3.481	0.402	0.429	0.062
Cluster 6	2.926	0.315	0.384	0.179

Ar = allelic richness, H_O = observed heterozigosity, H_E = expected heterozygosity, F_{IS} = within population coefficient of inbreeding.
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Table 4. F_{ST} values among genetic clusters (p-values for all pairs<0.001).

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Cluster 1	—	0.203	0.171	0.177	0.165	0.166
Cluster 2		—	0.237	0.275	0.297	0.311
Cluster 3			—	0.101	0.153	0.230
Cluster 4				—	0.108	0.235
Cluster 5					—	0.129
Cluster 6						—

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occurred mostly in eastern (LapL, LecL, PuiH), and central populations (CamI, VicH, VicL) and in one of the westernmost populations (TecL).

Alpine origin of the two Pyrenean *R. ferrugineum* lineages

The *Rhododendron* genus is known to originate from Asia [27] and colonized Europe from east to west. Our hypothesis was that migration from the Alps to the Pyrenees occurred with a loss of genetic diversity along the colonization routes. In our study, we identified two lineages in the Alps: the first, found only in the northern population (AlpI), was related to western Pyrenean populations. This lineage would possibly be found in more populations with more extensive sampling in the Alps. The second lineage, related to the eastern and central Pyrenees, was found in the same population AlpI but also in south and central-eastern populations from the Alps and in the Jura Mountains. At K = 6, populations from the Jura Mountains and the Alps are found in the same cluster, that may indicate the occurrence of recent gene flow between the Alps and the Jura Mountains, possibly in low land refugia during glaciations. It would be interesting to complete our data by a large sampling of populations from the Alps and the Jura Mountains in order to detail the European phylogenetic pattern of the species. Compared to the Alpine populations, Pyrenean populations were characterized by a substantial loss of genetic diversity (Ar, H_E, H_O) suggesting bottlenecks during the migration events [54], [16]. Cold, dry steppe/tundra occupied most of the land between the Alps and the Pyrenees during the last maximum glaciation [55] and could have allowed migration of Alpine species such as *R. ferrugineum* either from the southwestern Alps [56], [57] or from northern Alpine populations through the French Massif Central which is considered to be a key area in the colonization of the Pyrenees by Alpine species [58].

Genetic pattern and phylogeographical history of *R. ferrugineum* in the Pyrenees

We found that genetic diversity of *R. ferrugineum* was not correlated to altitude or population size; instead there was a sharp longitudinal split between the two genetic lineages. Other studies in alpine ecosystems similarly found no changes in genetic diversity depending on population size or along altitudinal gradients [59]. Along environmental gradients, several parameters such as overlap of generations or recruitment frequency may influence intrapopulation genetic variation and gene flow among populations may be sufficient to maintain relatively high genetic diversity even in the smallest populations.

The longitudinal genetic pattern seemed to result from the migration of two Alpine lineages and from the establishment of populations in refugia along the Pyrenean chain due to glaciation events. The strong differentiation between the eastern and western Pyrenean lineages and the low level of gene flow observed between the two suggested that very few admixtures occurred since the two lineages had become established. This may result from topographical barriers (high summits, glaciated areas), seed dispersion by gravity and short pollinator travels hampering gene flow among populations and genetic homogenization. An east-west genetic separation had also been observed in other Pyrenean species (i.e. *Papaver alpinum,* [57], *Trifolium alpinum*, [19]). The populations of the eastern lineage were little differentiated even though they were distant from one another (35 km between the two undifferentiated populations CamI and EynH), were of very different sizes, grew in contrasting environments, and experienced a low level of among-population current gene flow. Very small and isolated populations (PraL, LecL, LapL) showed a relatively high level of intrapopulation heterozygosity (H_E, H_O) and exhibited private alleles (PraL, LapL) despite relatively lower allelic richness likely due to the loss of rare alleles by genetic drift, common in such small populations [60]. These populations survive in rather unusual ecological conditions for this species, i.e. on north facing slopes in deep, shaded valleys and mainly beneath beech forest (*Fagus sylvatica*) or sometimes in the vicinity of Mediterranean *Quercus ilex* forests. These data suggested that they were more likely to be rear-edge remnant populations [12] left there after the recolonization of highland areas (HYP 3) rather than the result of a secondary colonization of lowland areas by high altitude individuals. Together, these findings and the absence of clear founder effects suggested that the eastern populations within CL2 have experienced recent and extensive gene flow. Such intense gene exchanges could have occurred in large lowland refugia during the cold periods of the Pleistocene and possibly later in genetically connected populations growing over high altitude plateaus, which are typical of eastern Pyrenees landscapes. These large high altitude populations could have then become fragmented possibly during the last 30 ky when climate became warmer and drier [26].

The four well separated and differentiated genetic clusters nested in the western Pyrenean lineage appeared as a sub-structure of the east/west configuration and presumably diverged more recently. Overall, we did not observe a gradient of genetic variability loss among these clusters as expected from a range-expansion wave [11]. Rather, we observed a consistent genetic heterozygosity and allelic richness and a significant isolation by distance (Mantel test) among them. Wright's isolation by distance model suggests that gene flow is reduced as geographical distance increases. This may explain the very low level of admixture between CL3/CL6. The patchy distribution of often homogenous and highly distinct clusters enclosing a similar level of within- and between-population genomic variability is compatible with the hypothesis of the survival of the species in numerous Pyrenean refugia as proposed for other Pyrenean species [19],[18].

Moreover, the level of differentiation, the sharp genetic boundaries between most clusters and the number of migrants per generation suggested a long isolation of populations and the action of genetic drift in several high altitude refugia (HYP 2) rather than the survival in large and genetically connected populations in low elevation unglaciated areas. The higher genetic diversity in SpoI likely accounts for such refugia as well as TouH (having one private allele) where genetic variability was greater than in other populations. However, the relatively higher level of admixture between CL5 and CL4 or CL6 suggested that they had recently exchanged genes. Because the admixture concerned mainly isolated and sometimes very distant (AraH and CL5 populations) populations at high and intermediate altitude, contemporary gene exchanges among them through pollen or seed dispersal was very unlikely. We suspected that, at least in this part of the Pyrenees, longitudinal gene exchanges could have occurred possibly in lowland refugia across Pyrenean piedmonts before the post-glacial recolonization of high altitude area through valleys (HYP 1). Such a recolonizing pattern has been observed in sessile oak [51] and suggested for *Aster pyrenaeus* [20]. Thus, the phylogeographical pattern in central and western Pyrenees could result both from population isolation and differentiation in nunataks and gene exchange in some lowland refugia. Among clusters, CL6 showed the lowest genetic variability (EspI, SomI and PieI) and genetic diversity gradually decreasing in this cluster along the sequence TecL, SomI and PieI. Thus, the genetic pattern of CL6 could be chiefly related to founder events and the loss of genetic diversity and heterozygosity [11] during westward expansion of the species.

Implications for conservation

The longitudinal genetic structuration found in *R. ferrugineum* and particularly the east/west separation between the two main genetic lineages has already been observed in other studies [57], [19]. This suggests that many other alpine species may have been subject to similar events as *R. ferrugineum* and, nowadays, could present a comparable genetic pattern. We state that these data now should be usefully considered in management projects for Pyrenean species particularly those having an extended east/west distribution. According to the European Economic Community laws (92/43/EEC habitat directives, 1992) *R. ferrugineum* heathlands must be locally preserved due to their high ecological and patrimonial value. We think that, henceforth, in aim to preserve genetic diversity of the species, the genetic pattern here revealed should be taken into consideration to choose sites that have to be protected. The eastern lowland isolated populations (LecL, PraL, LapL) grow in very specific habitat and present rare alleles. According to the "rear edge population hypothesis", they harbor all together the bulk of species' genetic diversity [12]. Scientific programs have to be developed to follow the population dynamic and the genetic diversity evolution of these very original populations and take an inventory of other currently unknown rear-edge populations. Moreover, studying population dynamics at the edge of a species' range is crucial to understand the response of species to environmental changes and their susceptibility to extinction [31]. Also the populations TouH (presenting a rare allele) and SpoI (having a high allelic richness) should be considered as populations of interest for conservation. However, other parameters should be taken into account such as surrounding plant community or pollinator diversity. For example, it is known that the number of bumblebees species, which are one of the main pollinators of *R. ferrugineum*, is high in the eastern Pyrenees, especially close to population EynH [61]. Consequently, it could be an interesting population to monitor.

In conclusion, the Pyrenees are usually considered to only be a latitudinal barrier to northward or southward species expansions. In agreement with previous findings [18,19], our study of the subalpine shrub *R. ferrugineum* highlights a much more complex role of the Pyrenees in species genetic pattern and biogeography. The species showed two genetically isolated lineages, which have probably colonized the Pyrenees from the Alps and Jura Mountains. Our findings suggest that the eastern lineage most likely survived in large lowland refugia and that low-altitude small and isolated rear-edge populations have survived until now after the upward recolonization of highland areas agreeing with our HYP 3. Extensive gene flow may have occurred in lowland refugia as well as among populations growing in high altitude plateaus. The sub-structure of the central and western lineage suggests that the species mostly survived glaciations in isolated nunataks (HYP 2) but that some lowland refugia could also have played a role locally (HYP 1). Our study provides a useful genetic reference framework to select *R. ferrugineum* heathlands in management projects in the Pyrenees and points out priority populations for protection.

Supporting Information

Table S1 Characteristics of 27 polymorphic microsatellite markers in *Rhododendron ferrugineum*.
(DOCX)

Table S2 Genetic parameters for the 27 microsatellite markers (non-significant values are in bold, $P<0.01$).
(DOCX)

Table S3 Pairwise F_{ST} comparisons between all sampling sites above the diagonal as calculated by Genetix. Non-significant F_{ST} are in bold.
(DOCX)

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Author Contributions

Performed the experiments: OC PD AP NE. Analyzed the data: OC PD AP NE. Contributed reagents/materials/analysis tools: OC PD AP NE. Wrote the paper: OC PD AP NE.

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CHAPITRE 2

- **Response of selfing, reproductive assurance and inbreeding depression to ecological gradients in an alpine species across the Pyrenees.**

RESPONSE OF SELFING, REPRODUCTIVE ASSURANCE AND INBREEDING DEPRESSION TO ECOLOGICAL GRADIENTS IN AN ALPINE SPECIES ACROSS THE PYRENEES

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SUMMARY

Climate changes exacerbate threats to plant-pollinator interactions and may have cascading effects on plant population persistence. In the absence of pollinators and/or mates, selfing may increase reproductive success and provide reproductive assurance (RA).

We investigated the potential for selfing to provide RA over the lifetime of a long-lived species and the relationships between selfing rate, RA and lifetime inbreeding depression (ID) along ecological gradients in seventeen populations of *Rhododendron ferrugineum* across the Pyrenees.

Both natural and pollinator-mediated seed sets decreased with lower mean temperatures. RA rose significantly with altitude and diminished with increasing *R. ferrugineum* floral display. However, microsatellite analysis of progeny revealed low selfing rates and considerable lifetime ID. Furthermore, similarly to RA, lifetime ID increased with hostile environmental conditions.

Thus, selfing does not seem to be adaptive in *R. ferrugineum* on a large spatial scale and RA does not appear to be currently involved in the maintenance of the mixed mating system. We suggest that selfing may have played a decisive role in population establishment and may have been conserved over time in populations with a slow individual turnover. However, obligate outcrossing populations could be endangered by the upward migration of their insect pollinators.

Key words: abiotic variables, altitude, Ericaceae, inbreeding depression, mountains, reproductive assurance, *Rhododendron ferrugineum,* selfing

INTRODUCTION

An unambiguous worldwide decline of pollinator abundance and diversity (Grixti *et al.,* 2009; Potts *et al.,* 2010) is ongoing. This decline results from major global change pressures such as agricultural intensification, non-native species invasion, spread of pathogens, landscape alteration increasing the isolation of populations and climate change (Gonzalez-Varo *et al.,* 2013). These threats on plant-pollinator interactions (Burkle *et al.,* 2013) lead to an increase in pollen limitation reducing seed set and thus have cascading effects on plant population persistence or crop production (Thomann *et al.,* 2013).

How plant–pollinator interactions are altered by a variety of disturbances has been widely studied, but this has primarily focused on how disturbance might affect pollinator populations and plant reproduction and demography, with little consideration of impacts on mating patterns (Aguilar *et al.,* 2008). Recurrent pollen limitation may result in strong selective pressure on plant mating systems (Eckert *et al.,* 2010) and promote evolution towards less reliance on pollinator and mate availabilities through selfing (Thomann *et al.,* 2013). A major tenet of mating system theories is that the evolution of selfing results from the balance of the 50% automatic gene transmission advantage, reproductive assurance (RA) and the extent of inbreeding depression (ID) that erodes the latter two advantages (Fisher, 1941; Lloyd, 1979). RA, commonly assessed by the production of seeds via autonomous selfing, occurs under chronic or unpredictable pollen limitation (Knight *et al.,* 2005) when pollinators or potential mates are scarce (Darwin, 1877; Lloyd, 1979). ID, the fitness reduction of inbred progeny relative to outbred progeny, is a widespread phenomenon in plants and is thought to be a major driving force in the evolution of plant mating systems (Charlesworth & Charlesworth, 1987). Low fitness of selfed progeny may compromise the benefit of selfers derived from RA and consequently the survival of predominantly selfed populations (Hedrick & Kalinowski, 2000).

The relationship between selfing rate, RA and ID is still a matter of discussion. In theory, in the absence of pollen limitation, plants would be predominantly fertilized by selfing with an ID lower than 0.5 (Lande & Schemske, 1985). However, because it increases the fitness of plants under pollen limitation, RA is expected to increase the ID threshold allowing evolution towards selfing (Thomann *et al.,* 2013). In species with repeated self-fertilization, purging of deleterious recessive genes is expected to decrease ID (Ohta & Cockerham, 1974). Indeed, Husband & Schemske (1996) and Winn *et al.* (2011) observed a negative relationship between selfing and ID in many species. However, high ID can be associated to high or intermediate selfing rates (Vaughton *et al.,* 2008; Ruan & Teixeira da Silva, 2012; Winn *et al.,* 2011).

Selfing, RA and ID appear to be context-dependent and thus for species within heterogeneous environments, shifts in the mating system that is favored may be observed (Herlihy & Eckert, 2002; Kalisz *et al.,* 2004). Studies assessing the potential of selfing to provide RA have given contradictory results. For instance, a general hypothesis in alpine plant populations states that selfing and thus RA should increase with increasing altitude (Garcia-Camacho & Totland, 2009). Indeed, small-scale heterogeneous abiotic conditions create a fragmented landscape and often small and more or less isolated populations. Additionally, as the climatic conditions get more hostile at higher altitudes, pollinator abundance and activity can be limiting factors for successful pollination and seed production which might increase selection for RA in the plants found there (Billings, 1974; Medan *et al.,* 2002). Through self-pollination experiments in field populations, Eriksen *et al.* (1993) obtained a higher seed set at high altitude than at low altitude while Gugerli (1998) and Arroyo *et al.* (2006) found no seed set changes with altitude. So, the prevailing opinion that the potential for selfing and RA become greater with increasing altitude is not always proved (Wirth *et al.,* 2010). It is important to note that pollination experiments reveal the capacity of a plant to self and not the realized mating system. Indeed, Gaudeul & Till-Bottraud (2003) found, from pollination experiment and genetic progeny analysis, a high potential for selfing in *Eryngium alpinum* whereas naturally pollinated offspring were predominantly outcrossed. Otherwise, ID varies between species, populations (Goodwillie & Knight, 2006), life-history stages (Husband & Schemske, 1996) and maternal families (Dudash *et al.,* 1997; Mutikainen & Delph, 1998), i.e. between seeds/seedlings belonging to the same mother plants (Gonzalez-Varo & Traveset, 2010). Recent studies on numerous taxa have revealed that stressful environments (Armbruster & Reed, 2005; Sedlacek *et al.,* 2013), competition (Cheptou *et al.,* 2000) or small population size often magnify ID (Michaels et al. 2008, Hirayama et al. 2007). Thus, both RA and ID are expected to increase as environmental-dependent stress increases due to global change (Beaumont *et al.,* 2011). Overall, these data call into question the reliability of selfing and RA to compensate for mate and pollinator scarcity and potential high ID and whether the evolution towards self-fertilization can be a safe way for most, especially perennial species, to cope with the ongoing global changes. To our knowledge, no studies have jointly measured selfing rate, RA and lifetime ID along large bioclimatic gradients to empirically estimate the actual long-term benefit of selfing in various natural ecological conditions. To our knowledge, no studies have jointly measured selfing rate, RA and lifetime ID along large bioclimatic gradients. Additionally, most investigations have been performed on herbaceous annual species and little is known about selfed progeny fitness in long-lived perennials, which can experience long lifetime ID (Morgan *et al.,* 1997). So, predicting the potential of selfing to provide actual RA in a changing world remains a major challenge in evolutionary biology and in ecology.

In this study, we focused on the alpine shrub *Rhododendron ferrugineum*, a mixed-mating system species (Escaravage *et al.,* 1997) in which reproduction occurs both by self- and cross-fertilization as in some other Rhododendron species (Kudo, 1993; Kameyama *et al.,* 2000; Hirao *et al.,* 2006). *Rhododendron ferrugineum* is frequent in the Pyrenees, a mountain range stretching across the isthmus that lies between the Iberian Peninsula and the rest of the European continent. Due to altitude shifts and oceanic influences to the west and Mediterranean influences to the east of the chain, the climate varies greatly along altitudinal and longitudinal gradients (Météo France data). This bioclimatic pattern offers the opportunity to address the potential of climate shifts to influence evolutionary processes and mating systems of the inhabiting species.

We investigated the effect of abiotic variables and *R. ferrugineum* floral display (as a measure of the species' pollinator attractiveness) on potential selfing rate, RA and lifetime ID in 17 populations across the Pyrenees. We first compared seed production in response to open pollination, autonomous self-pollination and pollinator-mediated services in seventeen populations along altitudinal and longitudinal bioclimatic gradients. From these seed-set data we then assessed the selfing capacity of the shrub and the RA. In order to investigate selfing rate and lifetime ID we finally performed a genetic progeny analysis in nine populations selected according to their representative altitudinal and longitudinal locations. We expected to find the highest selfing rates and RA at high altitude especially in the westernmost populations with a small floral display and the lowest in large populations at low altitude especially in easternmost populations. Otherwise, ID should be lower in populations experiencing high potential selfing rate as a result of the selection against inbred individuals and subsequent purging effects by strong environmental stresses. Overall, we expected to find a negative relationship between selfing and ID (Winn et al. 2011). Alternatively, a high level of ID could be observed, despite a significant level of selfing, in the absence of strong purging events and especially in small populations where 'drift load' could have occurred. ID could also be higher in more stressful environments (westernmost high-altitude population) or in populations with a high floral display if behind it, competition processes have occurred.

MATERIALS AND METHODS

Species of interest

Rhododendron ferrugineum L. (Ericaceae) is an evergreen shrub with a mean height of 70 cm that dominates subalpine landscapes mainly in the Alps and in the Pyrenees between 1500 to 2200 m a.s.l. In the Pyrenees, infrequent isolated populations can be found lower down, between 900–1500 m a.s.l. This species is protandrous (pollen grains mature before the stigma) and reproduces both sexually and vegetatively through layering. *R. ferrugineum* produces inflorescences with 5–28 bright-red nectariferous tubular flowers; in places it can produce more than 3000 flowers per m². The flowering period of a population lasts approximately one month, occurring from end of May to mid-July depending on the altitude. The seeds mature four to five weeks after the end of the blooming period. Previous studies conducted in the Alps showed that honeybees and bumblebees are the main pollinators of this species (Escaravage *et al.,* 1997; Escaravage & Wagner, 2004). *Rhododendron ferrugineum* has previously been described as a self-compatible species and spontaneous selfing occurs in the absence of pollinators (Escaravage *et al.,* 1998; Delmas *et al.,* 2014b). Geitonogamy is possible and is facilitated by pollinator foraging behavior.

Study sites and population characteristics

Seventeen populations were sampled along a longitudinal transect running the length of the Pyrenees (Figure 1). The populations sampled ranged between 1220 m and 2080 m a.s.l (Table 1). Geographical distances between populations across the Pyrenees ranged from 3 to 250 km with an average of 150 km. The climate varies greatly along longitudinal and altitudinal gradients in the Pyrenees. We investigated the potential long-term effects of climate on the *R. ferrugineum* mating system by using climatic data recorded from 24 stations distributed along the chain between 1991 and 2013 (Météo France data). At every study site, we calculated the mean monthly temperature (mean long-term temperature; MLT) during the flowering period (May to July depending on the altitude of the populations) from the three closest Météo France stations rather than one in order to be more representative of the surrounding climate. To adjust MLT from the three stations to each study site we used the lapse rate of ± 0.6°C/100 m of increasing or decreasing altitude (Ozenda & Borel 1991). We also investigated the current effect of climate on the realized mating system at every study site by recording mean current temperatures (MCT) during the flowering period of 2011 (May 21st to June 21st for populations at low altitude and June 26th to July 28th for the populations from high altitude). For this purpose in each population we fixed a "Lascar logger" weather meter in the canopy of a *R. ferrugineum* individual; at the same height and light conditions in all populations. Additionally to MLT and MCT, we investigated the effect of altitude and longitude because these two variables integrate many factors other than temperatures such as precipitation, snow cover, wind, variation in co-flowering community or the proximity to forests.

To describe *R. ferrugineum* floral display in each of the seventeen populations, we integrated inflorescence density, the cover of the species and the area occupied by the population as done by Delmas *et al.* (2014a,b). More specifically, *R. ferrugineum* floral display was estimated through the product of (i) the mean density of inflorescences per m² assessed from a 0.25 x 0.25 m plot placed on 20 randomly chosen individuals per population and (ii) the area (m²) covered by *R. ferrugineum* within each population estimated from the total area of the population (population perimeter obtained from geographic coordinates recorded every 5 meters) and the proportion of this total area occupied by *R. ferrugineum*. This latter was estimated by summing perimeters of all *R. ferrugineum* individuals in a 400 m² plot haphazardly placed at the population core.

Selfing capacity and reproductive assurance

In order to assess natural reproductive success, the level of potential selfing and RA, three pollination treatments were carried out in 2011 during the flowering season and were replicated twice on each of the 15 individuals of the 17 Pyrenean populations. As reported by Eckert *et al.* (2010) the treatments were: (i) intact inflorescences left to natural pollination (IN; control treatment), (ii) bagged intact inflorescences hand self-pollinated (IS) and (iii) emasculated inflorescences left to natural pollination (EN). The inflorescences were randomly selected on each individual, five flowers per inflorescence were manipulated and we gently removed the other flowers as done by Escaravage *et al.* (1997). The method is fully described in Delmas *et al.* (2014b).

Fruits were harvested five weeks after the treatments just before dehiscence to ensure the full development of the seeds. Two fruits per manipulated and control inflorescences were randomly selected, dissected under the stereomicroscope and filled seeds were counted. To assess seed set per treatment, we quantified the mean number of ovules per ovary per individual following the method described in Delmas *et al.* (2014a, b).

Potential selfing was estimated through the proportion of seed set from IS treatment. RA was calculated according to Lloyd & Schoen (1992) by comparing seed production from emasculated flowers left to natural pollination (EN) with natural seed production (IN), here

RA = 1 – *EN*/*IN*. RA gives information about the number of seeds attributed to autonomous self-pollination rather than outcross pollination. For each individual, RA can vary from 1 (no outcrossing had occurred) to 0 (fertilization comes entirely from outcrossing).

Mean (± SE) parameters per population were obtained by averaging the value of each parameter of individuals from the same population.

Progeny array analysis and mating system estimates

These analyses were performed in nine selected populations among the 17 considered for pollination experiments growing at low *vs* high altitude along the Pyrenean chain (Figure 1). In each population, we collected young leaves from 20 adult individuals and conserved it in silica gel until DNA extraction. For progeny array analyses, we randomly collected unmanipulated mature fruits on 10 adult individuals to obtain open-pollinated seeds in each population.

Seed germination was performed on moistened filter paper in 5 cm diameter Petri dishes in a greenhouse (24°C, 14h/10h). The greenhouse conditions minimized ID at the germination stage (Cheptou *et al.,* 2000) and thus reduced the potential bias in outcrossing rate estimates. We considered that seed germination had taken place when cotyledons were fully emerged. Ten seedlings per family were randomly collected and frozen until DNA extraction. Total sample size was 90 families including 900 progeny for mating system analysis.

DNA was extracted from leaves and seedlings with the DNeasy Plant Kit (QIAGEN, Courtaboeuf, France), following the manufacturer's protocol. We followed 15 polymorphic microsatellite markers (Table S1) previously developed using pyrosequencing technologies (454 FLX Titanium, Roche Applied Science, Meylan, France) as described in Delmas *et al.* (2011) and Charrier *et al.* (2012). Each primer was labeled with fluorochromes (Eurofins MGW Operon, Courtaboeuf, France) and used in three multiplexes optimized using Multiplex manager 1.1 (Holleley & Geerts, 2009). Amplifications were carried out in a 10 µl mix containing 10 ng of template DNA, 0.7 x Qiagen Multiplex PCR Master Mix, 10 µM of each primer and RNase-free water. Cycling conditions were 15 min at 95°C, 30 x (30 s at 95°C, 90 s at 56°C, 45 s at 72°C) and 30 min at 60°C. Each set of reactions included a negative (water) and a positive (known genotype) control. All the loci were unambiguously sized using an ABI 3730 sequencer (Applied Biosystems, Courtaboeuf, France) with the GeneScan™ – 600 LIZ® size standard and GENEMAPPER version 4.0 software (Applied Biosystems, California, USA). Genotype errors due to stutter bands, allele dropout and null alleles were estimated using the MICRO-CHECKER software (van Oosterhout *et al.,* 2004).

Mating system parameters were estimated using the software MLTR 3.1 based on Ritland's mixed-mating model (Ritland & Jain, 1981; Ritland, 2002). The program calculates maximum likelihood estimates of both outcrossing rates (Ritland & Jain, 1981) and correlated mating parameters based on the correlated matings model of Ritland (1989). The mating system parameters estimated the outcrossing rate (estimated from multilocus genotypes, t_m, and from the average single-locus estimate, t_s; Ritland, 2002). Multilocus selfing rates (s_m) were calculated as $s_m = 1 - t_m$. The program can estimate allelic frequencies in the pollen pool and in the ovule pool separately. As pollen and ovule allele frequencies did not differ significantly, we constrained the equality of frequencies to increase the statistical power of other estimates (Ritland, 2002). Using seedlings from the control treatment, the proportion of self-fertilized seeds (i.e. the selfing rate) was assessed. Standard errors and statistical significance of these mating system parameters were estimated from 1000 bootstraps. The analyses were performed with the population as a group factor and mating system estimates (t_s and t_m) were obtained for each population (n=17).

Lifetime inbreeding depression

Lifetime ID in field populations was determined by comparing the inbreeding coefficients (F) of mature plants to the expected F of progeny based on selfing rate ($s = 1 - t_m$), (Ritland, 1990; Goodwillie *et al.,* 2005). The difference between seedling and adult inbreeding proportion gave the strength of ID. To assess inbreeding coefficients in adult plants in each population (N = 9), we collected leaf samples from 20 chosen individuals and DNA extraction and amplification were performed as described above (see also Charrier *et al.,* 2014). Inbreeding coefficients (F) were computed using GenAlEx 6.5 software (Peakall & Smouse, 2006) and significance of F values were tested by applying 10000 randomizations. In the absence of ID, the expected equilibrium value of F for mature plants is $Fe = s / (2 - s)$. As described in Goodwillie *et al.* (2005), ID reduces F to $Fe = s\omega s / (2 - 2s + s\omega s)$, where ω is the fitness of selfed offspring compared to outcrossed offspring (i.e., ω s=1-ID). ID can, therefore, be estimated using Ritland's (1990) equilibrium estimator as:

$$ID = 1 - 2\left(\frac{(1-s)F}{s(1-F)}\right)$$

This ID estimator assumes that populations are at inbreeding equilibrium, selfing is the only form of inbreeding, and the marker polymorphisms are neutral and not physically linked to polymorphic loci affecting fitness and finally that there is no identity disequilibrium (Ritland, 1990; Eckert & Barrett, 1994).

When the fitness of selfed offspring is higher than the fitness of outcrossed offspring ($\omega_s > \omega_o$), the distribution of ID can be asymmetrical and show irregularity. In order to create a distribution of ID that is symmetrical and to be able to sum ID estimates in an unbiased fashion in the case where self progeny outperform outcross progeny, an alternative estimate of ID derived from Agren & Schemske (1993) can be used as done by Johnston & Schoen (1994), Dudash *et al.* (1997), Chang & Rausher (1999) or Mutikainen & Delph (1998). When $\omega_s > \omega_o$ (i.e. Ritland's equilibrium estimator was < 0), we used the following ID estimate: ID = $(\omega_o - \omega_s) / \omega_s$ instead of the usual ID = $(\omega_o - \omega_s) / \omega_o$ (when $\omega_s > \omega_o$) according to Agren & Schemske (1993). Considering the fitness of outcrossed progeny to be 1: ID = $(1/\omega_s)$-1. The estimate of ID used in such cases is therefore:

$$\mathrm{ID} = \left[\frac{s_m(1-F)}{2F(1-s_m)}\right] - 1$$

We estimated 95% bootstrap percentile confidence intervals for ID estimates of each patch based on 1000 ID bootstrap values generated from the bootstrap distributions of F and s_m.

Statistical analysis

Population and treatment effects on seed set were tested by a two-way analysis of variance and then Tukey's HSD post-hoc tests (R software). We used Pearson correlation to test the relationship between potential selfing, RA and ID.

To test the effect of altitude, longitude, MLT, MCT and total floral display, General Linear Models (GLMs) were conducted on natural reproductive success (IN), potential selfing (IS), pollinator-mediated pollination (EN), selfing rate (s), RA and ID coefficients for each populations using R v. 2.14.0 and the selection of the best model was done according to the Akaike information criteria (AIC). The model that minimizes AIC provides the most reliable fit to the data (Sakomoto *et al.,* 1986). We ranked models based on δAIC estimated as the difference between each AIC value and the smallest value. Models with $\delta\text{AIC} \leq 2$ were considered to have substantial support (Burnham *et al.,* 2002). The five explaining variables were tested and the model with the lowest AIC criteria was selected.

RESULTS

Mean long-term temperature (1991 to 2013 period) and mean current temperature (data logger, year 2011) during the flowering period (Table 1) ranged respectively from 9.47°C to 14.59°C and 10.72°C to 13.35°C depending on the sites. These two temperature variables were correlated ($R^2 = 0.48$, P value = 0.041) and both were correlated to longitude (MLT: $R^2 =$

0.59, P value = 0.01; MCT: $R^2 = 0.48$, P value = 0.04) increasing significantly from west to east. In the lowland populations MLT varied from 12.70°C (TecL) at the western part to 14.59°C (LecL) at the eastern part of the Pyrenees. The values were 9.47°C (TouH; west part) and 12.99°C (EynH; east part) for high altitude populations. MLT and MCT were not correlated to altitude (P value: 0.11 and 0.77 respectively). The total floral display (Table 1) varied greatly among populations and was the highest in SouH (2.94 10^8 flowers) and the lowest in LecL (2.85 10^5 flowers).

Estimates of R. ferrugineum mating system

Both pollination treatments and populations led to significant differences in seed set (Table 2). The mean seed set of treatments decreased significantly (Kruskal and Wallis post-hoc tests, P > 0.05) in following sequence: natural pollination seed set (IN: 0.61 ± 0.007 SE) > mean pollinator-mediated seed set (EN: 0.48 ± 0.007 SE) > hand self-pollination seed set (IS: 0.34 ± 0.009 SE). Significant differences for all treatments among populations are represented in Figure 2.

Mean IN seed set ranged from 0.41 (BouH) to 0.76 (TecL) (Fig. 2). The lowest EN values were found at ChiL (0.32) and TroH (0.38) and the highest at EynH (0.64). Populations ChiL and VicH had the lowest IS values (0.12 and 0.18 respectively) and PuiH, which was significantly different from all populations except EynH, had the highest (0.62). Finally RA increased seed production by 29% (mean RA = 0.29 ± 0.009 SE), with the highest values at PuiH, ChiL and TecL (0.39, 0.41 and 0.46 respectively) and the lowest at EynH (0.13) and SouL (0.15).

Genetic diversity, selfing and lifetime inbreeding depression

In the adult plants, 2-14 alleles per locus were detected, with a mean of 5.2 alleles while 2-9 alleles (mean 4.7) were detected for offspring. At several loci alleles appearing in the offspring genotypes were absent from the adults. The observed inbreeding coefficient of mature plants (F) ranged from -0.11 (LecL) to 0.08 (SouL) and differed significantly from zero in three populations (LecL: -0.11, PuiH: -0.10, SouL: 0.08). Overall, *R. ferrugineum* showed a predominantly outcrossed mating system with a mean selfing rate reaching 0.24 and ranging from -0.2 (LecL, PraL, ChiL, SouH, TroH) to 0.429 (PuiH).

Estimates of lifetime ID ranged from -0.21 to 1.24 (mean of 0.85 ± 0.21 SE). In two populations (LecL and SouL) inbreeding in maternal plants was higher than the progeny selfing rate resulting in negative ID values (see methods for the appropriate ID estimate used).

Selfing, reproductive assurance and inbreeding depression along ecological gradients

Mean IN seed set augmented significantly and mean IS seed set diminished while increasing MLT (Table 3). Mean EN seed set was significantly lowered with the increase in altitude and increased with the enhancement of floral display while results for RA were the opposite. Thus, low altitude populations with a large floral display had higher mean pollinator-mediated seed set (EN) and lower RA. In contrast, populations at high altitude with low floral display had lower mean pollinator-mediated seed set (EN) and higher RA. Longitude and MCT had no impact on seed set in any situation or on RA.

Selfing rate (s) did not vary through altitude, longitude, floral display or MLT and MCT. ID estimate increased with altitude and decreased while increasing MLT. We did not find any significant relationship between RA ($R^2 = 0.54$, P value = 0.12) or tm ($R^2 = 0.45$, P value = 0.21) and ID nor between selfing capacity (IS) and RA ($R^2 = 0.029$, P value = 0.912).

DISCUSSION

There are few empirical studies focusing on the relationships between selfing, RA and ID along large bioclimatic environmental gradients and empirical works lag behind theory (Winn *et al.,* 2011). So, it is difficult to anticipate the response of most species to the ongoing profound environmental changes. According to the criteria of Schemske & Lande (1985), *Rhododendron ferrugineum* has a mixed-mating system and RA through selfing significantly increases seed set and compensates for the lower pollinator effectiveness in high altitude small populations. However, microsatellite analysis of seedling progeny revealed very low mean selfing rates and very high lifetime ID. Moreover, lifetime ID increased with altitude in parallel to RA. Consequently, selfing does not provide RA over long time and the species relies almost exclusively on pollinator services for its seed reproduction. These findings question the actual role of selfing in the *R. ferrugineum* mating system and whether populations will be able to respond to global changes.

Estimates of R. ferrugineum mating system

We found that *R. ferrugineum* had lower natural seed set (IN: 0.61) than in other Pyrenean (0.7 on average; Delmas *et al.,* 2014b) or Alpine populations (0.74 - 0.99; Escaravage *et al.,* 1997). This could result from a lower selfing capacity (hand self-pollinated seed set IS: 0.34) compared to other populations (0.49, Delmas *et al.,* 2014b; > 0.77, Escaravage *et al.,* 1997) limiting all sources of self-fertilization (autonomous selfing, pollinator-facilitated selfing, geitonogamy). Our data showed that emasculated flowers produced fewer seeds than intact control flowers (0.48 *vs* 0.61) suggesting that autonomous selfing significantly contributed to total seed production as already shown in this species (Delmas *et al.,* in revision) or others (Elle & Carney, 2003; Kennedy & Elle, 2008; Zhang & Li, 2008; Vaughton & Ramsey, 2010; Brys *et al.,* 2011).

RA reached 29% on average. This implied that at least 30 % of natural seed set would have arisen from selfing. Using progeny analysis, Delmas *et al.* (in revision) found that selfing rates were higher than expected from RA alone (mean of 0.49 *versus* 0.27 respectively) as an avoidable consequence of geitonogamy in a mass-flowering species with a large number of flowers open at the same time (van Kleunen & Johnson, 2007). However, due to the selfing capacity of individuals, the selfing rate would not be expected to exceed 30% in our populations. Except in one population we found incredibly low selfing rates indicating that more than 80% of selfed seeds failed to give viable seedlings. This suggested strong early-acting ID (Waser & Price, 1991) limiting self-fertility in *R. ferrugineum*. This implied that self-pollination occurs at an immediate fecundity cost by reducing the number of developed seeds (increased lethality among selfed embryos) and the number of ovules available for cross-pollination (Waser & Price, 1991). Early-acting ID has been previously observed in the Ericaceae family (Mahy & Jacquemart, 1998; Hokanson & Hancock, 2000; Delmas *et al.,* in revision).

Despite having a mixed-mating system *R. ferrugineum* has a high lifetime ID (mean of 0.85) similar to those reported in the same species (mean of 0.90; Delmas *et al.,* in revision) or in long-lived species such as gymnosperm trees or clonal species (Sorensen, 1999; Gonzalez-Varo & Traveset, 2010; Winn *et al.,* 2011) and much higher than those recorded for primarily outcrossing species which are known to express very substantial ID (Winn *et al.,* 2011). Our data confirm the assumption that long-lived perennial species (at least 300 years for *R. ferrugineum*; Escaravage *et al.,* 1998) accumulate somatic mutations that are not or only weakly purged through its lifetime resulting in high ID levels (Morgan *et al.,* 1997; Petit & Hampe, 2006). They are also consistent with the selective inference hypothesis (Lande *et al.,* 1994), which states that deleterious mutations at multiple loci prevent purging processes (Lande *et al.,* 1994). It could also explain that lifetime ID was not negatively related to selfing (Winn *et al.,* 2011).

Response of mating system, reproductive assurance and lifetime inbreeding depression to ecological gradients

IN seed set decreased with decreasing MLT but was not sensitive to MCT. This suggested that long-term mechanisms favorable to the reproduction of the shrub had taken place at the warmest sites. Otherwise, no correlation among altitude and temperatures was detected; this is explained by the lag between the blooming period at low (June) and at high altitudes (July). The decline of pollinator-mediated seed set (EN) along with altitude showed that pollinator effectiveness might be involved. Indeed, the altitudinal decrease of pollinator effectiveness likely resulted from the generally harsh environmental conditions in alpine habitats (seasonal low temperature, high precipitation, short growing season, strong winds and long distance from forest) unfavorable to pollinator populations and their activity (Medan *et al.,* 2002; Arroyo *et al.,* 2006). This may reduce the possibility of cross-pollination (Medan *et al.,* 2002). As observed in other systems (Brys *et al.,* 2011), pollinator-mediated seed set increased with increasing floral display. Indeed large floral display is more attractive for pollinators and may thus increase visitation rates (Kunin, 1997, Dauber *et al.,* 2010).

We hypothesized that RA was significantly higher in small populations at high altitude. While neither hand self pollination seed set (IS), nor natural seed set was found to change with altitude or floral display, the increase in RA can be attributed to a decrease in pollinator services rather than in selfing capacity. These data support earlier findings in the same species (Delmas *et al.,* in revision; mean RA: 27%, decreasing with plant patch size) or other species showing that RA is context-dependent (Kalisz *et al.,* 2004), augmenting total seed set in populations with low floral display (Elle & Carney, 2003; Jacquemyn & Brys, 2008; Moeller & Geber, 2005), low rates of pollinator visitation (Kennedy & Elle, 2008; Kalisz *et al.,* 2004; Brys *et al.,* 2011) and growing in harsh habitats (Zhang & Li, 2008; Vaughton & Ramsey, 2010). We did not detect significant effect of abiotic factors or floral display on selfing rate. These results are in marked contrast to the general and our own hypothesis of higher selfing rates in small populations at higher altitudes (Garcia-Camacho & Totland, 2009). Moreover, we did not observe lower selfing rates and RA in the easternmost populations (no longitudinal effect) where the main pollinators of *R. ferrugineum* (bumblebees) are known to be especially diverse and abundant (Iserbyt *et al.,* 2008). The discrepancies between our results and expectations could come from the almost total counteraction of early-acting ID against selfed seed germination in any populations.

Lifetime ID significantly increased with altitude and low temperatures and that supports the idea that stressful environments (Armbruster & Reed, 2005; Sedlacek *et al.,* 2013) magnify ID. According to the findings of Delmas *et al.* (in revision) we did not detect any effect of floral display on the level of lifetime ID. This signifies that neither small population size (Michaels *et al.,* 2008, Hirayama *et al.,* 2007) nor intraspecific competition in large populations (Pujol & McKey 2006) magnified ID. Overall, the results did not confirm our hypothesis of a lower ID associated to higher selfing in small populations growing at high altitude. ID was even greater at high altitude populations experiencing higher RA.

Relationships between selfing, reproductive assurance and inbreeding depression

RA is a widely accepted hypothesis to explain the evolution of selfing (Jain, 1976; Lloyd, 1992; Herlihy & Eckert, 2002; Brys *et al.,* 2011). Indeed, RA may outweigh the disadvantages of ID when low pollinator and/or mate availability limit outcross success (Darwin, 1877; Stebbins, 1957; Jain, 1976; Lloyd, 1979; Holsinger, 1996). Overall, RA hypothesis has been put forward to resolve the disparity in ID and selfing relationships by explaining how selfing can be advantageous in species with strong ID (Ruan & Teixeira da Silva, 2012). However, our results challenged the above statement. Indeed, despite RA increased seed set by 29% on average, its gain was completely eroded by considerable early-acting and lifetime ID (see also Herlihy & Eckert, 2002). The two populations with negative lifetime ID had also a low (SouL) or negative s (LecL) indicating that selfed seedlings had already been eliminated by early-acting ID and that lifetime ID actually had no room to work in those populations. The ineffectiveness of RA to produce viable individuals could even be a widespread phenomenon in *R. ferrugineum*. Indeed, Charrier *et al.* (2014) found inbreeding coefficients (F_{IS}) close to zero in 22 of 33 populations investigated in Pyrenees, Alps and Jura mountains indicating that selfed progeny contributed little to adult populations. Five of the nine remaining populations had a significant homozygote deficit ($F < 0$) confirming the strong selection against inbred individuals. Finally, they recorded significant inbreeding ($F > 0$) only in four populations (13%). Thus, there is compelling evidence that, (i) although it possesses a mixed-mating system, *R. ferrugineum* behaves mainly as an obligate pollinator-dependent outcrossing species, (ii) the populations studied do not seem to take advantage from RA. Therefore, it is doubtful that RA currently plays a significant role in the evolution of selfing and mixed-mating overall. By contrast, our estimation of early-acting and lifetime ID, far above the threshold value favoring selfing (Lande & Schemske, 1985) helps to explain why both selfing capacity and selfing rate are lower in our populations compared to other populations from the Pyrenees (Delmas *et al.,* in revision) or Alps (Escaravage *et al.,* 1997).

Selfing in *R. ferrugineum* does not seem to be adaptive but rather results from the inability to prevent self-pollen reaching the stigma associated to ineffective mechanisms of self-incompatibility (Delmas *et al.,* 2014b). Selfing could in fact represent an incidental by-product of adaptation toward pollinator attraction. If not opposed by lifetime ID, selfing and clonality (Escaravage *et al.,* 1998; Pornon *et al.,* 2000), would both impoverish genetic diversity of populations (Charlesworth & Charlesworth, 1995).

Nevertheless, a pending question is why all *R. ferrugineum* populations studied to date continue selfing despite its apparent limited use. We propose that this intriguing incongruence is linked to the role of selfing in the history of the populations. The present data highlight that low selfing is able to work in some rare populations. Interestingly, Charrier *et al.* (2014) have shown that the four populations with significant levels of inbreeding ($F_{IS} > 0$) were all marginal, exhibiting especially low genetic diversity, a genetic pattern confidently related to founder events accompanied by a loss of genetic diversity and heterozygosity during the expansion of the species. Therefore, these data suggest that ID was lower in these populations and that selfing and RA likely played a decisive role in establishment of the populations. The high incidence of selfing permitting uniparental reproduction in marginal populations has been previously evoked (Pujol *et al.,* 2009) and seems confirmed here. Because *R. ferrugineum* has a long lifespan and the genotypic structure of populations are mainly established at early successional stage and persists over time through clonality, (Pornon *et al.,* 2000) colonizing selfing phenotypes could have been conserved until now in populations.

Recent studies have revealed a faster upward migration of insects relative to plants (Roth et al., 2014). This could threaten populations fully dependent on pollinator services for seed production. Only rare populations able to self and tolerating a certain level of inbreeding could respond to climate change and become source populations for the colonization of new higher altitude areas.

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TABLES

Table 1. Geographical information and characteristics of *R. ferrugineum* populations. MLT = mean long-term temperatures (from 1991 to 2012), MCT = mean current temperatures (data loggers), s = selfing rate, F = within population coefficient of inbreeding, (values in bold are significantly different from 0), ID = lifetime ID with 95% bootstrap percentile confidence intervals.

Moutain range	**Sites**	**Population code**	**Location**	**Altitude (m)**	**Floral display**	**MLT (°C) from 1991 to 2012**	**MCT (°C) 05/21-06/21 at low altitude 06/26-07/28 at high altitude**	**s**	**F**	**ID (95% CI)**
Eastern Pyrenees	Léca	LecL	42°28'07"N 02°31'40"E	1250	$2.84\ 10^5$	14.59 ± 0.59	13.10	-0.2	**-0.11**	-0.18 (-0.43 - 0.01)
	Prats de Mollo	PraL	42°25'21"N 02°24'38"E	1450	$1.32\ 10^6$	13.39 ± 0.59	12.70	-0.2	0.02	1.244 (-0.47 - 2.83)
	Eyne	EynH	42°28'27"N 02°07'05"E	1915	$5.59\ 10^6$	12.99 ± 1.05	13.03	--	--	--
	Puigmal	PuiH	42°23'26"N 2°05'0"E	2045	$4.21\ 10^7$	12.21 ± 1.00	13.35	0.429	**-0.10**	1.242 (-0.21 - 2.56)
	Lac des Bouillouses	BouH	42°33'31"N 01°59'42"E	2005	$1.96\ 10^8$	12.45 ± 1.06	13.08	--	--	--
Central Pyrenees	Soulcem	SouH	42°39'28"N 01°27'27"E	2035	$2.94\ 10^8$	10.55 ± 0.97	11.97	-0.2	-0.01	0.88 (-0.11 - 1.67)
	Soulcem	SouL	42°41'28"N 01°27'07"E	1240	$3.02\ 10^6$	13.99 ± 1.76	12.04	0.125	**0.08**	-0.21 (-0.73 - 0.31)
	Etang de Lhers	VicL	42°48'23"N 01°22'31"E	1277	$2.49\ 10^7$	13.76 ± 1.76	12.65	--	--	--
	Etang de Lhers	VicH	42°47'36"N 01°24'16"E	1940	$1.21\ 10^8$	11.14 ± 0.97	10.72	--	--	--
	Bethmale	BetL	42°51'42"N 01°04'02"E	1385	$1.11\ 10^7$	13.20 ± 2.11	11.69	0.2	-0.06	1.45 (0.27 - 2.68)
	Bethmale	BetH	42°50'24"N 01°03'42"E	1920	$6.14\ 10^8$	11.84 ± 0.95	12.87	--	--	--
	Le Peyras	PeyL	42°59'15"N 00°10'46"E	1220	$4.83\ 10^7$	12.00 ± 0.58	11.42	0.208	0.03	0.76 (-0.02 - 1.40)
	Col du Tourmalet	TouH	42°53'57"N 00°07'54"E	1950	$1.90\ 10^7$	9.47 ± 0.43	12.00	--	--	--
	Cirque de Troumouse	TroH	42°43'41"N 00°05'40"E	2080	$2.95\ 10^7$	10.83 ± 0.52	11.33	-0.2	0.02	1.244 (-0.14 - 2.55)
	Chiroulet	ChiL	42°57'38"N 00°04'17"E	1240	$1.75\ 10^6$	11.91 ± 0.63	11.91	-0.2	0.02	1.244 (0.48 - 1.95)
	Lac du Tech	TecL	42°54'40"N 00°15'32"W	1240	$1.48\ 10^5$	12.70 ± 0.65	13.17	--	--	--
	Luz Ardiden	LuzH	42°52'45"N 00°03'34"W	1905	$8.18\ 10^7$	10.94 ± 2.69	11.54	--	--	--

Table 2. Results of two-way analysis of variance to detect population and treatment effects on seed set. df: degree of freedom; MS : mean square; ***, *P* <0.001

	df	**MS**	**F statistic**
Treatment	2	16.81	263.87***
Population	16	0.83	13.07***
Treatment x Population	32	0.38	6.08***

Table 3. Statistical results for the GLMs testing the effects of ecological gradients on estimates of mating system and lifetime inbreeding depression in *R. ferrugineum*. IN: control treatment; IS: bagged intact inflorescences hand self-pollinated; EN: emasculated inflorescences left to natural pollination. Significant P values are in bold.

IN seed set (AIC = -117.55)	**Estimate**	**Standard error**	**P value**
Intercept	0.297	0.126	**0.01***
Longitude	-0.03	0.01	0.10
Mean long-term temperature	0.028	0.010	**0.009****
IS seed set (AIC = -73.93)			
Intercept	1.235e-01	2.421e-01	0.61
Mean long-term temperature	-6.99e-02	1.245e-02	**5.22e-08*****
Longitude	3.84e-02	1.83e-02	0.37
Mean current temperature	8.21e-02	2.08e-02	0.18
Floral display	5.36e-10	1.98e-10	0.72
EN seed set (AIC = -87.84)			
Intercept	9.424e-01	2.534e-01	**0.0002*****
Altitude	-1.07e-04	4.53e-05	**0.018***
Mean current temperature	-2.94	1.90e-02	0.12
Floral display	9.22e-10	2.24e-10	**5.89e-05*****
Reproductive assurance (AIC = -15.19)			
Intercept	-5.207e-01	3.21e-01	0.1062
Altitude	9.829e-05	5.84e-05	**0.019***
Longitude	-3.92e-02	2.49e-02	0.11
Mean current temperature	6.29e-02	2.68e-02	0.09
Floral display	-7.97e-10	2.79e-10	**0.0048****
Lifetime inbreeding depression (AIC = 40.95)			
Intercept	6.74	3.46	0.064
Altitude	1.311e-03	4.16e-04	**0.004****
Mean long term temperature	-2.264e-01	8.76e-02	**0.017***
Floral display	-3.11e-09	1.96e-09	0.12
Longitude	4.05e-01	2.67e-01	0.14
Mean current temperature	-4.54e-01	2.67e-01	0.18
Selfing rate (AIC = 4.94)			
Intercept	3.90	1.74	**0.03***
Longitude	2.05e-01	1.37e-01	0.14
Mean current temperature	-2.97e-01	1.71e-01	0.09
Altitude	3.31e-04	2.15e-04	0.13
Floral display	-1.39	1.00e-09	0.18

FIGURES

Figure 1. Location of the seventeen *R. ferrugineum* populations in the French Pyrenees. Black dots indicate the populations used for genetic analysis.

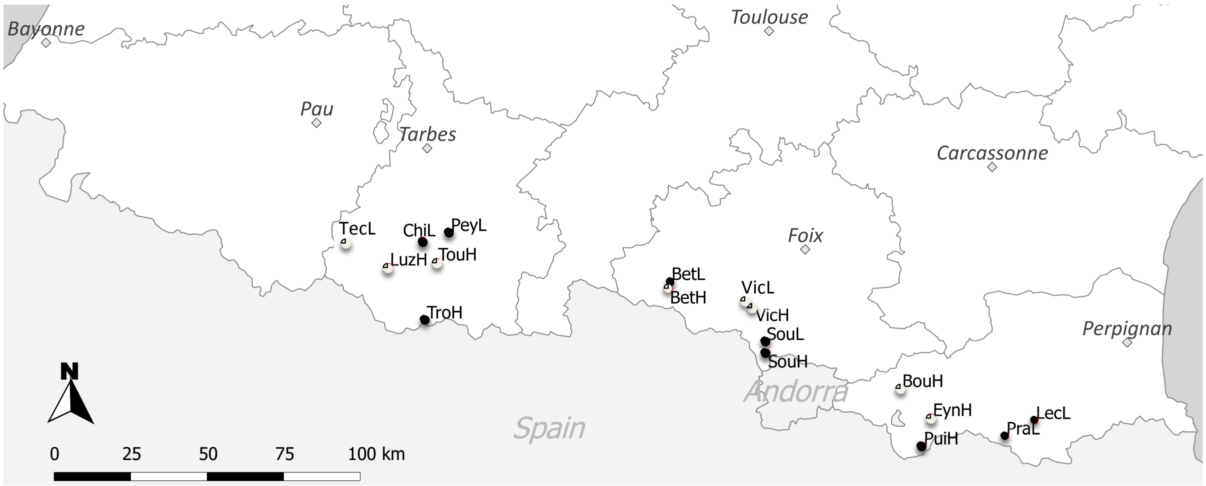
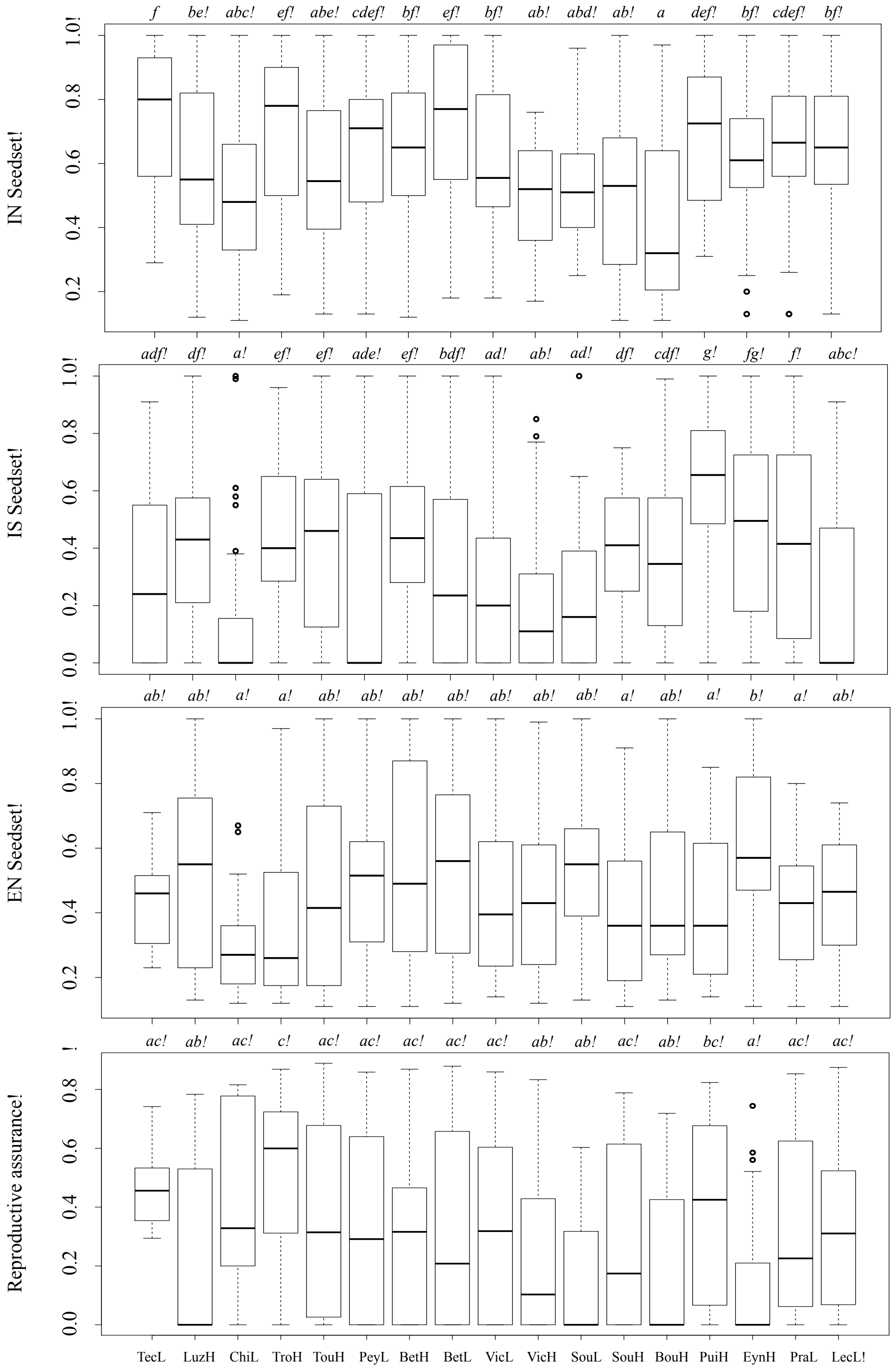


Figure 2. Seed set of the three pollination treatments (IN: control treatment; IS:_bagged intact inflorescences hand self-pollinated; EN: emasculated inflorescences left to natural pollination) and reproductive assurance (RA) in *R. ferrugineum* populations. Different letters indicate significant differences among populations.



ANNEXES

Table S1. Characteristics of 15 polymorphic microsatellite markers in *Rhododendron ferrugineum*.

Locus	GenBank accession number	Repeat motif	Allele size range (bp)	Primer sequences (5'-3')	Multiplex markers set	Fluorolabel
RF56P1	HQ822283	$(AC)_6$	73-85	F: AATGTATTTATTGTCTTTATCCCCA R : CTTGAGGTGTGCAGCTTTGA	1	Yakima Yellow
RF87P2	KC008594	$(CAC)_7$	133-142	F : CCTTTCCTCGCAATGAAGAA R : AGGAAGGTGATGAGGAGGGT	1	Fam
RF74P1	HQ822278	$(GA)_{13}$	152-182	F : ATGCCACCAAATCTATTGCC R : TTTCTCTCCTGCACGCTTCT	1	Atto565
RF140	KC008584	$(AC)_{13}$	196-208	F : ATGGCTAGCTTTGTGCTGCT R : TGCACATGAGTTCCTCAACA	1	Yakima Yellow
RF153	KC008583	$(GA)_7$	207-235	F : CCACACGCTAGGGAACTTTT R : TCAGCGTCGAAGAATCTCAA	1	Fam
RF202	KC008590	$(TG)_{15}$	86-104	F : CATTTTCCAGCACAAAACTAAATG R : TGTGACGGTGTATCGGAAGA	2	Atto565
RF182	KC008595	$(CT)_{12}$	150-164	F : TCTGTCCGACCGAATCTGTA R : CAGCAGCCATTAGACAGAAAAA	2	Yakima Yellow
RF128	KC008591	$(GA)_{11}$	146-156	F : ATAAACGGCTCTCAAATGCG R : GATGTGTTTCCGGCGTAGTT	2	Fam
RF41P1	HQ822282	$(TC)_9$	184-234	F: TCCAAGTGGTTATGTAATTCTATCG R : CTATGCTTCGGCAAAAGGAA	2	Atto550
RF113P2	KC008589	$(GTT)_8$	312-327	F : TCGTCAAATGCAGCCAAAC R : CAACGAACAATCATGCTCAAA	2	Atto550
RF81P1	HQ822275	$(GA)_{11}$	140-154	F : CGAAGGACCAACTGAAAAGG R : ACCATGGCCACTGTTTTACC	3	Yakima Yellow
RF85	KC008582	$(CAA)_5$	148-159	F : TGGTGCTGTGACAAAGGGTA R : TCCGAGGAAATCAATGAAGG	3	Atto550
RF163P1	KC008587	$(GA)_{12}$	179-191	F : TGAATGGTTCAAGCACCAAA R : GCTGTTCTTGCTGACATGGA	3	Fam
RF157P1	KC008592	$(TC)_{12}$	200-216	F : CTTCCTCTCCCATGCCATAA R : CCTTTCCGAAGCACCAATAA	3	Atto550
RF175	KC008585	$(TG)_{10}$	282-318	F : TTCTGAACCCTGCACTTCCT R : CCAGCGGAGACAGTATGGAT	3	Atto565

CHAPITRE 3

- **Spatial and temporal variability in visitor assemblages of *Rhododendron ferrugineum* in the Pyrenees.**

- **Variability of pollinator assemblages effectiveness and impact on plant mating system of *Rhododendron ferrugineum.***

SPATIAL AND TEMPORAL VARIABILITY IN VISITOR ASSEMBLAGES OF *RHODODENDRON FERRUGINEUM* IN THE PYRENEES.

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ABSTRACT

Pollination by animals is critical to sexual reproduction of most angiosperms. Despite widespread concern about declines in pollination services, little is known about the patterns of changes in most pollinator assemblages. Mountains have been considered as model systems due to their heterogenic habitats that may lead to variation in pollinator assemblages in space and time.

We monitored flower visitors during two years of reproduction along ecological gradients in seventeen populations of *Rhododendron ferrugineum* across the Pyrenees.

R. ferrugineum appears to be a generalist species in that it is able to exploit a wide variety of potential pollinators. Indeed, the visitor assemblages (richness, composition, diversity and visitation abundance) varied among populations. Visitor assemblages were impacted by biotic (*R. ferrugineum* flower density, plant diversity and plant richness of the surrounding community) and abiotic (longitude and temperature) parameters. Surprisingly, total floral display (estimated as a proxy of population size) and altitude had no impact on visitor communities.

These results should add to a growing appreciation that pollination services are variable at temporal and spatial scales and are sensible to environmental conditions. The ability to exploit a wide variety of visitor is particularly advantageous on the context of global changes.

KEY WORDS: altitude, ecological gradients, Ericaceae, floral display, mountains, plant-pollinators interactions, *Rhododendron ferrugineum* visitation, visitor assemblages

INTRODUCTION

As a facet of global changes, the recent decline of insect pollinator communities is currently a major concern (Winfree et al. 2011; Thomas et al. 2004) because of its impact on ecological and agricultural systems. Indeed, almost 90% and 75% of native and cultivated plant species, respectively, rely on animals for reproduction (Ollerton et al. 2006). Although many animal-pollinated plants can self-fertilize to some degrees, they require pollinators for genetic exchanges among individuals (Winfree et al. 2011). Pollination systems are under increasing threats from anthropogenic sources, such as habitat fragmentation, changes in land use, modern agricultural practices, use of chemicals and invasions of non-native plants and animals (Biesmeijer et al. 2006). If relationships between plants and their pollinators have been subject of much interest because of their implications in the evolution of floral traits or pattern of gene flow (Kay and Schemske 2003), little is known about the variability of visitor assemblage in space and time. It is crucial to understand how environmental variables impacts composition, richness, diversity and abundance of pollinator species in order to infer how global changes will affect plant-pollinator interactions in space and time. Variation in pollination service has the potential to profoundly influence the ecological dynamics of plant populations (Ashman et al. 2004).

Investigations on pollination systems suggest that plant-pollinator associations are diffuse because plants are generally visited by a large spectrum of animals and likewise, pollinators often use a wide array of plant taxa (Herrera 1996; Waser et al. 1996). Following these ideas, a large number of community-level studies had concluded that generalization is the rule whereas specialization is rare (e.g. Olesen and Jordano 2002). However, it has recently been proposed that pollination systems could exhibit “functional specialization”, a given plant species or a given plant functional group being primarily pollinated by a given functional group of pollinators (Ollerton et al. 2007). The concept implies that pollinators are clustered into functional groups (e.g., long-tongued *vs* small-tongued insects) that behave in similar ways on a type of flower (for instance flowers with easily accessible *vs* hidden floral rewards; Fontaine et al. 2006) and thus exert similar selection pressure (Fenster et al. 2004). Redundancy of species contributing to the same function is a guiding principle of biodiversity-ecosystem function theories and biological insurance hypothesis (Lawton and Brown 1993; Naeem 1998). Applied to plant-pollinator networks, this hypothesis suggests that the extinction or the extirpation of a given pollinator species would have limited consequences on its plant interaction because of the redundant functionality among pollinator species. Both theoretical and empirical work supports this hypothesis for functions such as productivity, resistance or resilience of ecosystems (Lehman & Tilman 2000; Balvanera et al. 2006; Ives & Carpenter 2007) although it has not been tested for pollination function.

Pollinators aim to maximize their energy gain per unit of time invested in foraging (Stephen and Krebs 1986). Consequently, availability, diversity and the degree of spatial intermixing between available food resources are major factors determining the distribution (Tscharntke et al. 1998; Keasar 2000; Wesselingh and Arnold 2000; McFrederick and LeBuhn 2005; Roulston and Goodell 2010; Natalis and Wesselingh 2012; Pakeman and Stockan 2013; Faria and Gonçalves 2013*)* and the behavior of pollinator by changing time costs associated with resource searching and handling (Stephens and Krebs 1986; Hersch and Roy 2007). Thus, the pollinator assemblage of a given species will depend on the attractiveness of its own population and the features of the surrounding plant communities. Small populations are less attractive to insects, consequently they may present less abundant and diverse pollinator assembles than large populations (Sih and Baltus 1987; Sowig 1989; Steffan-Dewenter and Tscharntke 1999). Co-flowering plants may either compete (exploitative competition) or facilitate (pollinator sharing) the pollination of a given species (Brown and Kodric-Brown 1979; Waser and Real 1979; Rathcke 1983). This implies that a plant embedded in the community may be either less *vs* more visited or visited by either less *vs* more efficient pollinators due to the surrounding community. Also, populations with high diversity in co-flowering species may attract a more diverse assemblage of pollinators (Schemske 1981; Rathcke 1983).

The activity, population size, diversity and composition of pollinator assemblages and subsequently services of pollinators are, at some extend, regulated by environmental factors such as temperature, relative humidity, solar radiation and/or wind (Vicens and Bosch 2000, Bolotov et al. 2013, Faria and Gonçalves 2013). It has been shown that pollinator assemblages may vary along spatial, temporal and ecological gradients (Herrera 1988; Eckhart 1992; Fenster and Dudash 2001; Devoto et al. 2005; Petanidou et al. 2008; Alarcon et al. 2008; Jacobs et al. 2009; Bolotov et al. 2013). For instance, in mountain areas Diptera grow in importance as pollinators with increasing altitude (Muller 1880; Mani 1962; Pojar 1974; Primack 1983) while Hymenoptera abundance decreases sharply with altitude (Arroyo et al. 1982). However, a more effective pollination, combined with a longer period of stigma receptivity may compensate for a decrease in pollination services (Bingham and Orthner 1998). Mountain ecosystems have been considered as model systems to test diverse physiological, ecological and evolutionary biology (Körner and Spehn 2002) because of the strong gradient found at relatively small spatial scale. Moreover, mountain systems world-wide are especially prone to the impact of changing climatic conditions and should therefore be a high priority in ecological studies (Franzen and Molander 2012).

In this study, we investigated the changes of *Rhododendron ferrugineum* population's visitor assemblages along altitudinal and longitudinal gradients in the Pyrenean chain during two reproductive seasons. In 17 populations of *R. ferrugineum*: (1) we gathered climatic, altitudinal and longitudinal data and measured *R. ferrugineum* floral display and floral density and diversity of the surrounding co-flowering community; (2) we investigated the composition, richness, visitation abundance (as a proxy of insect abundance) and diversity of visitor assemblages and identified the main visitors of *R. ferrugineum* and (3) we studied the impact of composition, richness and diversity of visitor assemblages on mean visit density per populations. These steps allow us to assess the effects of the local ecological context on the visitor assemblages across the Pyrenees. We first hypothesized that in any ecological contexts *R. ferrugineum* relies on the same visitor assemblage and thus is specialized upon it. This would signify that *R. ferrugineum* floral display has a driving effect on visitors. Because of this specialization, the pollination of the species would be threatened by extirpation or spatial and/or temporal shifts of species pollinator (Roth et al. 2014) due to global changes, except if redundant species can buffer global change impacts. In the second hypothesis, *R. ferrugineum* is able to efficiently use specific local visitor assemblages, which composition richness, abundance and diversity depend primarily on local ecological context. If there were redundancy among visitors, variation in pollinator assemblage would have no impact on visitation efficiency. This would prove the species would be able to cope with temporal or spatial changes in pollinator assemblages. In the third hypothesis, *R. ferrugineum* is using local assemblage but the variability in pollinator assemblage lead to variability in visitation efficiency and that may have an impact on plant reproduction.

MATERIAL AND METHODS

Species studied

Rhododendron ferrugineum L. (Ericaceae) is an evergreen shrub with a mean height of 70 cm that dominates subalpine landscapes mainly in the in the Alps and Pyrenees between 1500 to 2200 m a.s.l. on north-to-west facing slopes, sometimes reaching 90-100% of the vegetation cover. In the Pyrenees, infrequent smaller and isolated populations can be found lower down, between 900–1500 m a.s.l. *Rhododendron ferrugineum* produces inflorescences with 5–28 bright-red nectariferous tubular flowers; in some places it can produce more than 3000 flowers per m². The flowering period of a population lasts approximately one month, the blooming period ranges from end of May to mid-July depending on the altitude and the seeds are mature four to five weeks after the end of the blooming period. Previous studies conducted in the Alps and in one Pyrenean population showed that flowers were mainly visited by honeybees and bumblebees (Escaravage et al. 1997; Escaravage and Wagner 2004; Delmas et al. 2014a). *Rhododendron ferrugineum* reproduces both vegetatively through layering and sexually. It has previously been described to be a self-compatible species and spontaneous selfing occurs in the absence of pollinators (Escaravage et al. 1998). Geitonogamy is possible and is facilitated by pollinator foraging behavior.

Geographic and climatic data

Seventeen populations were studied along a longitudinal transect running the length of the Pyrenees (Figure 1). The sampled populations ranged between 1220 m and 2080 m a.s.l (Table 1). The climate varies greatly along longitudinal and altitudinal gradients in the Pyrenees.

We investigated the potential long-term effects of climate on *R. ferrugineum* visitor assemblages by using climatic data recorded from 24 stations distributed along the chain between 1991 and 2013 (Météo France data). In each population, we calculated the mean monthly temperature (mean long-term temperature; MLT; Figure 2) during the flowering period (May to July depending on the altitude of the populations) from the three closest Météo France stations rather than one in order to be more representative of the surrounding climate. To adjust MLT from the three stations to each population we used the lapse rate of ± 0.6°C/100 m of increasing or decreasing altitude (Ozenda and Borel 1991). We also investigated the current effect of climate on visitor assemblages in every population by recording mean current temperatures (MCT) during the flowering periods of 2011 and 2012 (in 2011: May 21st to June 21st for populations at low altitude and June 26th to July 28th for the populations from high altitude, in 2012: June 4st to June 25st for populations at low altitude and June 26th to July 27th for the populations from high altitude). In 2012, MCT could not be recorded in four populations (TouH, TecL, PraL, VicL). In each population, we fixed a "Lascar logger" weather meter in the canopy of a *R. ferrugineum* individual; at the same height and light conditions in all populations.

We focused on temperatures because these variables play a key role in species distribution in mountain systems, where there are strong thermal gradient with elevation (e.g. Gutierrez-

Illian et al. 2010; Stefanescu et al. 2011). Additionally to MLT and MCT, we investigated the effect of altitude and longitude because these two variables integrate many other influences that those of temperatures only as precipitations, snow cover, wind or distance to tree line limit.

Rhododendron ferrugineum floral display and flower density

To describe *R. ferrugineum* floral display in each of the 17 populations, we integrated inflorescence density, the cover of the species and the area occupied by the population as done by Delmas *et al.* (2014a, b). More specifically, *R. ferrugineum* floral display was estimated, in 2011, through the product of (i) the mean density of inflorescences per m^2 assessed from a 0.25 x 0.25 m plot placed on 20 randomly chosen individuals per population and (ii) the area (m^2) covered by *R. ferrugineum* within each population estimated from the total area of the population (population perimeter obtained from geographic coordinates recorded every 5 meters) and the proportion of this total area occupied by *R. ferrugineum*. This latter was estimated by summing perimeters of all *R. ferrugineum* individuals in a 400 m^2 plot haphazardly placed at the population core. We then calculated the flower density (number of *R. ferrugineum* open flowers) per m^2.

Surrounding flowering community

The co-flowering species of the 17 populations were determined in 2011 during the flowering period of *R. ferrugineum*. To estimate the abundance of each species, each flowering species were identified and the number of open flowers per species was counted in 20 quadrats of 0.55 x 0.55 meters. The diversity and equitability indexes of Shannon-Wiener were computed for each population. Shannon-Wiener index was calculated such as $H' = -\sum_{i=1}^{R} pi \ln pi$ where p_i is the number of open flowers from a given species on the total number of open flowers.

Visitor assemblages

The composition, richness, diversity and visitation abundance of visitor assemblages of *R. ferrugineum* were assessed by conducting visitor counts in 2011 and 2012. Each year, the estimation of the number of visits consisted in 30 sessions of 10 min observation in 0.55 x 0.55 meters quadrats in each population (a total of 85 h of observation per year). The sessions were spread over the entire flowering period and between 10 am and 4 pm and sampling days were separated by 4-5 days. Insects were classified as Hymenoptera (10 *Bombus* using the color pattern proposed by Rasmont (1999) for Pyrenean bumblebees, *Apis mellifera*, solitary bees and other Hymenoptera), Diptera (Empididae, Syrphidae and other Diptera), Coleoptera and Lepidoptera (*Macroglossum stellatarum* and other Lepidoptera). In each quadrat, every flowers of *R. ferrugineum* visited by insects were recorded and the number of *R. ferrugineum* open flowers was counted, we then estimated the mean number of visits per quadrat per population (hereafter referred as visit density). Surveys were carried out during maximum flower blooming to avoid differences in the composition of assemblage between populations as a result of variations in food-resource availability. ANOVA were used to test for differences in mean visit density among years, populations, groups of visitors and interactions between years and groups of visitors and between populations and groups of visitors.

In each population, visitation abundance was estimated as the sum of all counts for each visitor groups. We calculated visitor richness (S_{obs}) as the number of visitor groups in each population. Diversity was calculated as the diversity and equitability indexes of Shannon-Wiener such as $H' = -\sum_{i=1}^{R} pi \ln pi$ where p_i is the abundance of visits from a visitor group on the total of visitor groups. We characterized the composition of visitor assemblage per population by means of detrended correspondence analysis (DCA). For each population, the composition was estimated through the scores of the first and second axis. We carried out DCA with the package Vegan in R software (v. 2.14.0; R core team).

General Linear Models (GLMs) were used to test the effect of composition, diversity and richness of visitors on mean visit density using R software and the selection of the best model was done according to the Akaike information criteria (AIC). The model that minimizes AIC provides the most reliable fit to the data (Sakomoto et al. 1986). We ranked models based on δAIC estimated as the difference between each AIC value and the smallest value. Models with δAIC ≤ 2 were considered to have substantial support (Burnham et al. 2002). Explaining variables were tested and the model with the lowest AIC criteria was selected.

In order to measure visitor traits, three sessions of 30 min capture were set up in all populations, in 3x3 meters quadrats. Capture sessions were spread over the entire flowering period and between 10 am and 4 pm. During each sessions every visitors of *R. ferrugineum* were captured using a net, killed with ethyl acetate, stored in individual tube and frozen until processed in the laboratory. Each visitor was then identified, and morphological traits such as total length, width, and mouthpiece length were measured.

The effects of visitor assemblage diversity, richness and composition on body size length and width and mouthparts length were tested by a one-way analysis of variance and then Tukey's HSD post-hoc tests (R software).

Impact of the environment on visitor assemblages

We used Pearson correlations to test the relationship between all couples of environmental variables (MCT, MLT, altitude, longitude, *R. ferrugineum* floral density and floral display).

To test the respective effect of altitude, longitude, MLT, MCT, *R. ferrugineum* floral display and floral density and diversity and richness of the surrounding plant community, General Linear Models (GLMs) were conducted on composition, diversity, richness and visitation abundance of visitor assemblages for each populations using R software and the selection of the best model was done according to the Akaike information criteria (AIC) as explained above.

RESULTS

Ecological context

MLT (1991 to 2013 period; Fig. 2), MCT 2011 and MCT 2012 during the flowering period (Table 1) ranged respectively from 9.47°C to 14.59°C, 10.72°C to 13.35°C, 12.94°C to 16.47°C depending on the populations. The three variables were positively correlated to longitude (MLT: $R^2 = 0.55$, Pvalue = 0.01; MCT 2011: $R^2 = 0.48$, Pvalue = 0.04; MCT 2012: $R^2 = 0.58$, Pvalue = 0.03). Mean temperatures increased significantly from west to east, indeed MLT was 12.70°C for population TecL at the western part of the Pyrenees and 14.59°C for population LecL at the eastern part of the Pyrenees for the lowland populations and was 9.47°C at TouH (west part) and 12.99°C at EynH (east part) for high altitude populations. Mean long-term and mean current temperatures were not correlated to altitude (Pvalue: 0.11, 0.77 and 0.29 respectively).

The total floral display (Table 1) varied greatly among populations and was the highest at SouH ($2.94\ 10^8$ flowers) and the lowest at LecL ($2.85\ 10^5$ flowers). Floral density per m^2 (Table 1) varied from 40.65 (LecL) to 862.79 (PeyL). Floral display and floral density were not correlated to each other (Pvalue= 0.31) or to temperatures, longitude and altitude (Pvalues > 0.05 in all cases).

At the Pyrenean mountain range, 134 plant species were determined in all populations (in average 17.6 species per population). The number of species found per population varied from 3 (LecL) to 31 (LuzH). Shannon index varied from 0.44 (TouH) to 1.72 (TroH) and the equitability index from 0.14 (TouH) to 1 (at LecL where the three determined species had an equal low abundance; Table 1). Plant richness was positively correlated to altitude ($R^2 = 0.48$, Pvalue = 0.004). Plant richness and diversity were not correlated to longitude or temperatures and plant diversity was not correlated to altitude.

Visitors of Rhododendron ferrugineum

Number of visits were estimated thanks to 10 200 minutes of observation (total of 2011 and 2012) for the 17 populations. 10455 visits were recorded, among which 7225 Hymenoptera (1790 *Apis mellifera*, 1557 wild bees, 2713 *Bombus lucorum*, 568 *Bombus pascuorum*, 332 *Bombus pratorum*, 154 *Bombus sylvestris* and 111 others Hymenoptera), 2521 Diptera (289 Empididae, 620 Muscidae, 472 Syrphidae and 1140 others Diptera), 488 Lepidoptera (among which 391 *Macroglossum stellatarum*) and 221 Coleoptera. According to these observations we clustered visitors into 14 groups: *Apis mellifera*, wild bees, *Bombus lucorum, Bombus pascuorum, Bombus pratorum, Bombus sylvestris*, others Hymenoptera, Empididae, Muscidae, Syrphidae, other Diptera, *Macroglossum stellatarum*, other Lepidoptera, Coleoptera.

Hymenoptera were the main visitors, representing 69 % of all visits in all populations. Both years, all populations were visited by at least two groups of Hymenoptera, except BetH and LuzH in 2012 (visited only by *Apis mellifera* in both cases). In all populations, Diptera were observed foraging on flowers (except at PraL where no Diptera were observed in the quadrats in 2011 (Fig. 3) but some of them were captured during the capture sessions). Diptera were the main visitors in two populations in 2011 (BouH and VicH) and in 2012 (LecL and BouH).

Temporal and spatial variation in visitor assemblages

The total number of visits (visit abundance) was higher in 2011 than in 2012 in all populations except EynH. It was lower from 0.24% (BetL) to 80% and 84% (VicH and TecL) in 2012 compared to 2011 (Fig. 4). Several populations experienced a drastic diminution of their main visitors. The number of visits from Hymenoptera dropped from 36% to 84% at LuzH, TecL, BouH, SouH, SouL, VicH and ChiL. In some populations this decrease in visitation from one insect group was compensated by the visits of other visitors. For example, the lower number of visits of *Bombus* species in 2012 compared to 2011 was compensated by a higher number of *Apis mellifera* visits at PuiH, BetL, BetH, VicL and TouH and a higher number of Diptera and Lepidoptera visits at LecL and PraL. Visitations from *Bombus* species, wild bees, Diptera, Lepidoptera and Coleoptera decreased in most of the populations whereas visitations from *Apis mellifera* increased in 10 populations.

Shannon Index varied from 1.18 (PraL) to 2.14 (LecL) in 2011 and from 0.42 (BetH) to 1.76 (SouL) in 2012 (Table 3). Equitability Index varied from 0.52 (BetL) to 0.93 (LecL) in 2011 and from 0.30 (BetH) to 0.95 (ChiL) in 2012. Conversely, S_{obs} varied from 5 (PraL) to 12

(SouL) in 2011 and from 4 (PuiH, VicL, BetH, TroH, LuzH) to 12 (ChiL) in 2012 up to the 14 groups of visitors (Table 3). S_{obs} decreased in 13 populations from 2011 to 2012 and increased in two populations (PraL and ChiL).

Composition of visitor assemblages is given by DCA (Table 2). In both years, despite the large variability in visitation abundance, we could observe almost the same insect pattern along the axis. The primary axis (Table 2a) separated mainly high altitude populations (except TroH) abundantly visited by *Apis mellifera*, wild bees and Muscidae (Fig. 5; Table 2b) from low altitude populations (except SouL) principally visited by *Bombus sp*. Low altitude populations segregated along the second axis from populations visited by *B. pratorum* and *B. pascuorum* (LecL) and/or an array of other visitors including *Macroglossum* (LecL, PeyL, VicL), Syrphidae (LecL, PeyL), Empididae (LecL) and low altitude populations abundantly visited by *Bombus lucorum* (BetL, TecL) and a set of others visitors such as *Bombus sylvestris* (TecL, ChiL) and Coleoptera (TecL). From 2011 to 2012, the main changes were observed at VicL and TroH where the composition changed from bumblebees to bees in both populations.

Impact of visitor assemblage on visitations

There were significant differences in the mean total number of visits per quadrat (visit density; Figure 3) between populations (P value < 2.2e-16), years (P value = 3.602 e-13) and the interaction between years and populations (P value = 2.89e-05). It was significantly higher in 2011 than in 2012. In 2011, PuiH, SouH, VicH and BetH had the lowest mean visit density while SouL had the highest. In 2012, VicH had the lowest and BetL the highest mean visit density.

Visit density decreased while increasing insect richness and increased when Coleoptera, *B. sylvestris*, Other Lepidoptera and *B. lucorum* were more abundant (second axis of composition; Table 4). Insect diversity and composition of the first axis had no impact on visit density. Thus, visit density is not significantly impacted whatever the main visitors of the assemblages were wild bees, *A. mellifera* and Muscidae or *Bombus* species and other Lepidoptera.

Visitor traits

Visitor functional traits were measured thanks to 1530 min of visitor' sampling (90 min per population in 2011). 522 visitors were sampled from the Hymenoptera, Diptera, Lepidoptera and Coleoptera orders (respectively 335, 161, 20 and 6 individuals sampled, see Table S1) and 53 different species were determined. Total length ranged from 4 mm (*Dasytes* *gonocerus;* Coleoptera) to 19.92 mm (*Bombus lucorum;* Hymenoptera), width ranged from 1.6 mm (*Platycheirus albimanus*; Syrphidae) to 8.95 mm (*Bombus lucorum*; Hymenoptera) and mouthpart ranged from 0.4 mm (*Apis mellifera;* Hymenoptera) to 42 mm (*Macroglossum stellatarum;* Lepidoptera). The body size gradient separated well the *Bombus* genus (with the highest body size) from *Apis mellifera* and wild bees (intermediate body size) and from the others genus (lowest body size). The mouthpiece lengths were highly variables among genus and even among species. Mean body length ranged from 8.47 mm (VicH) to 13.42 mm (BetL), mean width ranger from 3.06 mm (EynH) to 5.37 mm (ChiL) and mouthpart length ranged from 2.17 mm (VicH) to 5.68 mm (BetL).

Visitor length, width and mouthparts lengths were impacted significantly by both insect diversity and insect richness (Table 5). Sizes increased while increasing insect richness and diversity. They also were higher in populations mainly visited by *Bombus* species and other Lepidoptera than wild bees, *A. mellifera* and Muscidae and in populations visited mainly by Empididae, other Hymenoptera, *Macroglossum* and Syrphidae than Coleoptera, *B. sylvestris* and other Lepidoptera. They were higher along the first composition axis (*B. pascuorum, B. pratorum* and other Lepidoptera) and lower along the second composition axis (Other Hymenoptera, Empididae, *Macroglossum sp*).

Impact of the environment on R. ferrugineum visitor assemblages

Visitor diversity decreased significantly with increasing *R. ferrugineum* floral density and plant richness of the surrounding community (Table 6). Visitor richness (S_{obs}) and visitation abundance decreased significantly with increasing longitude; it was higher at the western part of the Pyrenees and increased significantly with increasing MLT. Visitation abundance also decreased while decreasing plant diversity of the surrounding community.

The first axis of assemblage composition was not significantly impact by any of the studied variables. The second axis of assemblage composition (*Bombus* species) was impacted by plant diversity. When plant diversity decreased there were less visitations from Empididae, Other Hymenoptera, *Macroglossum* and Syrphidae while when plant diversity increased there were more visitations from Coleoptera, *B. sylvestris*, Other Lepidoptera and *B. lucorum*.

DISCUSSION

R. ferrugineum appears to be a generalist species in that it is able to exploit a wide variety of potential pollinators, indeed 53 species were identified visiting it. However, as shown in previous studies conducted in the Alps and in one Pyrenean population, the main visitors of *R. ferrugineum* at the Pyrenean scale were Hymenoptera (Escaravage et al. 1997; Escaravage and Wagner 2004, Delmas et al. 2013). The abundance-richness relationship is frequent in pollinator assemblages (Steffan-Dewenter et al. 2002), *i.e.* few abundant species and high number of scarce species.

Spatial and temporal variations of visitor assemblages

Visitor assemblages were variable in space and time in the 17 studied Pyrenean populations of *R. ferrugineum*. We found spatial and temporal differences in term of diversity, richness, composition and visitation abundance in visitor assemblages. These results invalidated our first hypothesis which was that *R. ferrugineum* rely on the same visitor assemblage at any environmental conditions.

At a spatial scale, visitor diversity and equitability, richness and composition varied greatly. Many authors had shown variation in pollinator assemblages of a plant species along geographical gradient (Eckert 2002; Moeller 2005; 2006). Almost all high altitude populations were mainly characterized by *Apis mellifera*, wild bees and Muscidae whereas low altitude populations were characterized by either *B. pascuorum, B. pratorum*, Syrphidae and Empididae or by *B. lucorum* and *B. sylvestris*. Many studies had shown an altitudinal shift from Hymenoptera to Diptera (Muller 1880; Mani 1962; Pojar 1974; Arroyo et al. 1982; Primack 1983), but our data showed the presence of Hymenoptera (especially *A. mellifera* and wild bees) at high altitude. The presence of domestic bees at high altitude may be due in some cases to hives at short distance from the study populations or to a competition between bees and bumblebees at low altitude (Roubik 1978). Also, it is important to note that many studies in alpine habitat were done in mountains with a larger altitudinal range than the Pyrenees.

At a temporal scale, we also observed variation in visitor richness and diversity. However, composition did not vary a lot from 2011 to 2012. We observed variations in bumblebee assemblages but bumblebees variations from one year to the next had been previously reported in the eastern Pyrenees by Iserbyt and Rasmont (2013). The number of visits decreased significantly in 2012 compared to 2011. This may be due to the weather conditions of the previous year. Indeed, the year 2011 was a particularly rainy year (in July 126 mm at Saint Girons – Central Pyrenees; 116 mm at Tarbes – Western Pyrenees, for example), it may have lead to a decrease in visitor reproduction and thus a decrease in visitor abundance of some species in 2012. We observed a drastic declined in *Bombus* species visitations while *Apis mellifera* visitations increased in most of the populations. This increase even compensated the decline in *Bombus* species in some populations. This compensation phenomenon between insect species has already been described. Indeed, depending on the weather conditions and the life cycle of *Bombus* species (period of nest founding, rapidity of family life, for example), some species will be advantaged certain years (Bolotov et al. 2013). It is possible that in our case, the abundance of *Apis mellifera* increases when the abundance of *Bombus* species decrease and consequently compensate the visitation decline.

Effect of visitor assemblage variations on visits

As shown in previous studies conducted in the Alps and in one Pyrenean population, the main visitors of *R. ferrugineum* at the Pyrenean scale were Hymenoptera (Escaravage et al. 1997; Escaravage and Wagner 2004; Delmas et al. 2013). Many studies described bees as the most pollinators of diverse species and specifically Ericaceae (Free 1970; Haslerud 1975; Reader 1977). Lepidoptera and Coleoptera were rarely observed in *R. ferrugineum* flowers, and seem to be of little importance as pollinators while many Diptera were observed and thus may be considered as relatively important pollinators.

Visit density was impacted by insect richness; indeed it increased while increasing richness but was not impacted by insect diversity. Previous study (Hoehn et al. 2014) showed positively relation between bee richness and pollen deposition. High visitor richness may lead to higher visitation efficiency and thus to higher seed set. Visit density was also impacted by visitor composition; it increased when Coleoptera, *B. lucorum, other Lepidoptera* and *B. sylvestris* were more represented in the assemblages. Coleoptera and other Lepidoptera were relatively rare in the corteges but *B. lucorum* and *B. sylvestris* appeared to be efficient visitors of *R. ferrugineum*. We detected no change in visit density between corteges mainly composed of wild bees, *A. mellifera* and Muscidae and corteges composed of *Bombus* species. It seems that in term of visits wild bees, *A. mellifera* and Muscidae behave in the same way as *Bombus* species. There is some redundancy in visit density for these groups of insects. The variability of visitor assemblage may or not lead to variations in visit density. When the main visitors change from wild bees, *A. mellifera* and Muscidae to *Bombus* species, there is no changes in visit density (according to our Hyp. 2), while when the main visitors change from Coleoptera, *B. sylvestris*, Other Lepidoptera and *B. lucorum* to Empididae, other Hymenoptera, *Macroglossum* and Syrphidae, there is a decreased in visit density (according to our Hyp. 3).

Visitor length, width and mouthparts lengths were impacted significantly by both insect diversity and insect richness (Table 5). Sizes increased while increasing insect richness and diversity. They also were higher in populations mainly visited by *Bombus* species and other Lepidoptera than wild bees, *A. mellifera* and Muscidae and in populations visited mainly by

Empididae, other Hymenoptera, *Macroglossum* and Syrphidae than Coleoptera, *B. sylvestris* and other Lepidoptera. This may have potential impact on plant reproduction. Additionally to the visitation frequency, Diptera are smaller than Hymenoptera, thus it is probable that they carry less pollen grain and consequently may be less efficient than Hymenoptera for pollination. However, Bingham and Orthner (1998) results showed that pollination in alpine and foothill populations was more comparable than would be expected from visitation rates and insect diversity alone. The most important factors contributing to these results are the dominant role of bumblebees as pollinators in alpine populations and the longer duration of stigmatic receptivity in high-elevation plants. In our case, wild bees and *A. mellifera* seem to compensate the *Bombus* decline at high altitude and could maintain cross-pollination in high altitude *R. ferrugineum* populations. It would be interesting to study if all Hymenoptera have the same effectiveness in pollination and the effect of these shifts on the reproductive success of *R. ferrugineum*.

Impact of biotic and abiotic variables on visitor assemblage

Environmental conditions and biotic parameters such as composition of the surrounding community or population size are known to directly affect abundance and foraging behavior of individual species that determine per capita visitation rates (Price et al. 2005).

Effects of biotic parameters

The increase in *R. ferrugineum* flower density and plant richness of the surrounding community decreased visitor diversity of *R. ferrugineum*. Some studies have documented relationship between pollinator attractiveness and plant density (Klinkhamer and de Jong 1990; Kunin 1997; Nielsen and Ims 2000), while others have not (Aizen 1997; Bosch and Waser 2001). If species richness, abundance and assemblage of visitors had already been closely related to plant species richness they usually increased while increasing species richness of the co-flowering community (Tscharntke et al. 1998; Roulston and Goodell 2011). However, in our case, we only studied the diversity of *R. ferrugineum* visitors. When the richness of the surrounding plant community increase visitors may be attracted by other plant species and thus decreased the visitor diversity of *R. ferrugineum.*

Plant diversity of the surrounding community affected particularly the *Bombus* composition of the assemblages; we observed fewer visitations from *B. lucorum* and *B. sylvestris* when plant diversity was low. Arhné et al. (2009) also showed that *Bombus* composition was affected by local factors such as plants from the surrounding community.

Total floral display of *R. ferrugineum* had no impact on visitor abundance, diversity, richness or composition while it is known that large population attract more pollinators (Ohashi and Yahara 1999). Bumblebee species are known to differ in their response to the size of floral display. It had been shown that individuals of *Bombus pratorum* and *Bombus terrestris* were attracted to plants with larger floral displays, while *Bombus pascuorum* exhibited no increase in recruitment according to display size (Goulson et al. 1998). Antonini et al. (2013) also shown that larger fragments presented no significant increase in the number of bee species when compared to smaller fragments and the abundance of the most common species was higher in smaller fragments than in larger ones. Floral display seems to have variables effects on visitor species and plant density appears to be as important for the attractiveness of pollinators than floral display (Grindeland et al. 2005).

Effects of abiotic parameters

No correlation among altitude and temperatures was detected; this is explained by the lag between the blooming period at low (June) and at high altitudes (July).

Abundance and richness of visitors were impacted by longitude. They were lowest at the eastern part of the Pyrenees and less *Bombus* individuals were observed. It is known that many species of bumblebees are found in this area (Rasmont et al. 2000). During our sessions, we observed very few bumblebees and a large part of the visits were done by Diptera. Bees seemed particularly sensible to climatic variation and it has been shown that their abundance can decrease drastically from a year to another (Iserbyt and Rasmont 2013). High MLT increased visitor richness and visitation abundance. Evidence of direct temperature mediated effects on the abundance of pollinators is relatively rare (Hegland et al. 2009). In mountain plant communities, the types and relative importance of different visitor groups are expected to shift over altitudinal gradients (Mani 1962, Arroyo, Primack and Armesto 1982). The activity levels of pollinators have been shown to decrease significantly with altitude (Arroyo, Armesto and Primack 1985), and the diversity of insect species declines rapidly in high, temperate mountains (Arroyo et al. 1982). In our study, visitor composition seemed to separate low and high altitude populations, however altitude did not impact significantly the visitor assemblage.

To conclude, *R. ferrugineum* is visited by a large variety of visitors in space and time. These results on *R. ferrugineum* should add to a growing appreciation that pollination services are variable, even on short temporal and spatial scales and are sensible to environmental conditions. Variability in visitor assemblages may lead to variation in visitation efficiency (Hyp. 3) or not (Hyp. 2). We observed a certain redundancy in term of visitation between wild bees, *A. mellifera* and *Bombus* species. Like other insect pollinators, many bumblebees and honeybees species, which are the main visitors of *R. ferrugineum*, have suffered severe declines in recent decades (Williams et al. 2007; Goulson et al. 2008; Cameron et al. 2011) and research suggests that climatic variations could be driving this widespread decrease (Goulson et al. 2008; Williams et al. 2009; Iserbyt and Rasmont, 2012). However, this redundancy among species may be particularly advantageous in the context of climate change. It is widely recognized that visitation rate is at best a crude index of pollination service because pollinator species vary in per visit effectiveness (Beatie 1971; Primack and Silander 1975; Motten et al. 1981; Schemske and Horvitz 1984; Wilson 1995), however it would be interesting to measure other component of effectiveness. Moreover, variation in pollination services has important potential implication for plant ecology, including seed production and population dynamics. We will thus investigate the relationship between pollination service and plant fitness.

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FIGURES AND TABLES

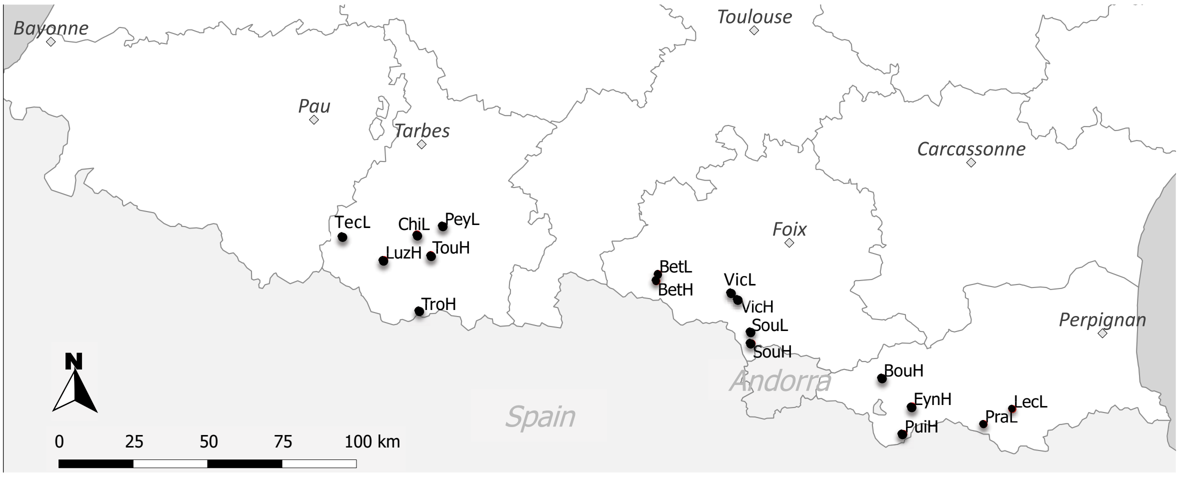

Figure 1. Location of the seventeen *R. ferrugineum* populations in the French Pyrenees.

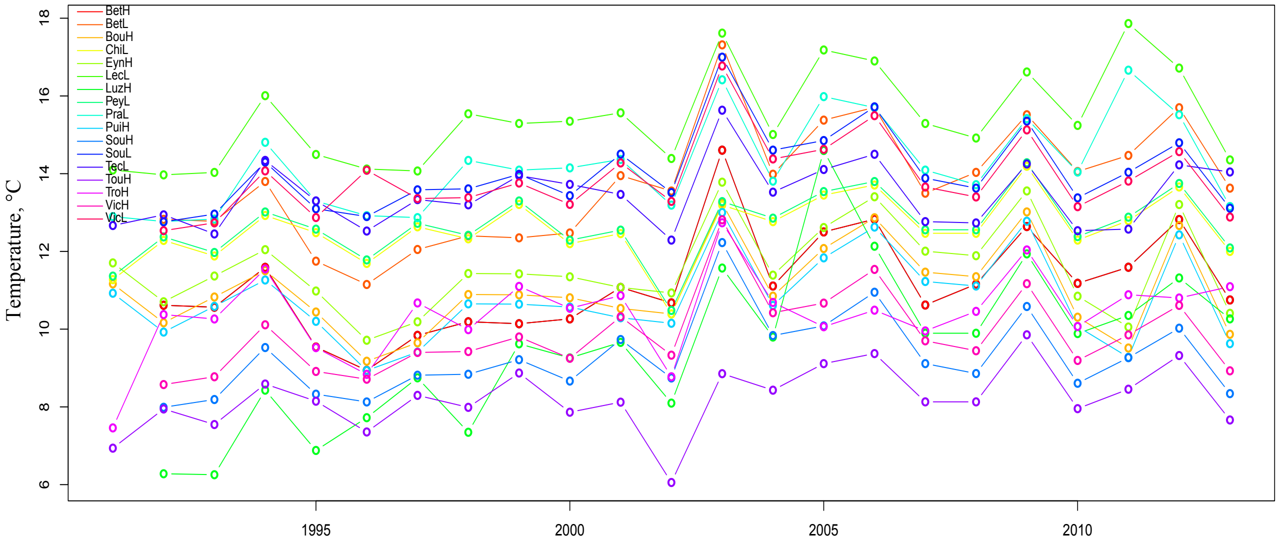

Figure 2. Mean long-term temperature (MLT) from 1991 to 2013 for the seventeen population of *R. ferrugineum*.

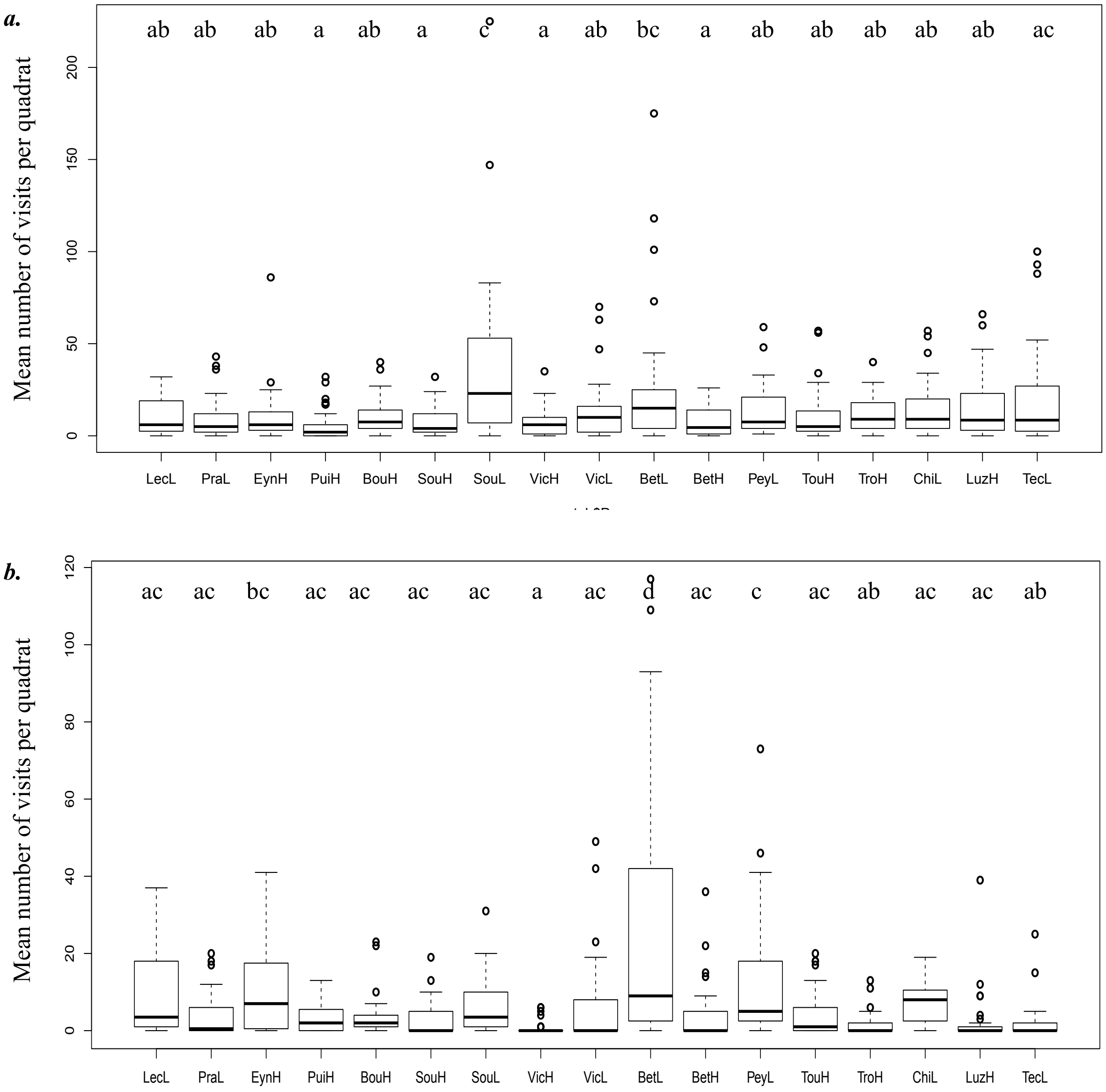

Figure 3. Mean density of visits per populations a. in 2011, b. in 2012.

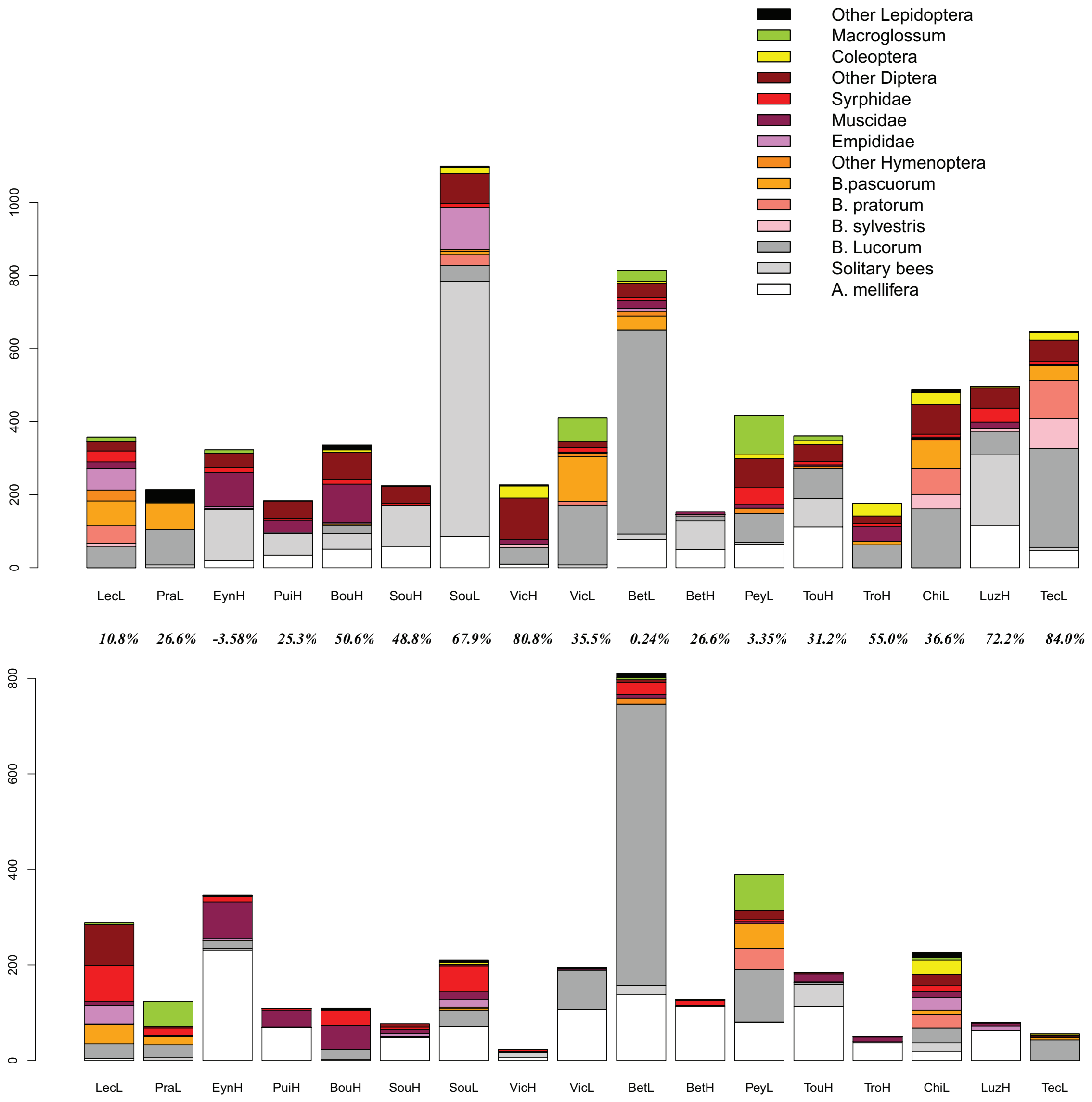

Figure 4. Total number of visits per populations in 2011 and 2012 and pourcentage of visitation decreased among years.

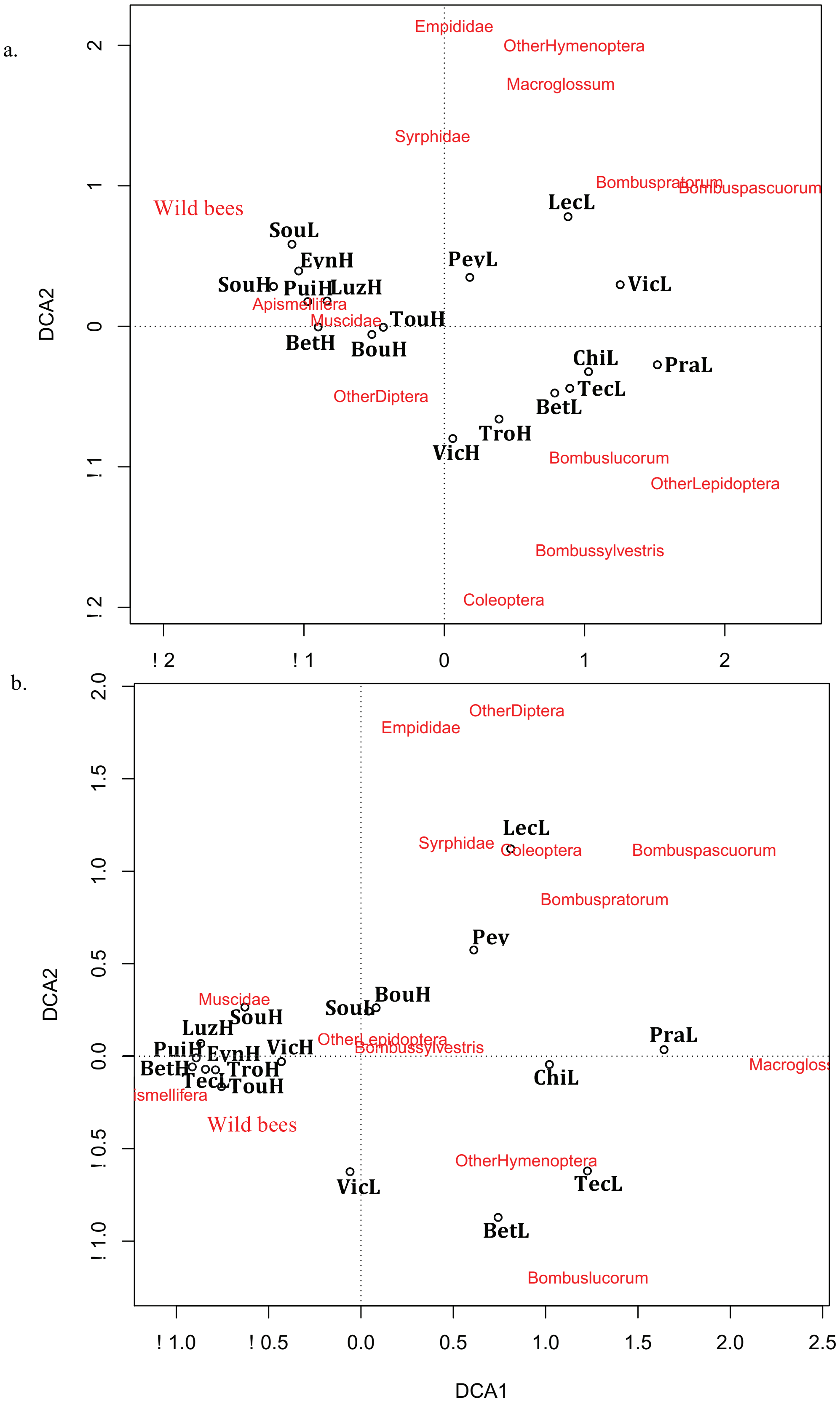

Figure 5. Results of the DCA with populations and groups of visitors.

Table 1. Geographical information and characteristics of *R. ferrugineum* populations. MLT = mean long-term temperatures (from 1991 to 2012), MCT = mean current temperatures (data loggers).

Mountain range	**Population**	**Code population**	**Location**	**Altitude (m)**	**Floral display**	**Flower density per m^2**	**MLT (°C) from 1991 to 2012**	**2011 MCT (°C)**	**2012 MCT (°C)**	**Abundance of co-flowering species**	**Richness of co-flowering species**	**Shannon Index of co-flowering plants**	**Equitability index**
Eastern Pyrenees	Léca	LecL	42°28'07"N 02°31'40"E	1250	2.84 10^5	40.65	14.59 ± 0.59	13.10	16.47	0.03	3	1.09	1
	Prats de Mollo	PraL	42°25'21"N 02°24'38"E	1450	1.32 10^6	147.02	13.39 ± 0.59	12.70	NA	1.01	17	0.87	0.30
	Eyne	EynH	42°28'27"N 02°07'05"E	1915	5.59 10^6	98.23	12.99 ± 1.05	13.03	14.37	15.02	12	1.27	0.51
	Puigmal	PuiH	42°23'26"N 2°05'0"E	2045	4.21 10^7	610.51	12.21 ± 1.00	13.35	14.99	65.62	19	1.20	0.40
	Lac des Bouillouses	BouH	42°33'31"N 01°59'42"E	2005	1.96 10^8	293.21	12.45 ± 1.06	13.08	14.16	15.51	17	1.53	0.54
Central Pyrenees	Soulcem	SouH	42°39'28"N 01°27'27"E	2035	2.94 10^8	585.75	10.55 ± 0.97	11.97	13.34	22.74	15	0.94	0.34
	Soulcem	SouL	42°41'28"N 01°27'07"E	1240	3.02 10^6	302.30	13.99 ± 1.76	12.04	14.43	70.22	16	0.76	0.27
	Etang de Lhers	VicL	42°48'23"N 01°22'31"E	1277	2.49 10^7	530.04	13.76 ± 1.76	12.65	NA	21.51	23	1.35	0.43
	Etang de Lhers	VicH	42°47'36"N 01°24'16"E	1940	1.21 10^8	325.16	11.14 ± 0.97	10.72	14.25	72.92	22	0.88	0.28
	Bethmale	BetL	42°51'42"N 01°04'02"E	1385	1.11 10^7	621.91	13.20 ± 2.11	11.69	13.71	48.03	18	1.19	0.41
	Bethmale	BetH	42°50'24"N 01°03'42"E	1920	6.14 10^8	109.97	11.84 ± 0.95	12.87	15.54	88.85	28	0.62	0.18
	Le Peyras	PeyL	42°59'15"N 00°10'46"E	1220	4.83 10^7	862.79	12.00 ± 0.58	11.42	12.94	2.36	10	1.44	0.62
	Col du Tourmalet	TouH	42°53'57"N 00°07'54"E	1950	1.90 10^7	347.12	9.47 ± 0.43	12.00	NA	82.89	24	0.44	0.14
	Cirque de Troumouse	TroH	42°43'41"N 00°05'40"E	2080	2.95 10^7	469.01	10.83 ± 0.52	11.33	14.07	9.72	21	1.72	0.55
	Chiroulet	ChiL	42°57'38"N 00°04'17"E	1240	1.75 10^6	125.38	11.91 ± 0.63	11.91	13.19	1.88	16	1.25	0.46
	Lac du Tech	TecL	42°54'40"N 00°15'32"W	1240	1.48 10^5	148.16	12.70 ± 0.65	13.17	NA	0.98	8	1.30	0.62
	Luz Ardiden	LuzH	42°52'45"N 00°03'34"W	1905	8.18 10^7	371.89	10.94 ± 2.69	11.54	14.3	8.86	31	1.05	0.30

Table 2. Results of the DCA (a) for 4 axis in 2011 and 2012 and (b) for the correlation among visitor groups and axis 1 and 2.

a.

2011	DCA1	DCA2	DCA3	DCA4
Eigenvalues	0.53	0.20	0.17	0.15
Decorana values	0.53	0.14	0.06	0.02
Axis lenghts	2.73	1.57	1.25	1.15

2012	DCA1	DCA2	DCA3	DCA4
Eigenvalues	0.55	0.27	0.17	0.18
Decorana values	0.56	0.19	0.07	0.03
Axis lenghts	2.55	1.99	1.45	1.42

b.

	2011		**2012**	
	DCA 1	**DCA2**	**DCA 1**	**DCA2**
A. mellifera	-1.02	0.14	-1.08	-0.21
Wild bees	-1.73	0.63	-0.38	-0.32
B. lucorum	1.17	-0.93	1.22	-1.19
B. sylvestris	1.10	-1.60	0.31	0.03
B. pratorum	1.53	1.01	1.31	0.83
B. pascuorum	2.18	0.97	1.85	1.10
Other Hymenoptera	0.92	1.98	0.89	-0.57
Empididae	0.07	2.12	0.32	1.76
Muscidae	-0.7	0.04	-0.68	0.30
Syrphidae	-0.08	1.34	0.51	1.14
Other Diptera	-0.45	-0.50	0.84	1.86
Coleoptera	0.42	-1.95	0.97	1.10
Macroglossum	0.83	1.71	2.39	-0.05
Other Lepidoptera	1.93	-1.12	0.11	0.08

Table 3. Visitor richness, Shannon and equitability index and mean length, width and mouthparts lengths of pollinators in 2011 and 2012 in 17 populations of *R. ferrugineum*.

Different letters indicate significant differences.

Moutain range	**Code population**	**Visitor richness 2011**	**Shannon Index of visitors 2011**	**Equitability Index of visitors 2011**	**Visitor richness 2012**	**Shannon Index of visitors 2012**	**Equitability Index of visitors 2012**	**Lenght**	**Width**	**Mouthparts length**
Eastern Pyrenees	LecL	10	2.14	0.93	9	1.74	0.79	9,50	3,45	3,10
	PraL	5	1.18	0.7	7	1.53	0.78	11,11	4,33	5,59
	EynH	8	1.48	0.71	8	1.01	0.48	9,05	3,06	2,98
	PuiH	7	1.56	0.80	4	0.79	0.57	11,24	4,07	2,68
	BouH	10	1.85	0.80	7	1.31	0.67	9,67	3,48	2,49
Central Pyrenees	SouH	7	1.19	0.61	6	1.25	0.69	10,14	3,59	2,31
	SouL	12	1.32	0.54	11	1.76	0.73	11,67	4,24	4,11
	VicL	10	1.54	0.67	4	0.80	0.58	12,21	4,96	4,75
	VicH	7	1.42	0.73	5	1.37	0.85	8,47	3,41	2,16
	BetL	11	1.25	0.52	9	0.95	0.43	13,42	5,82	5,67
	BetH	7	1.47	0.75	4	0.42	0.30	11,20	4,02	2,65
	PeyL	9	1.87	0.85	9	1.77	0.80	9,09	3,20	2,28
	TouH	9	1.73	0.78	8	1.14	0.55	10,79	3,77	2,56
	TroH	6	1.56	0.87	4	0.80	0.58	10,02	4,14	2,33
	ChiL	11	1.86	0.77	12	2.37	0.95	12,55	5,37	4,47
	TecL	12	1.78	0.71	5	0.85	0.52	12,56	4,98	5,20
	LuzH	9	1.64	0.74	4	0.70	0.50	10,75	3,95	2,32

Table 4. Statistical results for the GLMs testing the effects of diversity, richness and composition of visitor assemblage on mean visit density (number of visits per quadrat). Significant P values are in bold.

	Estimate	**Standard error**	**pvalue**
Intercept	-3.66	3.08	0.23
Composition axis 2	-3.32	1.39	**0.01***
Insect richness	2.01	0.30	**9.14e-11**

Table 5. Statistical results for the GLMs testing the effects of composition, richness and diversity of visitor assemblage on mean visitor length, width and mouthparts length. Significant P values are in bold.

Visitor lenght		**Estimate**	**Standard error**	**pvalue**
	Intercept	10.68	0.93	**<2e-16*****
	Composition axis 1	0.44	0.12	**0.0003*****
	Composition axis 2	-0.41	0.12	**0.0008*****
	Insect diversity	-2.5	0.59	**3.11e-05*****
	Insect richness	0.37	0.07	**1.16e-06*****
Visitor width		**Estimate**	**Standard error**	**pvalue**
	Intercept	4.05	0.46	**<2e-16*****
	Composition axis 1	0.38	0.06	**9.92e-103*****
	Composition axis 2	-0.37	0.06	**2.72e-09*****
	Insect diversity	-1.36	0.29	**5.37e-06*****
	Insect richness	0.18	0.03	**6.96e-07*****
Visitor mouthparts lenght		**Estimate**	**Standard error**	**pvalue**
	Intercept	0.78	0.62	0.21
	Composition axis 1	0.41	0.08	**1.02e-06*****
	Composition axis 2	-0.41	0.08	**9.54e-07*****
	Insect diversity	-1.24	0.40	**0.002****
	Insect richness	0.43	0.05	**<2e-16*****

Table 6. Statistical results for the GLMs testing the effects of ecological gradients on diversity, richness, visitation abundance and composition of visitor assemblages of *R. ferrugineum* populations. Significant P values are in bold.

Visitor diversity		**Estimate**	**Standard error**	**pvalue**
	Intercept	5.40	9.05	**5.23e-06*****
	Longitude	-1.63e-01	8.36e-02	0.06
	Floral display	2.58e-09	1.55e-09	0.11
	Floral density	-6.00e-04	2.86e-04	**0.04***
	Plant richness	-5.40e-02	1.37e-02	**0.0007*****
	Plant diversity	-4.53e-01	3.29e-01	0.18
Visitor richness		**Estimate**	**Standard error**	**pvalue**
	Intercept	-2.77	3.46	0.43
	Longitude	-1.22	0.42	**0.008****
	MLT	1.06	0.30	0.56
Visitor abundance		**Estimate**	**Standard error**	**pvalue**
	Intercept	-174.58	449.30	**0.02***
	Longitude	-104.74	68.67	**0.01***
	Plant diversity	-235.95	21.95	**0.03***
	MLT	1.21e02	2.78e01	**0.003****
Visitor composition Axis 1		**Estimate**	**Standard error**	**pvalue**
	Intercept	1.86	2.28	0.42
	Longitude	-0.31	0.19	0.11
	MLT	0.28	0.15	0.07
	Plant richness	-0.05	0.03	0.09
Visitor composition Axis 2		**Estimate**	**Standard error**	**pvalue**
	Intercept	2.48	5.23e-01	**7.31e-05*****
	Floral display	3.01e-09	1.79e-09	0.10
	Plant richness	-4.24e-02	1.44e-02	0.0069
	Plant diversity	-7.82e-01	3.65e-01	**0.04***

ANNEXE

Table S1. Determination and number (n) of insects captured on each *R. ferrugineum* population.

Population	Order	Family	Genus	Species	n
LecL	Hymenoptera	Apidae	*Bombus*	*lucorum*	6
		Apidae	*Bombus*	*pascuorum*	2
		Apidae	*Bombus*	*pratorum*	6
		Apidae	*Lasioglossum*	*calceatum*	1
		Other Hymenoptera	NI	NI	2
	Diptera	Empididae	*Empis*	*abdominalis*	1
		Syrphidae	*Melanostoma*	*scalare*	1
		Syrphidae	*Platycheirus*	*albimanus*	2
		Syrphidae	*Episyrphus*	*balteatus*	2
		Syrphidae	*Sphaerophoria*	*scripta*	2
		Muscidae	NI	NI	2
		Other Diptera	NI	NI	5
PraL	Hymenoptera	Apidae	*Bombus*	*pascuorum*	10
		Apidae	*Bombus*	*pratorum*	4
		Other Hymenoptera	NI	NI	2
	Diptera	Syrphidae	*Eristalis*	*tenax*	3
		Syrphidae	*Meliscaeva*	*auricollis*	1
		Syrphidae	*Episyrphus*	*balteatus*	2
		Syrphidae	*Eristalis*	*similis*	1
		Syrphidae	*Sphaerophoria*	*scripta*	3
		Syrphidae	*Platycheirus*	*albimanus*	2
		Muscidae	NI	NI	6
	Lepidoptera	Pieridae	*Pieris*	*brassicae*	1
		Sphingidae	*Macroglossum*	*stellatarum*	3
EynH	Hymenoptera	Apidae	*Bombus*	*lucorum*	3
		Apidae	*Apis*	*mellifera*	6
		Other Hymenoptera	NI	NI	1
	Diptera	Muscidae	NI	NI	13
		Empididae	*Empis*	*pandellei*	3
		Syrphidae	*Meliscaeva*	*cinctella*	1
		Syrphidae	*Eupeodes*	*nielseni*	1
		Muscidae	NI	NI	1
	Lepidoptera	Papilionoidea	*Clossiana*	*euphrosyne*	5
PuiH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	3
		Apidae	*Bombus*	*lucorum*	1
		Apidae	*Bombus*	*pratorum*	1
		Apidae	*Apis*	*mellifera*	21
		Apidae	*Halictus*	*rubicundus*	1
		Apidae	*Apis*	*NI*	1
	Diptera	Syrphidae	*Parasyrphus*	*vittiger*	2
		Syrphidae	*Platycheirus*	*scutatus*	1
		Syrphidae	*Platycheirus*	*albimanus*	2
		Syrphidae	*Lapposyrphus*	*lapponicus*	1
		Syrphidae	*Eupeodes*	*luniger*	1
		Syrphidae	*Eristalis*	*similis*	1
	Coleoptera	Cantharidae	*Rhagonycha*	*gallica*	1
BouH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	4
		Apidae	*Bombus*	*pratorum*	2
		Apidae	*Apis*	*mellifera*	10

		Apidae	*Apis*	*NI*	1
		Other Hymenoptera	NI	NI	1
	Diptera	Syrphidae	*Parasyrphus*	*vittiger*	1
		Syrphidae	*Platycheirus*	*albimanus*	1
		Syrphidae	*Melanostoma*	*mellinum*	1
		Syrphidae	*Sphaerophoria*	*laurae*	1
		Syrphidae	*Eupeodes*	*corrolae*	1
		Syrphidae	*Sphaerophoria*	*scripta*	1
		Muscidae	NI	NI	13
SouH	Hymenoptera	Apidae	*Apis*	*mellifera*	18
		Other Hymenoptera	NI	NI	1
	Diptera	Muscidae	NI	NI	8
	Lepidoptera	Geometridae	*Colostygia*	*turbata*	1
		Adelidae	*Adela*	*reamurella*	1
SouL	Hymenoptera	Apidae	*Bombus*	*pascuorum*	1
		Apidae	*Apis*	*mellifera*	17
	Diptera	Syrphidae	*Sphaerophoria*	*scripta*	1
		Muscidae	NI	NI	1
	Coleoptera	Oedemeridae	*Oedemera*	*virescens*	1
	Lepidoptera	Noctuoidea	*Euclidia*	*glyphica*	2
		Papilionoidea	*Coenonympha*	*pamphilus*	1
		Papilionoidea	*Clossiana*	*euphrosyne*	1
VicH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	4
		Apidae	*Bombus*	*lucorum*	2
		Apidae	*Bombus*	*pratorum*	1
	Diptera	Muscidae	NI	NI	6
		Other Diptera	NI	NI	2
VicL	Hymenoptera	Apidae	*Bombus*	*lucorum*	3
		Apidae	*Bombus*	*pratorum*	7
		Apidae	*Bombus*	*pascuorum*	13
		Apidae	*Apis*	*mellifera*	2
	Diptera	Syrphidae	*Platycheirus*	*albimanus*	4
		Syrphidae	*Sphaerophoria*	*scripta*	1
		Syrphidae	*Melanostoma*	*scalare*	1
BetL	Hymenoptera	Apidae	*Bombus*	*pascuorum*	21
		Apidae	*Bombus*	*pratorum*	3
		Apidae	*Bombus*	*lucorum*	1
		Apidae	*Bombus*	*sylvestris*	1
		Apidae	*Apis*	*mellifera*	1
		Other Hymenoptera	NI	NI	2
	Diptera	Syrphidae	*Lapposyrphus*	*lapponicus*	1
		Syrphidae	*Parasyrphus*	*macularis*	1
		Syrphidae	*Platycheirus*	*albimanus*	1
		Muscidae	NI	NI	2
	Lepidoptera	Noctuidae	*Euclidia*	*glyphica*	1
		Sphingidae	*Macroglossum*	*stellatarum*	1
BetH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	1
		Apidae	*Bombus*	*lucorum*	1
		Apidae	*Bombus*	*sylvestris*	1
		Apidae	*Apis*	*mellifera*	12
		Apidae	*Apis*	*NI*	1
	Diptera	Syrphidae	*Platycheirus*	*tatricus*	1
		Syrphidae	*Eristalis*	*tenax*	1
		Muscidae	NI	NI	4
PeyL	Hymenoptera	Apidae	*Bombus*	*lucorum*	3

		Apidae	*Bombus*	*pascuorum*	1
		Symphite		*tenthrede*	1
		Apidae	*Apis*	*mellifera*	3
	Diptera	Syrphidae	*Platycheirus*	*albimanus*	8
		Muscidae	NI	NI	3
	Coleoptera	Staphylinidae	*Philonthus*	*marginatus*	1
TouH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	2
		Apidae	*Apis*	*mellifera*	28
		Apidae	*Apis*	*NI*	1
		Apidae	*Andrene*	*NI*	2
		Other Hymenoptera	NI	NI	2
	Diptera	Muscidae	NI	NI	6
		Syrphidae	*Scaeva*	*selenitica*	1
		Syrphidae	*Eristalis*	*similis*	1
		Other Diptera	NI	NI	4
TroH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	12
		Apidae	*Bombus*	*pratorum*	2
		Apidae	*Bombus*	*lucorum*	5
		Apidae	*Halictus*	*rubicundus*	1
		Other Hymenoptera	NI	NI	2
	Diptera	Syrphidae	*Scaeva*	*selenitica*	3
		Syrphidae	*Platycheirus*	*tatricus*	1
		Muscidae	NI	NI	4
		Other Diptera	NI	NI	2
	Coleoptera	Dasytidae	*Dasytes*	*gonocerus*	2
ChiL	Hymenoptera	Apidae	*Bombus*	*sylvestris*	3
		Apidae	*Bombus*	*pascuorum*	5
		Apidae	*Bombus*	*lucorum*	2
		Apidae	*Bombus*	*pratorum*	6
	Diptera	Syrphidae	*Eristalis*	*tenax*	1
		Muscidae	NI	NI	1
	Lepidoptera	Geometridae	*Ematurga*	*atomaria*	1
TecL	Hymenoptera	Apidae	*Bombus*	*lucorum*	5
		Apidae	*Bombus*	*pascuorum*	6
		Apidae	*Bombus*	*sylvestris*	1
		Apidae	*Bombus*	*pratorum*	8
		Apidae	*Apis*	*mellifera*	1
		Apidae	*Eucera*	*NI*	1
		Other Hymenoptera	NI	NI	2
	Diptera	Muscidae	NI	NI	1
		Syrphidae	*Eupeodes*	*luniger*	1
		Syrphidae	*Chrysotoxum*	*marginatum*	1
		Syrphidae	*Merodon*	*rufus*	1
		Empididae	*Empis*	*pandellei*	3
		Empididae	*Empis*	*testacea*	1
	Coleoptera	Scarabaeidae	*Trichius*	*fasciatus*	1
	Lepidoptera	Sphingidae	*Hemaris*	*fuciformis*	1
		Geometridae	*Ematurga*	*atomaria*	1
LuzH	Hymenoptera	Apidae	*Bombus*	*sylvestris*	1
		Apidae	*Bombus*	*pascuorum*	3
		Apidae	*Bombus*	*lucorum*	2
		Apidae	*Apis*	*mellifera*	15
		Apidae	*Andrene*	*NI*	1
		Apidae	*Lasioglossum*	*fratellums*	1
		Apidae	*Apis*	*NI*	1

		Apidae	*Lasioglossum*	*calceatum*	1
		Other Hymenoptera	NI	NI	3
	Diptere	Muscidae	NI	NI	2
		Syrphidae	*Platycheirus*	*albimanus*	1

VARIABILITY OF POLLINATOR ASSEMBLAGES EFFECTIVENESS AND IMPACT ON PLANT MATING SYSTEM OF *RHODODENDRON FERRUGINEUM.*

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ABSTRACT

Taxonomic variation in pollination effectiveness is usual. As pollinator assemblages can vary spatially, the effectiveness of pollinator assemblages may be highly variable. For plant species with a mixed mating system, variation in pollinator assemblage could influence the degree of self-fertilization versus outcrossing and thus influence the genetic structure of species.

We compared several components of pollinator effectiveness (proportion of visited flowers, quantity and quality of insect and stigmatic pollen load) in 17 *R. ferrugineum* populations in the Pyrenees. We investigated how pollinator effectiveness varied depending on visitor diversity, richness and composition and what were the links between insect pollen load, stigmatic pollen load, seed set and selfing rates.

Pollinator effectiveness varied greatly among groups of pollinators and populations. *Bombus lucorum*, *Apis mellifera* and wild bees appeared to be the most efficient pollinators of *R. ferrugineum* while other Hymenoptera and Diptera were less efficient. Some groups of visitors showed redundancy in term of effectiveness and if one species tend to disappear it may be replaced by another one to maintain the pollination of *R. ferrugineum*. Although they were a high variability in pollinator assemblage effectiveness we did not detect link between effectiveness, seed set and selfing rates.

Key words: altitude, effectiveness, Ericaceae, insect pollen load, *Rhododendron ferrugineum,* selfing, stigmatic pollen load, visitation, visitor assemblages

INTRODUCTION

There are strong interests to know how taxonomic composition of pollinator assemblages affect plant mating system, especially with associated widespread concern over the disruption of pollinators faunas by human activity (Buchmann and Nabhan 1996; Kearns et al. 1998; Kremen and Ricketts 2000). Indeed, pollinators are threatened by global changes (Biesmeijer et al. 2006) and consequently the pollination services of plant community may endure drastic change and have strong impact on plant fitness. However, few studies have determined whether and how variation in pollinator assemblage is associated with pollination effectiveness and what are the impacts on plant mating system (Herrera 2000; Schimdt-Adam et al. 2000; Eckert 2002). For plant species with a mixed mating system, variation in pollinator assemblage could influence the degree of self-fertilization versus outcrossing and thus influence the genetic structure of species.

Several studies have shown that composition of pollinator assemblage can vary significantly in space and time (Pellmyr 1986; Herrera 1988; Schimdt-Adam et al. 2000; Fenster and Dudash 2001; Devoto et al. 2005; Petanidou et al. 2008; Alarcon et al. 2008; Jacobs et al. 2009). Local pollinator assemblage pattern is determined by a set of biotic and abiotic factors such climatic conditions (Jacobs et al. 2009), characteristics of the surrounding community and its own population pattern (Goulson et al. 1998). Insect taxa differ in plant species preference, in foraging strategies, behavior and response to resource pattern (Natalis and Wesselingh 2012). They may show variations in visitation rates, pollen removal deposition abilities and/or variable handling time (Schemske and Horvitz 1984; Tepedino et al. 1999; Ivey et al. 2003; Adler and Irwin 2006; Sahly and Conner 2007; Ferrero et al. 2011) and thus vary in their effectiveness in pollination and in their contribution to plant fitness (Schemske and Horvitz 1984; Herrera 1987; Kandori 2002; Artz et al. 2010). The effectiveness of a pollinator or an assemblage of pollinators can be decompose in various components such as pollen load (Primack and Silander 1975; Kephart 1983) visit duration (Fishbein and Venable 1996; Pellmyr and Thompson 1996), visit frequency (Primack and Silander 1975; Utelli and Roy 2000), pollen removal (Young and Stanton 1990; Conner et al. 1995), pollen deposition (Thomson 1986) or effect on fruit/seed set (Spears 1983; Dieringer 1992). For example, social bees are generally viewed as systematic foragers that visit a high proportion of flowers in an area and often return to the same area between foraging boots (Handel 1983; Waddington 1983), whereas butterflies often search for mates and oviposition sites while foraging and thus forage in short, often interrupted, bouts and move greater distances between flowers (Murawski and Gilbert 1986; Herrera 1987). Consequently, variation in pollinator assemblage may lead to variation in pollinator effectiveness. However, decrease in variation effectiveness may be buffered if there is redundancy among visitor species in pollinator assemblages *i.e.* several species have comparable effectiveness for the pollination a given plant species. For example, if two different pollinator species (A and B) have the same effectiveness, a shift from species A to species B will lead to no change in effectiveness of the pollinator assemblage. At a higher level, redundancy can exist between two different pollinator assemblages if they have comparable effectiveness for a given plant species.

The challenge in pollination system is to evaluate the individual and collective effectiveness of visitors in pollination and the degree of redundancy among visitor species. These needs to measure and compare the effectiveness of different visitors and assemblages in achieving pollination and consequent seed set have been a longstanding problem in pollination biology (Motten et al. 1981; Snow 1982). It is under a great interest in the context of global change. Indeed, authors suggest that pollinator assemblages that contain a wide range of species with different ecological requirements could maintain pollination services as environmental conditions change over time because decline in abundance of some taxa can be offset by increases in others (Yachi and Lorreau 1999; McCann 2000; Elmqvist et al. 2003). However, the maintenance of pollination service would be maintained if declining and increasing pollinators are at some extent functionally redundant. Thus, the degree of redundancy into and between pollinator assemblages could determine the potential of a plant species to cope with global changes.

In a previous study, we showed a high variability in visitor assemblages of *Rhododendron ferrugineum* in space and time (Charrier et al. in prep). Here, we investigated how the variability of pollinator assemblages affects their effectiveness and what are the impacts on pollination and mating system in various environmental conditions. In 17 populations of *R. ferrugineum* distributed along the Pyrenean chain: (1) we analyzed the effectiveness of visitor groups and the effectiveness of the entire pollinator assemblages in pollen transport. Pollinators with the highest proportion of visited flowers and carrying the highest proportion of *R. ferrugineum* pollen grains will be the most efficient groups. We have shown that high altitude populations were characterized by *Apis mellifera*, wild bees and Muscidae while low altitude populations were mainly visited by *Bombus* species. Diptera were the main visitors of few populations. Hymenoptera are known to be more efficient pollinators and due to their large size, they are expected to carry more pollen than other pollinators such as Diptera. Consequently, populations with a high proportion of Hymenoptera should have a more efficient pollinator assemblage. However, there are differences in visitation constancy among pollinator groups (Heinrich et al. 1976), leading to differences in proportion of conspecific pollen carried by pollinators and thus in effectiveness among them. (2) We studied how the effectiveness of visitor assemblages changes with richness, diversity, visitation abundance and composition of visitor assemblage. Effectiveness is supposed to increase with richness, diversity and visitation abundance. Populations with the highest proportion of the most efficient pollinator groups should have the most efficient assemblages. (3) We investigated the links between effectiveness, pollen deposition, seed set and selfing rates. We hypothesize that, stigmatic pollen loads and seed sets would increase and selfing rates decrease with effectiveness of pollinator assemblage. Inversely, a higher selfing rate is expected to compensate for a low pollinator assemblage's effectiveness. Theoretical models have demonstrated that pollen limitation due to poor or variable pollination environments can select for high selfing rates principally because the reproductive assurance value of selfing minimizes the ability of inbreeding depression, pollen discounting, and seed discounting to maintain outcrossing (Morgan and Wilson 2005; Porcher and Lande 2005).

MATERIAL AND METHODS

Species studied

Rhododendron ferrugineum L. (Ericaceae) is an evergreen shrub with a mean height of 70 cm that dominates subalpine landscapes mainly in the Alps and in the Pyrenees between 1500 to 2200 m a.s.l. on north-to-west facing slopes, sometimes reaching 90-100% of the vegetation cover. In the Pyrenees, infrequent smaller and isolated populations can be found lower down, between 900–1500 m a.s.l. *Rhododendron ferrugineum* produces inflorescences with 5–28 bright-red nectariferous tubular flowers; in some places it can produce more than 3000 flowers per m^2. The flowering period of this species lasts approximately one month, the blooming period ranges from end of May to mid-July depending on the altitude and the climatic conditions. Seeds are mature four to five weeks after the end of the blooming period. *Rhododendron ferrugineum* reproduces both vegetatively through layering and sexually. It has previously been described to be a self-compatible species and spontaneous selfing occurs in the absence of pollinators (Escaravage et al. 1998). Geitonogamy is possible and is facilitated by pollinator foraging behavior.

Seventeen populations were studied along a longitudinal transect running the length of the Pyrenees (Figure 1). The sampled populations ranged between 1220 m and 2080 m a.s.l (Table 1).

Characteristics of pollinator assemblages

Visitor assemblages were previously determined for the 17 *R. ferrugineum* populations (Charrier et al. in prep). Insects were classified as Hymenoptera (10 *Bombus* using the color pattern proposed by Rasmont (1999) for Pyrenean bumblebees, *Apis mellifera*, wild bees, other Hymenoptera), Diptera (Empididae, Syrphidae, other Diptera), Coleoptera and Lepidoptera (*Macroglossum stellatarum* and other Lepidoptera).

The composition, richness and diversity of *R. ferrugineum* visitor assemblages were assessed by conducting visitor counts during the 2011 flowering season. The estimation of the number of visits consisted in 30 sessions of 10 min observation on 0.55 x 0.55 meters quadrats in each population (a total of 85 h of observation). In each quadrat, we counted the number of visits per insect and the number of open flowers of *R. ferrugineum*. We then estimated the proportion of visited flowers on the total number of flowers per quadrat; it varied from 0 (no flower visited) to 1 (all flowers of the quadrat were visited). The sessions were spread over the entire flowering period and between 10 am and 4 pm and sampling days were separated by 4-5 days. Surveys were carried out during maximum flower blooming to avoid differences in the composition of assemblage between populations as a result of variations in food-resource availability. We calculated visitor richness (S_{obs}) as the number of visitor groups in each population. Diversity was calculated as the diversity and equitability indexes of Shannon-Wiener such as $H' = -\sum_{i=1}^{R} pi \ln pi$ where p_i is the abundance of visits from a visitor group on the total of visitor groups. We characterized the composition of visitor assemblage per population by means of detrended correspondence analysis (DCA). For each population, the composition was estimated through the values of the first and second axis (Charrier et al. in prep). We carried out DCA with the package Vegan in R software (v. 2.14.0; R core team).

Visitor and stigmatic pollen loads

In order to determine visitor pollen load, three sessions of 30 min capture were set up in all populations, in 3x3 meters quadrats. Capture sessions were spread over the entire flowering period and between 10 am and 4 pm. During each sessions every visitors of *R. ferrugineum* were captured using a net, killed with ethyl acetate, stored in individual tube and frozen until processed in the laboratory. Each visitor was then identified. To remove pollen grain from the insect's body, each captured insect was dabbed with a small gelatin-fuchsin cub (see Dafni 1992). The gelatin cub was then placed on a slide, heated to melting point and covered with a cover slip. We avoided pollen storage such as pollen baskets on bees as they contain pollen unlikely to be available for pollination. We performed pollen counts on each slide under a binocular microscope. We differentiated between conspecific *R. ferrugineum* pollen (tetrad pollen were multiplied by four to obtain the number of pollen grains) and pollen from co-flowering plant species and calculated the purity of pollen load as the proportion of *R. ferrugineum* pollen grain. During *R. ferrugineum* flowering period, no other Ericaceae with tetrad pollen was in bloom allowing the direct assessment of conspecific pollen.

To assess pollen delivery onto stigmas by pollinators we performed flower emasculation during the flowering season. We excised the stamens before anthesis to avoide self-pollen deposition on two inflorescences (five flowers per inflorescences) of 15 individuals in each of the 17 populations. The emasculated flowers were left to natural pollination and at the end of the flowering period we randomly collected two styles from each of the manipulated inflorescences and put them into individual tubes. Each stigma was then placed on a microscope slide with a drop of fushin and the stigmatic pollen load was counted.

Visitor effectiveness in pollen transport

We evaluated the pollination effectiveness (PE) of each visitor based on visitation surveys. We estimated (1) the proportion of flowers visited per quadrat (P_{Flower}), (2) the quantity of pollen (Q_{Pollen}) as the number of pollen grain carried over by visitor and (3) the proportion of *R. ferrugineum* pollen (P_{Rf}) transported by each visitor of the group. PE was then computed as: mean P_{Flower} x mean Q_{Pollen} x mean P_{Rf} for each group of pollinator and for each population.

Plant mating system

To assess the contribution of visitors to the mating system of *R. ferrugineum*, we performed two pollination treatments on 15 individuals in each of the 17 populations. In the first treatment (EN), two inflorescences were randomly selected on each individual. Five flowers per inflorescence were emasculated and the other flowers were gently removed as described in Delmas *et al.* (2014). The second treatment (IN) consisted of unmanipulated flowers used as control. Fruits were harvested five weeks after the treatments, just before dehiscence to ensure the full development of the seeds. Two fruits per manipulated and control inflorescences were randomly selected, dissected under a binocular and filled seeds were counted. To assess seed set per treatment, we quantified the mean number of ovules per ovary per individual following the method described in Delmas et al. (2014).

In order to study the realized mating system, analyses were performed in nine selected populations among the 17 considered for pollination experiments growing at low *vs* high altitude along the Pyrenean chain (Figure 1). Seed germination was performed on moistened filter paper in 5 cm diameter Petri dishes in a greenhouse (24°C, 14h/10h). Ten seedlings per family were randomly collected and frozen at -20°C until DNA extraction. Total sample size was 90 families including 900 progenies for mating system analysis.

DNA was extracted from leaves for the adults and cotyledons for the seedlings with the DNeasy Plant Kit (QIAGEN, Courtaboeuf, France), following the manufacturer's protocol (see Charrier et al. 2014) and 15 polymorphic microsatellite markers previously developed using pyrosequencing technologies (454 FLX Titanium, Roche Applied Science, Meylan, France) were used as described in Delmas et al. (2011) and Charrier et al. (2012).

Selfing rates were estimated using the software MLTR 3.1 based on Ritland's mixed-mating model (Ritland & Jain, 1981; Ritland, 2002).

Statistical analysis

ANOVA were used to test for differences in P_{Flower}, Q_{Pollen}, P_{Rf}, pollen on stigma, PE and seed sets among populations and Tukey's HSD post-hoc tests were done if results were significantly different (R software).

To test the effects of visitor assemblage diversity, richness and composition, General Linear Models (GLMs) were conducted on PE using R software and the selection of the best model was done according to the Akaike information criteria (AIC). The model that minimizes AIC provides the most reliable fit to the data (Sakomoto et al. 1986). We ranked models based on δ AIC estimated as the difference between each AIC value and the smallest value. Models with δ AIC ≤ 2 were considered to have substantial support (Burnham et al. 2002). Explaining variables were tested and the model with the lowest AIC criteria was selected.

The effects were tested by a one-way analysis of variance and then Tukey's HSD post-hoc tests (R software).

RESULTS

Visitor assemblages

Visitor diversity (Shannon Index) varied from 1.18 (PraL) to 2.14 (LecL) and equitability index varied from 0.52 (BetL) to 0.93 (LecL). S_{obs} varied from 5 (PraL) to 12 (SouL) out of the 14 insects groups. Composition of visitor assemblages is given by DCA (Table 2). The primary axis separated mainly high altitude populations (except TroH) abundantly visited by *Apis mellifera*, wild bees and Muscidae (Fig. 2; Table 2) from low altitude populations (except SouL) principally visited by *Bombus sp*. Low altitude populations segregated along the second axis from populations visited by *B. pratorum* and *B. pascuorum* (LecL) and/or an array of other visitors including *Macroglossum* (LecL, PeyL, VicL), Syrphidae (LecL, PeyL), Empididae (LecL) and low altitude populations abundantly visited by *Bombus lucorum* (BetL, TecL) and a set of others visitors such as *Bombus sylvestris* (TecL, ChiL) and Coleoptera (TecL).

Visitors and stigmatic pollen load

522 visitors were captured and among them 53 species were determined (15 Hymenoptera, 24 Diptera, 5 Coleoptera and 9 Lepidoptera species; see Table S1). Among all groups examined (Table 3), other Hymenoptera carried the highest mean Q_{Pollen} (9020 ± 141) and *Macroglossum stellatarum* and other Lepidoptera the lowest (23 +/-1 and 29 ±1 respectively), significant differences among groups are in Table 3 (Pvalue < 0.001). We detected no significant difference between *Apis mellifera*, wild bees and *Bombus* species. For *Apis mellifera, Bombus sylvestris, Bombus pratorum* and *Macroglossum stellatarum* P_{Rf} was superior to 80% but was only 21% for the other Diptera and 29% for the Syrphidae. P_{Rf} varied significantly between taxa (Pvalue< 0.001), population (Pvalue = 1.48^{e}-05) and the interaction between taxa and population (Pvalue = 3.36e-07). Visitors from PeyL carried the lowest P_{Rf} (36%) and SouH, BetL, ChiL and TouH had the highest (Table 1).

The total quantity of pollen on stigma (mean 162 pollen grain ± 0.81) and the proportion of *R. ferrugineum* pollen grains per stigma (mean 0.88 ± 0.001) did not vary among populations (Pvalue = 0.488 and 0.133 respectively; Table 1).

Insect pollen load and stigmatic pollen load were not correlated (Pvalue = 0.11).

Visitor effectiveness

We observed significant variations in the proportion of visited flowers (Table 1 and 3) among populations (Pvalue: 2.2e-16), groups (Pvalue: 2.2e-16) and interaction between populations and taxa (Pvalue: 2.2e-16). SouL, ChiL and TecL (mean proportion: 0.01) had the highest proportion of visited flowers and PuiH the lowest (mean proportion: 0.001).

Mean PE per visitor groups varied significantly (Pvalue < 2^{e}-16) from 21.24 for *B. lucorum* to 0.008 for the other Lepidoptera in 2011 (Table 3). We detected no significant difference in PE between *A. mellifera*, wild bees, *B. pascurorum*, *B. sylvestris* and *B. pratorum*.

Mean PE of pollinator assemblages varied significantly among populations (Pvalue <0.001). TecL and BetL had the highest effectiveness (19.04 and 13.50 respectively) and PuiH the lowest (0.80).

Mean PE increased significantly while increasing visitor richness and when *Bombus* visitation increased.

Plant mating system

Mean IN seed set (Table 1) was 0.61 (± 0.007 SE) and ranged from 0.41 (BouH) to 0.76 (TecL). Mean EN seed set was 0.48 (± 0.007 SE), the lowest values were found at ChiL (0.32) and TroH (0.38) and the highest at EynH (0.64). Significant differences in IN and EN seed sets among populations were detected and are shown in Table 1 (Pvalue: 2.14e-10).

R. ferrugineum showed a predominantly outcrossed mating system with a mean selfing rate reaching 0.24 and ranging from -0.2 (LecL, PraL, ChiL, SouH, TroH) to 0.429 (PuiH).

Stigmatic pollen load, mean EN seed set and selfing (s) were not correlated to PE (Pvalue > 0.05 in all cases).

DISCUSSION

R. ferrugineum is visited by a large range of insect species. If Hymenoptera appeared to be the main visitors, Diptera were also well represented. The effectiveness of pollinators is known to vary among groups and thus have an impact on plant mating system. Few studies have investigated geographical variation in the mating system of widespread plant irrespective of variation in the pollinator assemblage (Barrett and Eckert 1990).

Pollen transport effectiveness variability of the cortege assemblages

Our work was consistent to Escaravage and Wagner (2004) and Charrier et al. (in prep), indeed Hymenoptera were the most efficient pollinators of *R. ferrugineum*. Taxonomic variation in pollination effectiveness is usual (Schemske and Horvitz 1984; Kandori 2002; Sahli and Connor 2007). Lepidoptera and Coleoptera were rarely observed in *R. ferrugineum* flowers, and seem to be of little importance as pollinators while many Diptera were observed and thus may be considered as relatively important pollinators. While we detected no difference in efficiency among Diptera, we observed strong differences among Hymenopteran taxa. Pollen loads were much lower in our study (11350 pollen grains in average for honeybees and 15270 in average for bumblebees in Escaravage and Wagner (2004) *vs* 2214 in average for honeybees and 1548 in average for bumblebees in our study). Pollen transportation may differ among years and thus lead to differences in efficiency for a given visitor group from a year to another. *B. lucorum*, honeybees and wild bees were the most efficient pollinators whereas other *Bombus* taxa had a lower efficiency. They transported a high number of pollen grains with a high proportion of *R. ferrugineum* pollen grain and visited the highest number of flowers. Bees carried a large part of conspecific pollen (> 60%) indicating a high constancy in flower visitation. Constancy is beneficial for the plant because less pollen is misplaced and thus it results in a higher rate of successful fertilization (Wilson and Stine 1996). Bumblebees, although known to be temporally specializing in one flower species, may occasionally forage on other minor species (Cruden 1971; Heinrich 1979). Only *B. pascuorum* and other Hymenoptera carried a low proportion of conspecific pollen (41% and 45% respectively).

B. lucorum was particularly abundant at ChiL and TecL, these two populations had the highest efficiency confirming that *B. lucorum* is the most efficient pollinator of *R. ferrugineum*. Population BouH and LecL were the only populations where the visitor corteges were predominantly composed of Diptera species (Charrier et al. in prep). Their effectiveness were relatively low but were not the lowest, thus Diptera may maintain a relatively high level of pollination.

Effectiveness increased with increasing visitor richness and increased with *Bombus* visitation. *Bombus* were efficient pollinators and particularly *B. lucorum* explaining the increase of the effectiveness along the two axis of composition. Even if *A. mellifera* and wild bees were efficient visitors we detected differences among populations mainly visited by *A. mellifera* and wild bees and populations visited by *Bombus*. PuiH had the lowest efficient assemblage even if it was mainly composed by *A. mellifera* but in this population the overall low number of visits decreased the effectiveness of the assemblage.

Our estimates of pollinator effectiveness may be misleading because it took into account only a part of all the component of the efficiency (Waser 1983). We did not take into account pollen deposition; it would be interesting to estimate it for each group of visitors. Ashman and Stanton (1991) detected differences among Hymenoptera, indeed *Bombus bifarius* had significantly higher pollen deposit than Bombyliidae. Thus, in order to be more accurate in the estimation of effectiveness it would be interesting to study more component of effectiveness such as behavior or number of pollen grain deposited on stigma. Also, we did not focus into the sequence of visitation and some groups of visitors may carry a large part of self-pollen (geitonogamy).

Effect of pollinator effectiveness on stigmatic pollen load and plant reproduction

We detected small number of pollen grain deposited on stigma. This may be due to the low number of visitation or visitor behavior. Indeed, *A. mellifera* are known to agglutinate the pollen load with regurgitated nectar and place it wet into corbiculae on the hind tibia (Simpson et al. 1977). This practice would reduce the chance of pollen being scooped into the stigma. Also, species that do not land on flowers such as *Macroglossum* are expected to deposit few pollen grains at each visit.

Surprisingly, even if there were significant differences between cortege effectiveness, there was no difference between populations in the number and proportion of *R. ferrugineum* pollen deposited on stigma and no relationship between these parameters and pollinator assemblage's effectiveness. We expected a variation in pollen deposit depending on the effectiveness of visitor assemblage. If we assume that every pollen grain that was deposited on the stigma must have been put there by a visitor, an increase in visitation rates must lead to an increase on pollen deposit (Galen and Stanton 1989; Young and Stanton 1990; Jones and Reithel 2001). Pollen deposit also depends on the quantity and proportion of conspecific pollen carried out by the visitors. Visitors may groom pollen from their bodies in successive floral visits (Conner et al. 1995) and thus lead to low pollen grain deposit.

We detected no relationship between insect pollen load and stigmatic pollen load. Visitation abundance and visitor pollen load did not inform precisely on pollination. As shown in Popic et al. (2013), only few visitors are pollinators. It would be interesting to determine which species on *R. ferrugineum* assemblage are effective pollinators.

The lowest EN seed set (emasculated flowers) than IN seed set (control flowers) indicated the selfing capacity of *R. ferrugineum* (Charrier et al. in prep). We did not observe correlation between mean effectiveness of visitor assemblages and EN and IN seed sets nor selfing rates. Seed set in coffee populations visited by many pollinator taxa was buffered against a decrease in the dominant honeybee because previously infrequent visitors increased in visitations rates with the loss of the dominant taxon (Ricketts 2004). Therefore, the high number of taxa that are effective pollinators of *R. ferrugineum* may buffer this plant against shifts in pollinator assemblages. Or differences in seed set are due to other parameters such as soil composition and nutrients availability.

Hymenoptera appeared to be the most efficient pollinators in term of visitation and pollen grain transport. However, we detected high variability in the effectiveness of Hymenoptera; *B. lucorum*, *A. mellifera* and wild bees were the most efficient pollinators. There is redundancy in effectiveness among these species, thus if one species tend to disappear it may be replaced by another and maintain pollination. Although the variability in visitor assemblage effectiveness was high among populations, we detected no difference in pollen deposit. *R. ferrugineum* mating variability does not seem to be a result from the effectiveness variability of pollinator assemblage. Some have argued that variation in pollinator effectiveness and abundance leads to selection for a generalized pollination system (Petterson 1991; Petanidou and Ellis 1993; Waser et al. 1996; Gomez and Zamora 1999; Johnston and Steiner 2000). The ability of *R. ferrugineum* to exploit a wide variety of pollinators that vary in abundance and effectiveness may in part explain its success over a highly heterogenic habitat.

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Figure 1. Location of the seventeen *R. ferrugineum* studied populations in the French Pyrenees. Black dots indicate the populations used for genetic analysis.

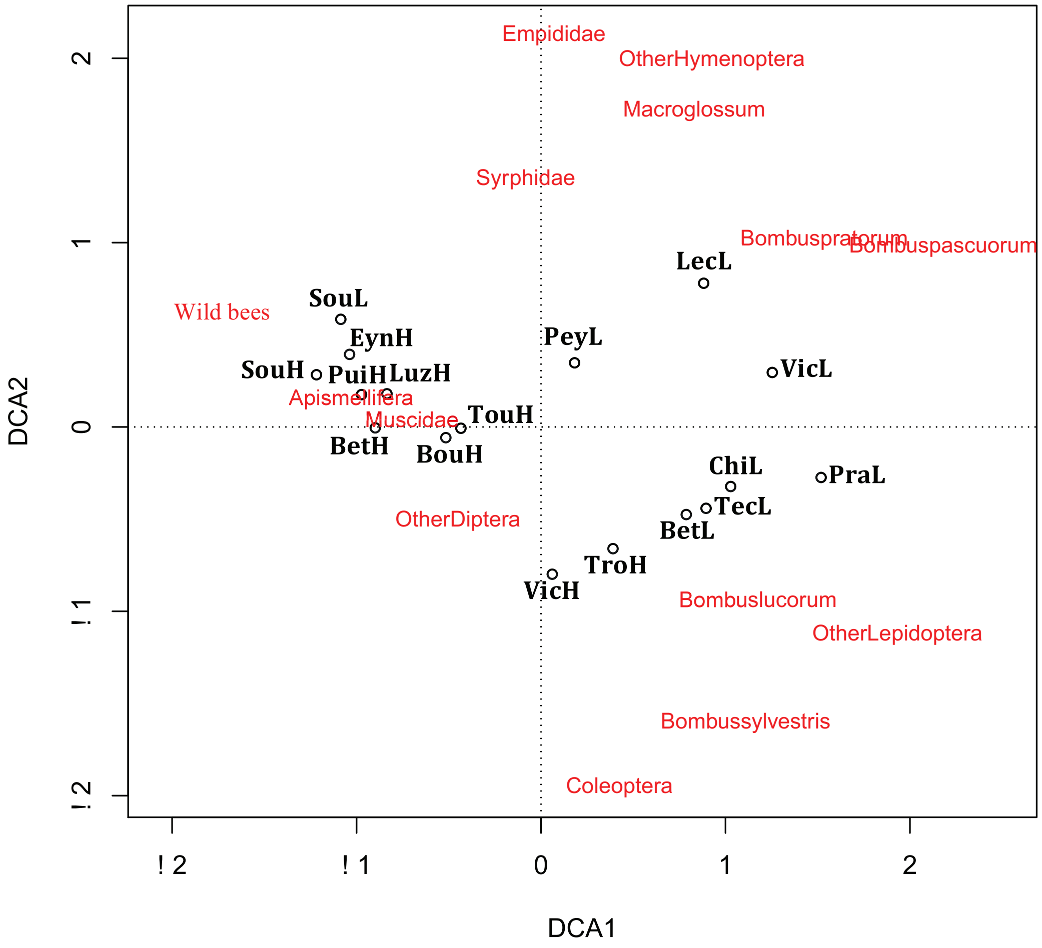

Figure 2. Results of the DCA with populations and groups of visitors.

Table 1. Geographical information on the studied populations and mean number of pollen grain and proportion of *R. ferrugineum* pollen grain per stigma, mean proportion of pollen carried by visitors, proportion of visited flowers, effectiveness, IN (control) and EN (emasculated inflorescences) seed set and selfing rates (s). Different letters indicate significant differences.

Sites	**Code population**	**Location**	**Altitude (m)**	**Mean number of pollen grain per stigma**	**Proportion of *R. ferrugineum* pollen grain per stigma**	**Mean proportion of *R. ferrugineum* pollen carried out by visitors**	**Proportion of visited flowers** (300 min observation)	**Effectiveness**	**IN seed set**	**EN seed set**	**s**
Léca	LecL	42°28'07"N 02°31'40"E	1250	192 ± 0.01	0.91 ± 0.01	0.44 ± 0.01 **ab**	0.009 ± 7.71e-05 **ab**	8.56 **c**	0.63 ± 0.003 **bf**	0.45 ± 0.003 **ab**	-0.2
Prats de Mollo	PraL	42°25'21"N 02°24'38"E	1450	109 ± 0.02	0.67 ± 0.02	0.67 ± 0.01 **ac**	0.003 ± 3.86e-05 **bc**	2.90 **ab**	0.67 ± 0.003 **cdef**	0.42 ± 0.003 **a**	-0.2
Eyne	EynH	42°28'27"N 02°07'05"E	1915	168 ± 0.02	0.72 ± 0.03	0.54 ± 0.01 **ac**	0.004 ± 5.65 e-05 **bc**	1.31 **a**	0.61 ± 0.003 **bf**	0.64 ± 0.003 **b**	--
Puigmal	PuiH	42°23'26"N 2°05'0"E	2045	113 ± 0.02	0.83 ± 0.02	0.73 ± 0.01 **bc**	0.001 ± 1.89e-05 **d**	0.80 **a**	0.69 ± 0.003 **def**	0.41 ± 0.003 **a**	0.429
Lac des Bouillouses	BouH	42°33'31"N 01°59'42"E	2005	41 ± 0.02	0.85 ± 0.02	0.51 ± 0.01 **ac**	0.004 ± 3.11e-05 **bc**	2.19 **ab**	0.41 ± 0.003 **a**	0.47 ± 0.004 **ab**	--
Soulcem	SouH	42°39'28"N 01°27'27"E	2035	149 ± 0.01	0.93 ± 0.01	0.81 ± 0.01 **c**	0.003 ± 3.51e-05 **bc**	2.34 **ab**	0.50 ± 0.004 **ab**	0.41 ± 0.004 **a**	-0.2
Soulcem	SouL	42°41'28"N 01°27'07"E	1240	160 ± 0.01	0.88 ± 0.01	0.62 ± 0.01 **ac**	0.01 ± 0.004 **a**	9.33 **c**	0.54 ± 0.002 **abd**	0.54 ± 0.003 **ab**	0.125
Etang de Lhers	VicL	42°48'23"N 01°22'31"E	1277	250 ± 0.01	0.84 ± 0.02	0.51 ± 0.01 **ac**	0.009 ± 8.64e-05 **ab**	9.50 **c**	0.61 ± 0.003 **bf**	0.45 ± 0.004 **ab**	--
Etang de Lhers	VicH	42°47'36"N 01°24'16"E	1940	250 ± 0.01	0.87 ± 0.03	0.44 ± 0.02 **ac**	0.004 ± 4.65e-05 **bc**	2.47 **ab**	0.50 ± 0.002 **ab**	0.44 ± 0.003 **ab**	--
Bethmale	BetL	42°51'42"N 01°04'02"E	1385	184 ± 0.002	0.97 ± 0.002	0.76 ± 0.007 **c**	0.009 ± 8.69 e-05 **ab**	13.50 **cd**	0.73 ± 0.004 **ef**	0.55 ± 0.004 **ab**	0.2
Bethmale	BetH	42°50'24"N 01°03'42"E	1920	70 ± 0.01	0.87 ± 0.04	0.65 ± 0.01 **ac**	0.004 ± 4.02e-05 **bc**	3.23 **ab**	0.65 ± 0.003 **bf**	0.55 ± 0.005 **ab**	--
Le Peyras	PeyL	42°59'15"N 00°10'46"E	1220	108 ± 0.01	0.88 ± 0.02	0.36 ± 0.02 **a**	0.006 ± 5.2e-05 **bc**	4.83 **ab**	0.66 ± 0.003 **cdef**	0.51 ± 0.004 **ab**	0.208
Col du Tourmalet	TouH	42°53'57"N 00°07'54"E	1950	198 ± 0.0007	0.99 ± 0.0006	0.74 ± 0.01 **c**	0.005 ± 4.72e-05 **bc**	5.44 **ab**	0.59 ± 0.004 **abe**	0.38 ± 0.004 **ab**	--
Cirque de Troumouse	TroH	42°43'41"N 00°05'40"E	2080	250 ± 0.0001	0.99 ± 0.0001	0.61 ± 0.01 **ac**	0.003 ± 2.87e-05 **bc**	2.94 **ab**	0.70 ± 0.004 **ef**	0.47 ± 0.004 **a**	-0.2
Chiroulet	ChiL	42°57'38"N 00°04'17"E	1240	137 ± 0.02	0.79 ± 0.02	0.83 ± 0.008 **c**	0.01 ± 9 7.59e-05 **a**	9.78 **c**	0.52 ± 0.003 **abc**	0.32 ± 0.003 **a**	-0.2
Lac du Tech	TecL	42°54'40"N 00°15'32"W	1240	230 ± 0.01	0.93 ± 0.01	0.56 ± 0.01 **ac**	0.01 ± 0.0001 **a**	19.04 **d**	0.76 ± 0.003 **f**	0.44 ± 0.002 **ab**	--
Luz Ardiden	LuzH	42°52'45"N 00°03'34"W	1905	149 ± 0.01	0.98 ± 0.001	0.61 ± 0.01 **ac**	0.007 ± 6.47e-05 **c**	5.89 **ab**	0.60 ± 0.004 **be**	0.54 ± 0.004 **ab**	--

Table 2. Results of the DCA (a) for 4 axis and (b) for the correlation among visitor groups and axis 1 and 2.

a.

2011	DCA1	DCA2	DCA3	DCA4
Eigenvalues	0.53	0.20	0.17	0.15
Decorana values	0.53	0.14	0.06	0.02
Axis lenghts	2.73	1.57	1.25	1.15

b.

	2011	
	DCA 1	**DCA2**
A. mellifera	-1.02	0.14
Other Apini	-1.73	0.63
B. lucorum	1.17	-0.93
B. sylvestris	1.10	-1.60
B. pratorum	1.53	1.01
B. pascuorum	2.18	0.97
Other Hymenoptera	0.92	1.98
Empididae	0.07	2.12
Muscidae	-0.7	0.04
Syrphidae	-0.08	1.34
Other Diptera	-0.45	-0.50
Coleoptera	0.42	-1.95
Macroglossum	0.83	1.71
Other Lepidoptera	1.93	-1.12

Table 3. Mean proportion of visited flowers, pollen quantity, proportion of *R. ferrugineum* pollen grain and effectiveness per group of visitor. Different letters indicate significant differences.

Group	**Q_{pollen}**	**P_{Rf}**	**Proportion of visited flowers**	**Effectiveness**
Apis mellifera	2120 ± 43 **b**	0.84 ± 0.001 **d**	0.01 ± 0.0001 **a**	8.02 ± 0.06 **ab**
Wild bees	2309 ± 362 **b**	0.61 ± 0.03 **bcd**	0.02 ± 0.0002 **a**	10.42 ± 0.07 **ab**
Bombus pascuorum	771 ± 47 **ab**	0.41 ± 0.03 **ab**	0.01 ± 0.0001 **a**	1.39 ± 0.01 **b**
Bombus sylvestris	1204 ± 137 **ab**	0.89 ± 0.01 **d**	0.004 ± 0.0001 **b**	1.08 ± 0.02 **b**
Bombus lucorum	2584 ± 40 **b**	0.72 ± 0.002 **cd**	0.03 ± 0.0002 **a**	21.24 ± 0.12 **a**
Bombus pratorum	1636 ± 166 **b**	0.80 ± 0.01 **cd**	0.007 ± 0.0001 **b**	3.00 ± 0.04 **b**
Other Hymenoptera	9020 ± 141 **c**	0.45 ± 0.02 **ab**	0.002 ± 4.84e-05 **b**	4.28 ± 0.08 **b**
Syrphidae	513 ± 23 **ab**	0.29 ± 0.005 **a**	0.004 ± 3.68e-05 **b**	0.35 ± 0.0002 **c**
Muscidae	72 ± 1 **a**	0.39 ± 0.005 **ab**	0.007 ± 4.49e-05 **b**	0.03 ± 0.0002 **c**
Empididae	79 ± 7 **a**	0.55 ± 0.05 **ab**	0.004 ± 6.17e-05 **b**	0.05 ± 0.0007 **c**
Other Diptera	37 ± 6 **a**	0.21 ± 0.02 **a**	0.03 ± 3.23e-05 **a**	0.001 ± 3.63e-06 **c**
Coleoptera	291 ± 73 **ab**	0.55 ± 0.05 **ab**	0.004 ± 4.53e-05 **b**	0.17 ± 0.001 **c**
Macroglossum	23 ± 1 **a**	0.87 ± 0.004 **d**	0.05 ± 7.67e-05 **a**	0.02 ± 0.0003 **c**
Other Lepidoptera	10.25 ± 4 **a**	0.60 ± 0.002 **bcd**	0.001 ± 2.22e-05 **bc**	0.008 ± 0.0001 **c**

Table 4. Statistical results for the GLMs testing the effects of diversity, richness and composition of visitor assemblage on mean PE and mean EN seed set. Significant P values are in bold.

Mean PE	**Estimate**	**Standard error**	**pvalue**
Intercept	-9.82	3.74	**0.008****
Composition axis 1	2.20	0.63	**0.0005*****
Composition axis 2	-3.65	2.40	**0.01***
Visitor richness	1.95	0.31	**3.56e-10****

Mean EN seed set	**Estimate**	**Standard error**	**pvalue**
Intercept	0.55	0.01	**<2e-16*****
Composition axis 1	-0.02	0.003	**7.43e-10*****
Composition axis 2	0.04	0.008	**1.61e-07*****
Visitor diversity	-0.09	0.01	**7.43e-12*****
Visitor richness	0.006	0.001	**5.39e-05****

ANNEXE

Table S1. Identification and number (n) of insects captured on each *R. ferrugineum* population.

Population	Order	Family	Genus	Species	n
LecL	Hymenoptera	Apidae	*Bombus*	*lucorum*	6
		Apidae	*Bombus*	*pascuorum*	2
		Apidae	*Bombus*	*pratorum*	6
		Apidae	*Lasioglossum*	*calceatum*	1
		Other Hymenoptera	NI	NI	2
	Diptera	Empididae	*Empis*	*abdominalis*	1
		Syrphidae	*Melanostoma*	*scalare*	1
		Syrphidae	*Platycheirus*	*albimanus*	2
		Syrphidae	*Episyrphus*	*balteatus*	2
		Syrphidae	*Sphaerophoria*	*scripta*	2
		Muscidae	NI	NI	2
		Other Diptera	NI	NI	5
PraL	Hymenoptera	Apidae	*Bombus*	*pascuorum*	10
		Apidae	*Bombus*	*pratorum*	4
		Other Hymenoptera	NI	NI	2
	Diptera	Syrphidae	*Eristalis*	*tenax*	3
		Syrphidae	*Meliscaeva*	*auricollis*	1
		Syrphidae	*Episyrphus*	*balteatus*	2
		Syrphidae	*Eristalis*	*similis*	1
		Syrphidae	*Sphaerophoria*	*scripta*	3
		Syrphidae	*Platycheirus*	*albimanus*	2
		Muscidae	NI	NI	6
	Lepidoptera	Pieridae	*Pieris*	*brassicae*	1
		Sphingidae	*Macroglossum*	*stellatarum*	3
EynH	Hymenoptera	Apidae	*Bombus*	*lucorum*	3
		Apidae	*Apis*	*mellifera*	6
		Other Hymenoptera	NI	NI	1
	Diptera	Muscidae	NI	NI	13
		Empididae	*Empis*	*pandellei*	3
		Syrphidae	*Meliscaeva*	*cinctella*	1
		Syrphidae	*Eupeodes*	*nielseni*	1
		Muscidae	NI	NI	1
	Lepidoptera	Papilionoidea	*Clossiana*	*euphrosyne*	5
PuiH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	3
		Apidae	*Bombus*	*lucorum*	1
		Apidae	*Bombus*	*pratorum*	1
		Apidae	*Apis*	*mellifera*	21
		Apidae	*Halictus*	*rubicundus*	1
		Apidae	*Apis*	*NI*	1
	Diptera	Syrphidae	*Parasyrphus*	*vittiger*	2
		Syrphidae	*Platycheirus*	*scutatus*	1
		Syrphidae	*Platycheirus*	*albimanus*	2
		Syrphidae	*Lapposyrphus*	*lapponicus*	1
		Syrphidae	*Eupeodes*	*luniger*	1
		Syrphidae	*Eristalis*	*similis*	1
	Coleoptera	Cantharidae	*Rhagonycha*	*gallica*	1
BouH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	4
		Apidae	*Bombus*	*pratorum*	2

		Apidae	*Apis*	*mellifera*	10
		Apidae	*Apis*	*NI*	1
		Other Hymenoptera	NI	NI	1
	Diptera	Syrphidae	*Parasyrphus*	*vittiger*	1
		Syrphidae	*Platycheirus*	*albimanus*	1
		Syrphidae	*Melanostoma*	*mellinum*	1
		Syrphidae	*Sphaerophoria*	*laurae*	1
		Syrphidae	*Eupeodes*	*corrolae*	1
		Syrphidae	*Sphaerophoria*	*scripta*	1
		Muscidae	NI	NI	13
SouH	Hymenoptera	Apidae	*Apis*	*mellifera*	18
		Other Hymenoptera	NI	NI	1
	Diptera	Muscidae	NI	NI	8
	Lepidoptera	Geometridae	*Colostygia*	*turbata*	1
		Adelidae	*Adela*	*reamurella*	1
SouL	Hymenoptera	Apidae	*Bombus*	*pascuorum*	1
		Apidae	*Apis*	*mellifera*	17
	Diptera	Syrphidae	*Sphaerophoria*	*scripta*	1
		Muscidae	NI	NI	1
	Coleoptera	Oedemeridae	*Oedemera*	*virescens*	1
	Lepidoptera	Noctuoidea	*Euclidia*	*glyphica*	2
		Papilionoidea	*Coenonympha*	*pamphilus*	1
		Papilionoidea	*Clossiana*	*euphrosyne*	1
VicH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	4
		Apidae	*Bombus*	*lucorum*	2
		Apidae	*Bombus*	*pratorum*	1
	Diptera	Muscidae	NI	NI	6
		Other Diptera	NI	NI	2
VicL	Hymenoptera	Apidae	*Bombus*	*lucorum*	3
		Apidae	*Bombus*	*pratorum*	7
		Apidae	*Bombus*	*pascuorum*	13
		Apidae	*Apis*	*mellifera*	2
	Diptera	Syrphidae	*Platycheirus*	*albimanus*	4
		Syrphidae	*Sphaerophoria*	*scripta*	1
		Syrphidae	*Melanostoma*	*scalare*	1
BetL	Hymenoptera	Apidae	*Bombus*	*pascuorum*	21
		Apidae	*Bombus*	*pratorum*	3
		Apidae	*Bombus*	*lucorum*	1
		Apidae	*Bombus*	*sylvestris*	1
		Apidae	*Apis*	*mellifera*	1
		Other Hymenoptera	NI	NI	2
	Diptera	Syrphidae	*Lapposyrphus*	*lapponicus*	1
		Syrphidae	*Parasyrphus*	*macularis*	1
		Syrphidae	*Platycheirus*	*albimanus*	1
		Muscidae	NI	NI	2
	Lepidoptera	Noctuidae	*Euclidia*	*glyphica*	1
		Sphingidae	*Macroglossum*	*stellatarum*	1
BetH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	1
		Apidae	*Bombus*	*lucorum*	1
		Apidae	*Bombus*	*sylvestris*	1
		Apidae	*Apis*	*mellifera*	12
		Apidae	*Apis*	*NI*	1
	Diptera	Syrphidae	*Platycheirus*	*tatricus*	1
		Syrphidae	*Eristalis*	*tenax*	1
		Muscidae	NI	NI	4
PeyL	Hymenoptera	Apidae	*Bombus*	*lucorum*	3

		Apidae	*Bombus*	*pascuorum*	1
		Symphite		*tenthrede*	1
		Apidae	*Apis*	*mellifera*	3
	Diptera	Syrphidae	*Platycheirus*	*albimanus*	8
		Muscidae	NI	NI	3
	Coleoptera	Staphylinidae	*Philonthus*	*marginatus*	1
TouH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	2
		Apidae	*Apis*	*mellifera*	28
		Apidae	*Apis*	*NI*	1
		Apidae	*Andrene*	*NI*	2
		Other Hymenoptera	NI	NI	2
	Diptera	Muscidae	NI	NI	6
		Syrphidae	*Scaeva*	*selenitica*	1
		Syrphidae	*Eristalis*	*similis*	1
		Other Diptera	NI	NI	4
TroH	Hymenoptera	Apidae	*Bombus*	*pascuorum*	12
		Apidae	*Bombus*	*pratorum*	2
		Apidae	*Bombus*	*lucorum*	5
		Apidae	*Halictus*	*rubicundus*	1
		Other Hymenoptera	NI	NI	2
	Diptera	Syrphidae	*Scaeva*	*selenitica*	3
		Syrphidae	*Platycheirus*	*tatricus*	1
		Muscidae	NI	NI	4
		Other Diptera	NI	NI	2
	Coleoptera	Dasytidae	*Dasytes*	*gonocerus*	2
ChiL	Hymenoptera	Apidae	*Bombus*	*sylvestris*	3
		Apidae	*Bombus*	*pascuorum*	5
		Apidae	*Bombus*	*lucorum*	2
		Apidae	*Bombus*	*pratorum*	6
	Diptera	Syrphidae	*Eristalis*	*tenax*	1
		Muscidae	NI	NI	1
	Lepidoptera	Geometridae	*Ematurga*	*atomaria*	1
TecL	Hymenoptera	Apidae	*Bombus*	*lucorum*	5
		Apidae	*Bombus*	*pascuorum*	6
		Apidae	*Bombus*	*sylvestris*	1
		Apidae	*Bombus*	*pratorum*	8
		Apidae	*Apis*	*mellifera*	1
		Apidae	*Eucera*	*NI*	1
		Other Hymenoptera	NI	NI	2
	Diptera	Muscidae	NI	NI	1
		Syrphidae	*Eupeodes*	*luniger*	1
		Syrphidae	*Chrysotoxum*	*marginatum*	1
		Syrphidae	*Merodon*	*rufus*	1
		Empididae	*Empis*	*pandellei*	3
		Empididae	*Empis*	*testacea*	1
	Coleoptera	Scarabaeidae	*Trichius*	*fasciatus*	1
	Lepidoptera	Sphingidae	*Hemaris*	*fuciformis*	1
		Geometridae	*Ematurga*	*atomaria*	1
LuzH	Hymenoptera	Apidae	*Bombus*	*sylvestris*	1
		Apidae	*Bombus*	*pascuorum*	3
		Apidae	*Bombus*	*lucorum*	2
		Apidae	*Apis*	*mellifera*	15
		Apidae	*Andrene*	*NI*	1
		Apidae	*Lasioglossum*	*fratellums*	1
		Apidae	*Apis*	*NI*	1
		Apidae	*Lasioglossum*	*calceatum*	1

		Other Hymenoptera	NI	NI	3
	Diptere	Muscidae	NI	NI	2
		Syrphidae	*Platycheirus*	*albimanus*	1

DISCUSSION GÉNÉRALE

SOMMAIRE – DISCUSSION GÉNÉRALE

Les milieux alpins sont des points chauds de biodiversité et apparaissent comme sensibles aux changements globaux. Ils constituent donc des milieux particulièrement intéressants pour des études scientifiques. Durant notre étude, nous nous sommes intéressés à une espèce largement distribuée dans les Pyrénées et dans les Alpes : *Rhododendron ferrugineum*. Cette espèce, qui constitue de vastes populations classées landes d'intérêt communautaire par le programme Natura 2000, présente un système mixte de reproduction (auto- et allo-gamie) et un assemblage varié d'insectes visiteurs.

Dans un premier temps, nous avons décrit la structuration génétique de populations de *Rhododendron ferrugineum*, ce qui nous a notamment permis de caractériser des zones refuges pour cette espèce dans les Pyrénées lors des dernières glaciations et de faire des hypothèses quant à la colonisation des Pyrénées par des individus provenant des Alpes. Par la suite, nous nous sommes concentrés sur 17 populations pour lesquelles nous avons décrit le système de reproduction (capacité à s'autoféconder, assurance reproductive et dépression de consanguinité) et nous avons étudié les relations entre ces paramètres du système de reproduction et l'environnement. Enfin, nous avons étudié les cortèges de pollinisateurs de *R. ferrugineum* pour savoir comment variaient les cortèges de pollinisateurs en fonction de gradients environnementaux et quel était l'impact de la variabilité en efficacité des pollinisateurs sur le système de reproduction de cette espèce végétale.

Populations de *R. ferrugineum* dans les Pyrénées

Impact des changements climatiques passés sur l'histoire biogéographique de Rhododendron ferrugineum

Le premier objectif de cette thèse a été d'étudier la structure biogéographique de *R. ferrugineum* dans les Pyrénées à l'aide de marqueurs microsatellites.

✓ *L'origine Alpienne de R. ferrugineum*

Le genre *Rhododendron*, originaire d'Asie (Goetsch et al. 2005), aurait colonisé l'Europe d'est en ouest. Des individus des Alpes auraient ensuite migrés dans les Pyrénées induisant une plus faible diversité dans les Pyrénées que dans les Alpes (Fig. 1). En effet, l'analyse microsatellite a révélé un niveau de diversité génétique relativement élevé dans les Pyrénées (Ar : 2-4.18 ; H_E : 0.29-0.44) mais cependant plus faible que dans les Alpes ou les Apennins (Ar : 2.71-5.57 ; H_E : 0.37-0.68 ; Bruni et al. 2012) ou que pour d'autres espèces Pyrénéennes (*Pinus uncinata* : H_E : 0.83-0.99 ; Dzialuk et al. 2009 ; *Quercus petraea* : H_E : 0.76-0.84 ; Alberto et al. 2010). Cette plus faible diversité génétique suggère des goulots d'étranglement le long des routes de colonisation (Demesure et al. 1996 ; Stehlik et al. 2002). La présence de steppes entre les Alpes et les Pyrénées (Burga et Perret 1998) ont pu permettre la migration d'espèces Alpiennes soit par le sud-ouest des Alpes (Schonswetter et al. 2005 ; Kropf et al. 2006) soit par le nord des Alpes puis par le Massif Central qui est considéré comme une route majeure de colonisation vers les Pyrénées (Braun-Blanquet 1948).

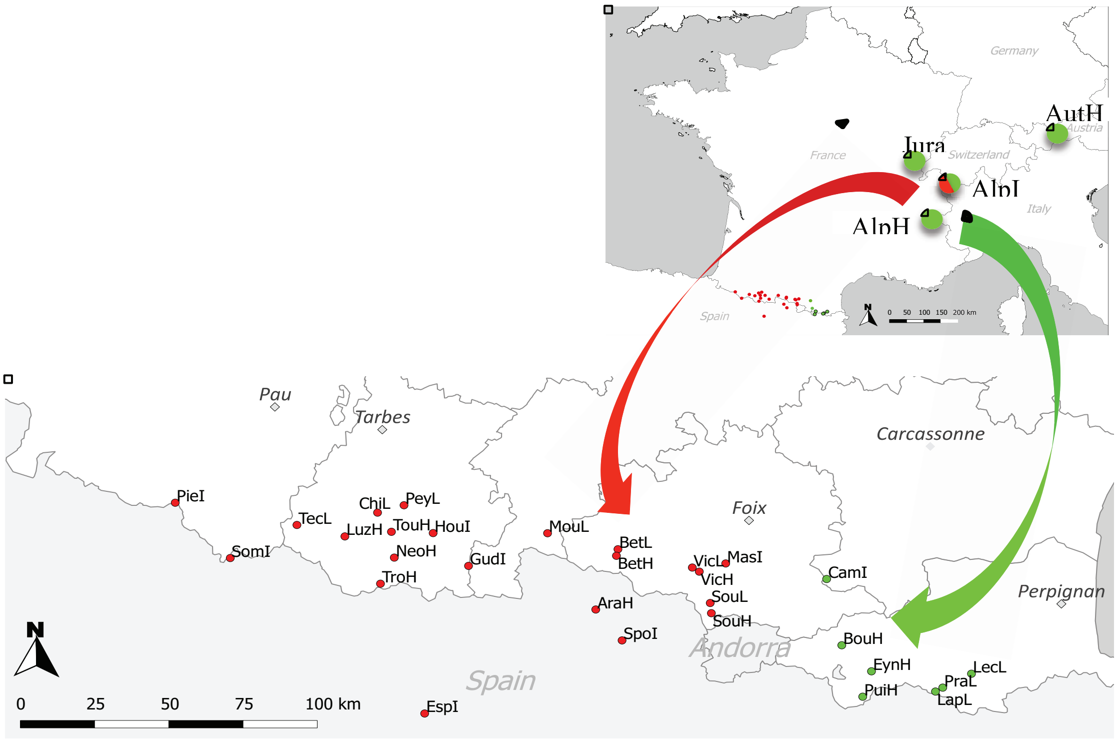

Fig. 1. Colonisation des lignées génétiques Alpiennes vers les Pyrénées.

- ✓ *Patron génétique et histoire phylogéographique de R. ferrugineum dans les Pyrénées*

Le patron génétique actuel des populations de *R. ferrugineum* semble résulter de la colonisation des Pyrénées par deux lignées génétiques Alpiennes, puis de l'isolement de certaines populations lors des évènements de glaciation. Nous avons identifiés deux lignées génétiques distinctes : la première trouvée seulement dans la population Alpienne AlpI et dans les populations du centre et de l'ouest des Pyrénées et la seconde également présente dans la population AlpI mais aussi dans les populations Alpienne AutH et AlpH, celle du Jura et dans les populations de l'est de la chaine Pyrénéennes. Au sein des Pyrénées, la forte différentiation et les faibles flux de gènes entre les deux lignées génétiques suggèrent qu'il y a eu peu d'introgression entre ces deux lignées. Cela est probablement du aux barrières topographiques (hauts sommets, grands glaciers), à la dispersion des graines par barochorie et aux faibles distances parcourues par les pollinisateurs limitant les flux de gènes entre les populations et leur homogénéisation génétique. Une séparation entre les lignées de l'est et de l'ouest de la chaine des Pyrénées est également observée chez d'autres espèces (*Papaver alpinum*, Kropf et al. 2006 ; *Trifolium alpinum*, Lauga et al. 2009).

Les populations de l'est de la chaine sont peu différenciées les unes des autres malgré une distance relativement importante entre elles. Par ailleurs, elles se développent dans des environnements très différents, sont de taille variable et présentent peu de flux de gènes. Cela suggère que ces populations ont récemment échangé des gènes. Ces échanges de gènes ont pu se faire dans de grands refuges de basse altitude durant les périodes glaciaires du Pléistocène, puis dans de grandes populations génétiquement connectées sur les grands plateaux d'altitude typiques des Pyrénées-Orientales. Les petites populations isolées (PraL, LecL, LapL) présentent un niveau relativement élevé d'hétérozygotie (PraL : $H_E = 0.32$, $H_O = 0.31$; LecL : $H_E = 0.30$, $H_O = 0.34$; LapL : $H_E = 0.34$, $H_O = 0.42$) ainsi que des allèles privés malgré la faible richesse allélique probablement due à la perte d'allèles rares par dérive génétique (Lesica et Allendorf 1995). Ces petites populations se développent dans des conditions environnementales inhabituelles *i.e.* dans des vallées profondes et très ombragées, principalement couvertes de hêtres (*Fagus sylvatica*) parfois à proximité de forêts de *Quercus ilex*. Ces données suggèrent que ces populations sont des populations relictuelles (Hampe et Petit 2005) ayant persisté à basse altitude à la faveur de conditions abiotiques micro-stationnelles favorables et isolées depuis la remontée à haute altitude de l'espèce.

Les quatre groupes génétiques de la lignée de l'ouest semblent être une sous structure de la séparation est/ouest des Pyrénées. On a pu observer des niveaux d'introgression élevés entre certains groupes ce qui suggèrent des échanges de gènes assez récents. Ces échanges de gènes pourraient avoir eu lieu dans de grands refuges de basse altitude le long du piedmont Pyrénéen avant la recolonisation des zones de haute altitude. Ce patron de colonisation a déjà été observé pour *Quercus Petraea* (Alberto et al. 2010) et suggéré pour *Aster pyrenaeus* (Escaravage et al. 2011). Par ailleurs, le patron de distribution en groupes génétiquement différents les uns des autres et ayant des niveaux de diversité génétique comparable est compatible avec l'hypothèse de survie d'une espèce dans différents refuges comme cela a été proposé pour d'autres espèces Pyrénéennes (Segarra-Moragues et al. 2007 ; Lauga et al. 2009). Les fortes diversités génétiques observées dans les populations SpoI et TouH (possèdent un allèle privé) pourraient indiquer la présence de refuges de haute altitude (nunataks) le long de la chaine Pyrénéenne. Le patron phylogéographique dans cette partie des Pyrénées pourrait à la fois résulter d'un isolement et d'une différentiation des populations dans des nunataks mais également d'échanges de gènes dans des refuges de basse altitude.

La diversité génétique relativement élevée à l'intérieure des populations peut résulter du mode de reproduction de *R. ferrugineum*. Bien que la multiplication végétative et l'autofécondation soient des facteurs diminuant la diversité génétique d'une espèce (Koelling et al. 2012), la durée de vie élevée des individus (Escaravage et al. 1998, Pornon et al. 2000) et la forte dépression de consanguinité permettent un maintien relativement élevé de la diversité génétique chez cette espèce.

Système de reproduction sexuée de R. ferrugineum le long de gradients environnementaux

Le deuxième objectif de cette thèse a été d'étudier la variabilité spatiale du système de reproduction sexuée de *R. ferrugineum* le long de gradients environnementaux. Cette espèce présente un système mixte de reproduction où l'assurance reproductive permet de maintenir la production de graines dans divers environnements.

- ✓ *Système de reproduction de R. ferrugineum*

Nous avons observé une proportion de graines produites plus faible pour le traitement contrôle (IN moyen: 0.61) que dans une autre étude également menée dans les Pyrénées (IN moyen : 0.7; Delmas et al. 2014) ou que dans des populations Alpiennes (IN : 0.74 - 0.99; Escaravage et al. 1997). Cette plus faible proportion de graines produites par les fleurs contrôles pourrait résulter d'une plus faible capacité d'autofécondation des individus (autopollinisation manuelle IS: 0.34) comparée aux autres études (IS : 0.49, Delmas et al. 2014; IS > 0.77, Escaravage et al. 1997). Les fleurs émasculées présentent une proportion de graines produites plus faible que les fleurs contrôles (0.48 *vs* 0.61) suggérant que l'autofécondation contribue à la production de graines chez *R. ferrugineum* (Delmas et al. 2014).

Les tests de pollinisation nous ont permis d'observer que près de 30% des ovules sont fécondés par de l'autopollen. Par la suite, par des techniques d'analyses de parenté, nous avons observé des taux d'autofécondation très faible (à l'exception de la population PuiH), en effet, près de 80% des graines issues d'autofécondation n'ont pas donné de descendants. Cela suggère une forte dépression de consanguinité (Waser and Price 1991) limitant l'autofécondation chez *R. ferrugineum*. L'autofécondation a donc un coût en réduisant le nombre d'ovules disponibles pour l'allofécondation (Waser and Price 1991). Des taux élevé de dépression de consanguinité ont déjà été observés chez d'autres espèces d'Ericaceae (Mahy et Jacquemart 1998; Hokanson et Hancock 2000; Delmas et al. 2014). Les tests de pollinisation permettent de mesurer la capacité d'un individu à produire une descendance issue d'autofécondation, cependant, il semble indispensable de compléter ces mesures par des analyses de paternité afin de déterminer l'impact de la dépression de consanguinité sur le devenir des descendants issus d'autofécondation.

Malgré un système mixte de reproduction, *R. ferrugineum* présente un taux de dépression de consanguinité élevé (0.85) similaire à ceux d'autres espèces végétales comme certains conifères ou des espèces se reproduisant principalement par clonalité (Sorensen 1999;

Gonzalez-Varo and Traveset 2010; Winn et al. 2011). Nos données confirment que les espèces pérennes à longue durée de vie (au moins 300 ans pour *R. ferrugineum*; Escaravage et al. 1998) accumulent des mutations somatiques et ne sont pas ou peu purgées au cours de leur vie ce qui résulte en un taux élevé de dépression de consanguinité (Morgan et al. 1997; Petit and Hampe 2006).

- ✓ *Variabilité du système de reproduction, de l'assurance reproductive et de la dépression de consanguinité le long de gradients écologiques*

La proportion de graines produites par les fleurs contrôles diminue avec la diminution de la température sur le long terme mais n'est pas sensible à la température saisonnière. Ceci suggère que les mécanismes favorables à la reproduction sexuée de *R. ferrugineum* se sont mis en place sur le long terme au niveau des sites les plus chauds. La diminution de la proportion de graines produites par les fleurs émasculées, dépendant donc uniquement des pollinisateurs pour leur fécondation, avec l'altitude montre que l'efficacité des pollinisateurs pourrait être impliquée dans la production de graines. En effet, la baisse de l'efficacité des pollinisateurs avec l'altitude est une conséquence des conditions climatiques difficiles qui sont défavorables pour les pollinisateurs (Medan et al. 2002; Arroyo et al. 2006) et peut entrainer une diminution de la fécondation croisée (Medan et al. 2002). Comme observé pour d'autres espèces (Brys et al. 2011), la production de graines des fleurs émasculées augmente avec l'augmentation en ressource florale (display floral) de *R. ferrugineum*. Cela pourrait indiquer que les populations de grande taille sont plus attractives pour les pollinisateurs (Kunin 1997 ; Dauber et al. 2010).

Notre hypothèse était que l'assurance reproductive serait significativement plus élevée dans les petites populations de haute altitude (Garcia-Camacho et Totland 2009). Cependant, la production de graines issues d'autofécondation manuelle ou de pollinisation naturelle n'ont pas été impacté par l'altitude ou la taille des populations. La proportion de graines produites par les fleurs émasculées a quant à elle diminué avec l'altitude et la disponibilité en ressource florale. L'augmentation de l'assurance reproductive peut donc être attribuée à une diminution des services de pollinisation. Ces résultats sont en accord avec une étude précédente sur la même espèce (Delmas et al. 2014) ou avec d'autres espèces montrant que l'assurance reproductive est dépendante du contexte environnemental (Kalisz et al. 2004). Elle permet la production de graines dans des populations avec de faibles ressources florales (Elle et Carney 2003; Moeller et Geber 2005), de faibles taux de pollinisation (Kennedy et Elle 2008; Kalisz et al. 2004; Brys et al. 2011) ou se développant dans des climats contraignants (Zhang et Li 2008; Vaughton et Ramsey 2010). En ce qui concerne les taux de dépression de consanguinité, ils augmentent avec l'altitude et les faibles températures, supportant l'idée que les stress environnementaux peuvent augmenter la dépression de consanguinité (Armbruster et Reed 2005; Sedlacek et al. 2013). En accord avec l'étude de Delmas et al. (2014) nous n'avons détecté aucun effet de la disponibilité en ressources florales sur les taux de dépression de consanguinité. Cela infirme notre hypothèse de faible dépression de consanguinité associée à de fort taux d'autofécondation dans les petites populations de haute altitude. La dépression de consanguinité était même plus élevée à haute altitude dans les populations ayant de fort taux d'assurance de reproduction.

✓ *Relations entre autofécondation, assurance reproductive et dépression de consanguinité*

L'assurance reproductive est une des théories expliquant l'évolution de l'autofécondation (Jain 1976; Lloyd 1992; Herlihy et Eckert 2002; Brys et al. 2011). En effet, l'assurance reproductive peut permettre le maintien de la production de graines quand il y a peu de pollinisateurs ou quand le faible nombre de partenaires conspécifiques limites les opportunités d'allofécondation (Darwin 1877; Stebbins 1957; Jain 1976; Lloyd 1979; Holsinger 1996).

Bien que l'assurance reproductive augmente la production de graine d'environ 30% chez *R. ferrugineum*, la descendance issue d'autofécondation semble ensuite complétement éliminée par la dépression de consanguinité (Herlihy et Eckert 2002). Les deux populations (LecL et SouL) ayant un taux de dépression de consanguinité négatif avaient également un faible ou négatif taux d'autofécondation indiquant que les descendants issus d'autofécondation ont été éliminés par dépression de consanguinité. L'inefficacité de l'assurance reproductive à produire des graines viables semble être un phénomène établi chez *R. ferrugineum*. En effet, nous avons détecté un indice de fixation (F_{IS}) proche de 0 dans 22 des 33 populations étudiées dans les Pyrénées, les Alpes et le Jura indiquant qu'il y a peu de descendants d'autofécondation.

Bien que possédant un système mixte de reproduction *R. ferrugineum* se comporte comme une espèce dépendante des pollinisateurs pour la reproduction croisée. Les populations ne semblent pas tirer profit de l'assurance reproductive. Il y a des doutes quand au rôle actuel de l'assurance reproductive dans l'évolution de l'autofécondation. Nos estimations de taux de dépression de consanguinité sont en dessous des seuils favorisant l'autofécondation (Lande et

Schemske 1985) ce qui pourrait expliquer pourquoi la capacité d'autofécondation et les taux de dépression de consanguinité sont plus faibles que dans une autre population Pyrénéenne (Delmas et al. 2014) ou dans des populations Alpiennes (Escaravage et al. 1997).

Chez *R. ferrugineum*, l'autofécondation ne semble pas être adaptative, mais résulte plutôt de l'incapacité d'empêcher l'autopollen d'arriver sur les stigmates et de mécanismes d'auto-incompatibilité inefficaces (Delmas et al. 2014b). S'ils n'étaient pas contrés par la dépression de consanguinité, l'autofécondation et la clonalité diminueraient la diversité génétique des populations (Charlesworth et Charlesworth 1995).

Une question que l'on peut se poser est pourquoi les populations *de R. ferrugineum* ont toujours cette capacité à l'autofécondation malgré le peu de descendants qui en résultent. Nous pensons que cela pourrait être lié au rôle de l'autofécondation dans l'histoire des populations. Nos données montrent qu'un faible taux d'autofécondation produit une descendance dans quelques populations. En effet, 4 populations marginales avec un taux significatifs de consanguinité ($F_{IS}> 0$) avaient des niveaux de diversité génétique particulièrement faible. Ce patron génétique est consistant avec des évènements de fondation accompagnés de perte de diversité génétique et d'hétérozygotie durant les périodes d'expansion de l'aire de répartition de l'espèce. Ces données suggèrent que la dépression de consanguinité dans ces populations devait être faible et que l'assurance de reproduction a joué un rôle fondamental dans l'établissement de ces populations.

Des études récentes ont montré une migration plus rapide des plantes que leurs insectes pollinisateurs (Roth et al. 2014). Ceci pourrait avoir un impact fort sur les espèces végétales dépendant complétement des pollinisateurs pour leur reproduction. Seule les populations capables d'autofécondation pourrait faire face aux changements globaux et coloniser de nouveaux milieux d'altitude.

Variabilité des cortèges de visiteurs et système de reproduction de R. ferrugineum

Visiteurs de R. ferrugineum.

Le dernier objectif de cette thèse a été de montrer la variabilité du cortège de visiteurs de *R. ferrugineum*, son efficacité et les effets de cette variabilité sur le système de reproduction de *R. ferrugineum*.

Dans le cadre des changements climatiques actuels, les pollinisateurs sont largement menacés. Les pollinisateurs étant largement impliqués dans la reproduction de nombreuses espèces de végétaux, il apparaît crucial de connaître leur degré de spécialisation envers leurs pollinisateurs pour déterminer l'impact des changements climatiques sur leur reproduction. La première étape dans la réalisation de cet objectif a été de déterminer les cortèges de visiteurs de *R. ferrugineum* dans 17 populations des Pyrénées.

Bien que visité par une grande diversité d'insectes (53 espèces déterminées), *Rhododendron ferrugineum* est principalement visité par des Hyménoptères (Fig. 2) et tout particulièrement des bourdons et des abeilles (Escaravage et al. 1997; Escaravage and Wagner 2004, Delmas et al. 2013) et ceux dans la quasi totalité des populations étudiées. Plusieurs études ont montré que les bourdons sont les pollinisateurs principaux de nombreuses espèces végétales et notamment des Ericacées (Free 1970; Haslerud 1975; Reader 1977). Et comme beaucoup d'espèces de pollinisateurs, ils sont largement menacés par les changements globaux (Williams et al. 2007; Goulson et al. 2008; Cameron et al. 2011).

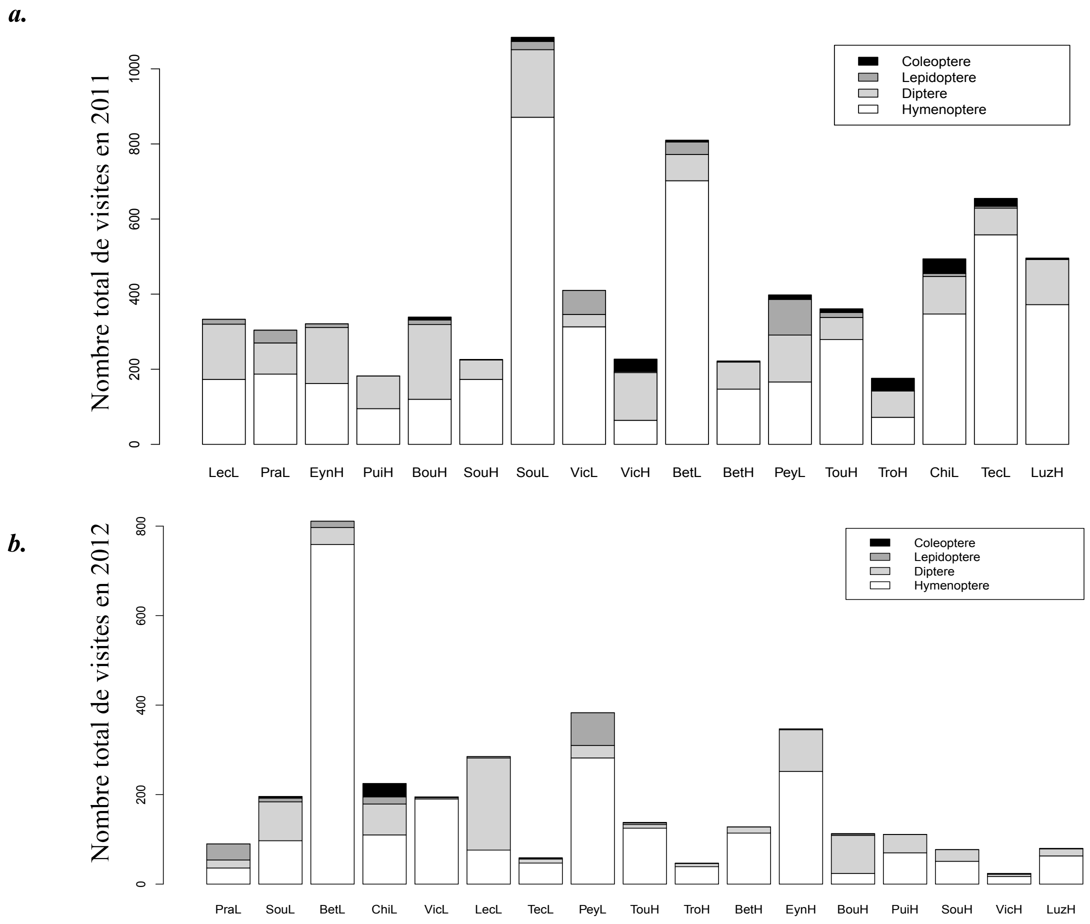

Fig. 2. Nombre de visites par ordre de visiteurs pour les 17 populations étudiées en 2011 et en 2012

✓ *Variations spatiales et temporelles des assemblages de visiteurs*

L'assemblage de visiteurs de *R. ferrugineum* présente des variations spatiales et temporelles en terme de diversité, de richesse, de composition et d'abondance dans les 17 populations étudiées. Des variations géographiques des cortèges de pollinisateurs ont déjà été montrées pour plusieurs espèces végétales (Eckert 2002; Moeller 2005; 2006). Dans notre étude, les populations de *R. ferrugineum* de haute altitude étaient principalement caractérisées par des visites de *Apis mellifera*, d'abeilles sauvages (Halictidae, Andrenidae) et de Muscidae alors que les populations de basse altitude étaient principalement visitées par *Bombus pascuorum, B. pratorum*, des Syrphidae et des Empididae ou par *B. lucorum*. La dominance des Hyménoptères ou des Diptères en fonction de l'altitude est étudiée depuis des décennies (Muller 1880; Mani 1962; Pojar 1974; Arroyo et al. 1982; Primack 1983), cependant dans notre étude nous observons des Hyménoptères à la fois à haute et basse altitude. La présence d'abeilles domestiques à haute altitude peut s'expliquer par la présence de ruches proches des

populations de *R. ferrugineum* étudiées ou à des phénomènes de compétition entre les abeilles et les bourdons à basse altitude (Roubik 1978).
De 2011 à 2012, nous avons observé une forte variation en terme de richesse et de diversité de l'assemblage de visiteurs mais peu de variation dans leurs compositions. Le nombre de visites était beaucoup plus faible en 2012 comparé à 2011. Ce plus faible nombre de visites pourrait être du aux mauvaises conditions climatiques de 2011 entrainant une baisse de la reproduction des visiteurs. Nous avons notamment observé une baisse importante des visites des bourdons tandis que les visites de *Apis mellifera* ont augmenté dans la plupart des populations. Cette augmentation a même, dans certaines populations, compensée le déclin des bourdons. Il est probable que les abeilles domestiques aient moins été touchées par les conditions climatiques de 2011 par la présence de ruches. Les décalages dans les cycles de vie des différentes espèces peuvent également avoir favorisé le développement des abeilles cette année là (Bolotov et al. 2013).

✓ *Impact des variables biotiques et abiotiques sur les assemblages de visiteurs*

Nous avons montré que la diversité en visiteurs de *R. ferrugineum* diminue avec une augmentation de la densité en fleurs de *R. ferrugineum* et de la richesse spécifique de la communauté environnante. Si généralement la diversité, la richesse et l'abondance en visiteurs a tendance à augmenter avec le nombre d'espèces végétales co-florissantes (Tscharntke et al. 1998; Roulston and Goodell 2011), il faut noter que dans notre étude nous avons seulement pris en compte les visiteurs de *R. ferrugineum*. Il est donc possible qu'un plus grand nombre d'espèces végétales attirent certains visiteurs de *R. ferrugineum* et de ce fait diminue la richesse et la diversité de l'assemblage de visiteurs de cette espèce.

La diversité des espèces végétales de la communauté affectait particulièrement la composition en bourdons. En effet, dans les populations avec de faible diversité végétale les visites de *Bombus lucorum* et *B. sylvestris* étaient moins importantes que dans les populations avec une diversité végétale plus élevée. Arhné et al. (2009) ont eux aussi montré que la composition en bourdons était liée aux espèces de la communauté végétale.

La disponibilité en ressource florale de *R. ferrugineum* n'a eu aucun impact significatif sur l'abondance, la diversité, la richesse ou la composition des cortèges de visiteurs alors qu'en théorie les grandes populations végétales sont plus attractives pour les pollinisateurs (Ohashi and Yahara 1999). Les bourdons sont connus pour leurs réponses variables face à la taille des populations. Il a été montré que des individus de *Bombus pratorum* et *Bombus terrestris* étaient attirés par des populations de grande taille tandis que le nombre de visites de *Bombus* *pascuorum* n'était pas lié à la disponibilité en ressources florales. Par ailleurs, Antonini et al. (2013) ont montré que la richesse spécifique en abeilles était plus élevée dans des populations de petites tailles. Les effets de la disponibilité en ressources florales semblent variables en fonction des espèces de visiteurs.

La diversité, l'abondance et la richesse en visiteurs de *R. ferrugineum* étaient plus faibles dans l'est des Pyrénées et peu de bourdons ont été observés. Les Pyrénées-Orientales sont connues pour leur forte diversité en bourdons (Rasmont et al. 2000). Ces espèces semblent particulièrement sensibles au climat et il a été montré que l'abondance de certaines espèces peut diminuer de manière drastique d'une année sur l'autre (Iserbyt et Rasmont 2013). Les températures les plus élevées sur le long terme augmentent la richesse et l'abondance en visiteurs. Les effets directs des températures sur l'abondance des pollinisateurs ont rarement été montrés (Hegland et al. 2009). Cependant de nombreuses études ont montré que les Diptères et notamment les mouches sont plus abondants dans les conditions les plus froides et humides alors que les bourdons et les abeilles sont plus abondants dans les habitats chauds et secs (Arroyo et al. 1982; McCall et Primack 1992; Totland 1993; Lázaro et al. 2008). En milieux de montagne, l'importance relative des différents groupes de visiteurs change généralement le long des gradients altitudinaux (Mani 1962 ; Primack et Armesto 1982). Dans notre étude, la composition des assemblages de visiteurs séparait les populations de basses et hautes altitude mais ne l'impactait pas de manière significative.

- ✓ *Variabilité de l'efficacité du cortège de visiteurs de R. ferrugineum*

Parmi la grande diversité d'espèces visitant *R. ferrugineum,* les bourdons et les abeilles sont les visiteurs les plus efficaces. Par exemple, *B. lucorum* transporte en moyenne 35 fois plus de grains de pollen que les Muscidae. Cependant, les charges polliniques des visiteurs étaient plus faibles que lors d'une précédente étude menée par Escaravage and Wagner (2004) dans les Alpes Autrichiennes. Dans les deux études, les visiteurs transportaient une grande quantité de pollen conspécifique. Les bourdons sont généralement spécialisés de manière temporelle envers certaines espèces végétales (Cruden 1971; Heinrich 1979). Durant la période de floraison de *R. ferrugineum*, seul *B. pascuorum* transportait 40% de pollen de *R. ferrugineum*, les 3 autres espèces de bourdons transportaient de 72 à 89% de pollen conspécifique montrant une forte spécificité envers *R. ferrugineum*. Ces quantités élevées de pollen conspécifique indique une certaine constance dans les visites, ce qui est bénéfique pour les espèces végétales car cela diminue les pertes de pollen et résulte en un succès de fécondation plus important (Wilson and Stine 1996). Les différences de charges polliniques entre les groupes d'insectes (transport de 21% de grains de pollen de *R. ferrugineum* pour les autres Diptères à 89% pour *B. sylvestris*) montrent les différences dans la récolte de pollen. Nous n'avons pas pris en compte dans notre étude les séquences de visites des visiteurs et ces derniers peuvent donc transporter de grande quantité d'auto-pollen ce qui pourrait réduire les chances d'obtenir des descendants.

Bien que les Hyménoptères soient les visiteurs les plus efficaces de *R. ferrugineum*, nous avons observé de fortes différences entre les groupes. *B. lucorum* est l'espèce la plus efficace avec *A. mellifera* et les abeilles sauvages. *B. pascuorum*, *B. sylvestris* et *B. pratorum* présentaient des efficacités plus faibles, en grande partie due à une proportion de fleurs visitées plus faible. Les Lépidoptères et les Coléoptères n'ont été que rarement observés sur *R. ferrugineum*, de plus ils transportent de faible quantité de pollen et semblent donc être des visiteurs peu efficaces. Certains Diptères, comme les Syrphidae, transportaient des quantités de pollen relativement importantes et bien que leur efficacité soit faible, leur forte abondance dans certaines populations en fait des visiteurs relativement efficaces.

L'efficacité des cortèges de visiteurs augmente lorsque que la richesse en groupes de visiteurs et l'abondance en bourdons augmentent. Ce résultat confirme l'efficacité la plus importante des bourdons. Cependant, dans notre étude nous ne prenons en compte que certains paramètres de l'efficacité, nous n'avons par exemple pas tenu compte des quantités de pollen déposé par chaque groupe de visiteurs. Stanton (1991) a montré que *Bombus bifarius* dépose une plus grande quantité de pollen que les Bombyliidae. Pour être plus précis dans cette mesure de l'efficacité il faudrait donc également prendre en compte les quantités de pollen déposées ainsi que les séquences de visites. De manière générale, l'efficacité des groupes de visiteurs était relativement faible, si ces derniers transportaient pour certains groupes de grandes quantités de grains de pollen de *R. ferrugineum*, très peu de fleurs étaient visitées, diminuant ainsi les chances de dépôts de grains de pollen.

Quelque soit l'efficacité des cortèges de visiteurs, les quantités de grains de pollen déposées sur les stigmates étaient relativement faibles. Cela peut être du aux comportements des insectes. Par exemple, *Apis mellifera* est connue pour agglutiner les grains de pollen avec du nectar et de les placer dans des corbeilles à pollen au niveau des pattes postérieures (Simpson et al. 1977). Cette pratique réduit les chances du pollen d'être déposé sur un stigmate. D'autres espèces de visiteurs, comme certains Lépidoptères, ne se posent pas sur les fleurs, par conséquent ils transfèrent généralement peu de grains de pollen sur les stigmates.

- ✓ *Impact de la variabilité des assemblages de visiteurs sur le système de reproduction de R. ferrugineum*

Nous n'avons détecté aucune corrélation entre l'efficacité des cortèges de visiteurs et la production de graines ou les taux d'autofécondation. La variabilité du système de reproduction de *R. ferrugineum* ne semble donc pas résulter de la variabilité de l'efficacité des cortèges de visiteurs. Dans notre étude, quelque soit l'efficacité du cortège de visiteurs, le nombre de grains de pollen déposés sur les stigmates de *R. ferrugineum* étaient très faible. Il semble donc qu'un cortège de faible efficacité soit potentiellement suffisant pour permettre la reproduction croisée de *R. ferrugineum*. D'autres variables comme la composition du sol ou les apports en eau doivent être des facteurs entrainants une variabilité dans la reproduction de *R. ferrugineum*.

Conclusions et perspectives

Dans le cadre des changements globaux actuels, les espèces végétales subissent de nombreuses contraintes. Les espèces végétales entomogames, qui dépendent en grande partie des pollinisateurs pour leur reproduction croisée, semblent particulièrement sensibles aux changements globaux. *R. ferrugineum* est une espèce visitée par une large diversité de pollinisateurs. La production de graines ne semble pas dépendre de l'efficacité du cortège de pollinisateurs, cette espèce sera donc potentiellement peu affectée par les changements de cortèges de pollinisateurs que les changements globaux peuvent entrainer. Il semblerait que même un cortège de visiteurs ayant une faible efficacité permette le maintien de la reproduction croisée chez *R. ferrugineum*. Cela peut expliquer que cette espèce se développe facilement dans les milieux alpins qui sont contraignants pour les pollinisateurs.

Bombus lucorum, Apis mellifera et les abeilles sauvages apparaissent comme les pollinisateurs principaux de *R. ferrugineum*. Il serait cependant intéressant d'étudier plus précisément la composition des cortèges de visiteurs de *R. ferrugineum* et d'estimer un plus grand nombre de paramètres de l'efficacité (séquence de visites ou quantité de grains de pollen déposée à chaque visite). En effet, très peu de grains de pollen sont déposés lors des visites par les insectes pollinisateurs et même si *R. ferrugineum* est visité par un très grand nombre d'espèces plus ou moins efficaces dans le transport de pollen, il est possible qu'un très petit nombre d'entre elles déposent efficacement du pollen sur les stigmates. Par ailleurs, nous avons étudié la variabilité spatiale des cortèges de visiteurs, nous pourrions également étudier leur variabilité temporelle. Il est probable que la variabilité spatiale des cortèges de

pollinisateurs soit également importante d'une année sur l'autre.
Quelque soit le contexte environnemental, nous avons observé une forte dépression de consanguinité précoce qui contrebalance les effets de l'assurance reproductive en conduisant à une faible production de graines par autofécondation. Cette forte dépression de consanguinité a permis un maintien relativement élevé de la diversité génétique dans les Pyrénées. Un tel niveau de dépression de consanguinité ne permet pas l'évolution de l'autofécondation et maintient ainsi le système mixte de reproduction chez *R. ferrugineum*. Le maintien de la capacité d'autofécondation peut permettre à *R. ferrugineum* de coloniser de nouveau milieu ou de survivre aux changements globaux.

Nous avons montré que lors de changements importants comme les dernières glaciations, *R. ferrugineum* a migré et a pu survivre dans divers types de refuges que ce soit dans de grands refuges de basse altitude ou des nunataks à haute altitude. Nos résultats montrent que la lignée de l'est a probablement survécu aux évènements de glaciation dans de grands refuges de basse altitude, laissant de petites populations relictuelles lors de la recolonisations des hautes altitudes. Au centre et à l'ouest de la chaine, la structuration génétique en groupes distincts suggère que l'espèce a survécu dans des nunataks mais également, possiblement, dans des refuges de basse altitude. Il serait par ailleurs intéressant d'étudier l'impact de ces lignées sur le système de reproduction de *R. ferrugineum*.

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ANNEXES

ANNEXES

Table S1. Determination of the surrounding co-flowering community in *R. ferrugineum* populations.

	LecL	**PraL**	**EynH**	**PuiH**	**BouH**	**SouH**	**SouL**	**VicL**	**VicH**	**BetL**	**BetH**	**PeyL**	**TouH**	**TroH**	**ChiL**	**TecL**	**LuzH**
Ajuga pyramidales																	**x**
Ajuga reptans							**x**	**x**		**x**	**x**				**x**		**x**
Alchemilla alpina						**x**							**x**	**x**			**x**
Alchemilla saxatilis		**x**				**x**			**x**								
Alchemilla sp				**x**						**x**	**x**						
Androsace carnea																	**x**
Antennanra dioica			**x**	**x**	**x**									**x**			**x**
Anthyllis vulneraria																	**x**
Arabis sp													**x**				
Astéracées sp.	**x**		**x**					**x**							**x**	**x**	
Barbarea vulgaris			**x**	**x**	**x**												
Bellis perennis									**x**								
Biscutella brevifolia													**x**				
Boraginacée sp.												**x**					
Calamintha alpina											**x**						
Calamintha sylvatica				**x**													
Caltha palutris											**x**						
Cardamine pratensis								**x**	**x**	**x**							
Cardamine hirsuta							**x**			**x**		**x**					
Cerastium arvense											**x**		**x**				
Cerastium alpinum						**x**											
Cerastium glomeratum						**x**											
Conopodium majus							**x**			**x**	**x**	**x**			**x**		**x**
Cruciata glabra		**x**	**x**	**x**			**x**	**x**	**x**	**x**			**x**		**x**		**x**
Cytisus scoparius			**x**	**x**	**x**		**x**										
Dactylorhiza maculata		**x**			**x**			**x**			**x**	**x**				**x**	
Daphne cneorum														**x**			
Daucus carota								**x**									
Dryas octopetala									**x**								
Euphorbia hyberna								**x**							**x**		
Fragaria vesca		**x**					**x**	**x**								**x**	
Galium odoratum										**x**							
Galium palustre															**x**	**x**	
Galium pumulium			**x**	**x**	**x**	**x**			**x**				**x**	**x**			
Galium verum		**x**															
Galium saxatile											**x**						
Genistella sagittalis			**x**														

	LecL	PraL	EynH	PuiH	BouH	SouH	SouL	VicL	VicH	BetL	BetH	PeyL	TouH	TroH	ChiL	TecL	LuzH
Genista villosa								x									
Gentiana alpina														x			
Gentiana kochiana		x						x									
Gentiana occidentalis														x			
Gentiana pyrenaica					x												
Gentiana verna														x			
Gentiana sp													x				x
Geranium cinereum													x				
Geum montanum									x				x				x
Geranium robertianum							x										
Globularia sp				x													
Helianthemum nummularium									x		x						
Hieracium murorum			x														
Hieracium pilosella				x	x	x								x			x
Hippocrepis comosa											x						
Homogyne alpina														x			x
Horminum pyrenaicum																	x
Hutchinsia alpina													x				x
Ilex aquifolium										x							
Jasione montana											x			x			
Lathyrus linifolius		x			x		x			x					x		x
Leontodon pyrenaicus				x	x												
Lathyrus linifolius	x																
Lathyrus montanus								x									
Leucanthemum alpinum				x													
Lonicera sp.							x										
Lotus alpinus													x				x
Lotus corniculatus	x	x	x	x	x	x	x	x	x	x			x		x		
Lotus pedunculatus									x								
Lychnis alpina											x			x			
Melampyrum pratense		x	x								x						
Melampyrum sylvaticum																	x
Meum athamenticum														x			
Myosotis alpestris													x				
Myosotis sylvatica															x		
Myosotis sp.		x															
Nigritella nigra								x									
Paronychia kapela ssp serpyllifolia											x						

	LecL	PraL	EynH	PuiH	BouH	SouH	SouL	VicL	VicH	BetL	BetH	PeyL	TouH	TroH	ChiL	TecL	LuzH
Pedicularis kerneri												x					
Pedicularis pyrenaïca				x	x												
Phyteuma hemisphaericum						x					x						
Phyteuma spicatum								x									
Pinguicula vulgaris		x						x						x			x
Plantago media																	x
Platanthera bifolia		x															
Polygala alpestris																	x
Polygala alpina									x		x						
Polygala vulgaris								x	x	x		x			x	x	
Polygonum alpinum				x													
Potentilla erecta					x		x	x	x	x	x	x	x	x	x	x	x
Potentilla aurea						x							x	x			
Potentilla pyrenaica					x				x								
Primula sp.		x										x					
Ranunculus brevifolius					x												
Ranunculus gouani																	x
Ranunculus nemorosus		x							x		x				x		
Ranunculus platanifolius				x													
Ranunculus pyrenaeus														x			
Ranunculus sp								x		x		x	x			x	
Rosa pendulina								x									
Rubus idaeus							x			x							
Sagina glabra								x	x								
Saxifraga cuneifolia											x						
Saxifraga granulata													x				
Saxifraga moschata									x								x
Saxifraga stellaris						x											
Saxifraga umbrosa																	
Sedum alpestre														x			
Sedum brevifolium											x						
Sempervivum montanum											x						
Scilla verna																x	x
Silene acaulis													x				
Silene rupestris		x	x	x	x		x				x			x			
Sorbus aria		x						x									
Sorbus aucuparia								x									
Stellaria holostea						x						x			x		x
Stellaria sp.													x				

	LecL	PraL	EynH	PuiH	BouH	SouH	SouL	VicL	VicH	BetL	BetH	PeyL	TouH	TroH	ChiL	TecL	LuzH
Sisymbrium pyrenaicum				x													
Taraxacum sp.									x								
Thalictrum aquilegifolium							x										
Thymus serpillum				x	x	x		x		x	x		x	x	x		x
Trifolium alpinum				x		x			x	x	x		x	x	x		x
Trifolium alpinum albinos											x						
Trifolium pratense		x			x	x			x				x				
Trifolium repens						x											x
Valeriana sp								x									
Veronica alpina													x	x			x
Veronica chamaedrys							x			x	x						
Veronica fruticulosa											x						
Veronica sp			x						x								
Vicia pyrenaica													x				x
Viola biflora											x						
Viola canina																	x
Viola reichenbachiana							x			x					x		

ABSTRACT: Recent global changes affect the physiology, distribution and phenology of species, also they impact population dynamic and interactions among species. Plant-pollinators interactions are particularly threated by global changes and perturbations of these interactions may lead to important changes in plant mating system. In this context of global changes, we determined the pollinator assemblages of *Rhododendron ferrugineum* and how the variability of their efficiencies affects the mating system. We also studied the biogeographic history of *R. ferrugineum* and how it responds to past climatic changes.

We have shown that *R. ferrugineum* is visited by a large variety of insects. Visitor assemblages efficiency varied along environmental gradients but did not seem to impact the mating system of *R. ferrugineum*. This species presents a mixed mating system, indeed it is able to self-fecundate but high levels of inbreeding depression are limiting the development of self-fecundate descendants. High levels of inbreeding depression maintain high genetic diversity in the Pyrenees. Also, it did not allow the evolution of selfing and maintain a mixed mating system. The ability to self-fecundate may favor the colonization of new habitats. During the last glaciation, *R. ferrugineum* survived in large lowland refugia and in nunataks at high altitude. Some marginal populations present a high level of selfing ($F_{IS} > 0$) and low genetic diversity. This genetic pattern is consistent with foundation events and loss of genetic diversity and heterozygosity along colonization rods. Our data suggest that inbreeding depression in these populations should have been low and the reproductive assurance played a key role in the establishment of these populations.

KEY WORDS: alpine habitats, autofecondation, biogeography, effectiveness, environmental gradients, inbreeding depression, microsatellite markers, plant-pollinators interactions, reproductive assurance

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RÉSUMÉ : Les changements globaux récents affectent la physiologie, la distribution et la phénologie des espèces, ainsi que la dynamique des populations et les interactions entre espèces. Les interactions plantes-pollinisateurs sont particulièrement menacées par les changements globaux et la perturbation de ces interactions peut avoir des conséquences importantes sur le système de reproduction des espèces végétales. Dans ce contexte des changements globaux, nous avons déterminé les cortèges de visiteurs de *Rhododendron ferrugineum* le long de gradients environnementaux et étudié comment la variabilité de leur efficacité affectait le système de reproduction de cette espèce. Nous nous sommes également intéressés à l'histoire biogéographique de cette espèce et comment elle a répondu à des changements passés tels que les dernières glaciations.

Nous avons mis en évidence que *R. ferrugineum* est visité par une large diversité d'espèces d'insectes. L'efficacité des cortèges de visiteurs varie le long de gradients environnementaux mais ne semble pas avoir un impact sur le système de *R. ferrugineum*. Cette espèce présente un système mixte de reproduction, avec une capacité à l'autofécondation mais les taux élevés de dépression de consanguinité limitent le développement des individus issus d'autofécondation. Cette forte dépression de consanguinité a permis un maintien relativement élevé de la diversité génétique dans les Pyrénées. Un tel niveau de dépression de consanguinité ne permet pas l'évolution de l'autofécondation et maintient ainsi le système mixte de reproduction chez *R. ferrugineum*. La capacité d'autofécondation de *R. ferrugineum* pourrait lui permettre de coloniser de nouveaux milieux ou de survivre aux changements globaux. Durant les dernières glaciations, *R. ferrugineum* a survécu dans de grands refuges de basse altitude ainsi que dans des nunataks (refuges de haute altitude). Quelques populations marginales présentent un taux significatifs de consanguinité ($F_{IS} > 0$) et des niveaux de diversité génétique particulièrement faibles. Ce patron génétique est consistant avec des évènements de fondation accompagnés de perte de diversité génétique et d'hétérozygotie durant les périodes d'expansion de l'aire de répartition de l'espèce. Ces données suggèrent que la dépression de consanguinité dans ces populations devait être faible et que l'assurance reproductive a joué un rôle fondamental dans l'établissement de ces populations.

MOTS CLÉS : autofécondation, assurance reproductive, biogéographie, dépression de consanguinité, efficacité, gradients écologiques, interactions plantes-pollinisateurs, marqueurs microsatellites, milieux alpins

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