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# Flower-foraging insects and their pollen loads in mountain permanent grasslands

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**Abstract.** Semi natural grasslands are considered as a vital habitat for wild pollinators, which in return contribute to preserve the floristic diversity of this environment. To study the interactions between pollinators and plants, flower-foraging insects were caught from beginning of May to end of June along walking transects in 6 mountain permanent grasslands in Cantal (France). We developed and test in parallel a method based on DNA barcoding analysis, allowing a quick identification of the insect and its pollen load at the same time. We collected thus 394 flower visitor insects, most of them belonging to Diptera (72%) of which comprised 32% Empididae and 20% Syrphidae, and 20 % belonging to Hymenoptera of which 80% were wild native and domestic bees. Three families of flowers (Asteraceae, Apiaceae and Ranunculaceae) comprised two thirds of the total flowering species from which the insects were collected. DNA barcoding of these insects showed that 87% of the collected insects were carrying pollen and 45% were carrying two genders of plants or more. Results suggest the important role of the Diptera as wild pollinators at this period in such mountain environment. Moreover, our results have demonstrated that the DNA barcoding is a powerful tool to study flower-foraging insects and their pollens loads which will be very soon operational.

**Keywords.** Mountain grasslands – Flower-foraging insects – Pollination – Interactions plants-insects – DNA barcoding.

## *Insectes butineurs et transport de pollen dans les prairies permanents de montagne*

**Résumé.** La pollinisation des plantes prairiales est un processus biologique essentiel qui assure le maintien de la diversité floristique des prairies permanentes. Pour étudier ces interactions plantes-insectes, nous avons capturé les insectes butinant les fleurs sur des transects dans 6 prairies permanentes du Cantal, contrastées du point de vue floristique. Nous avons développé et testé en parallèle une méthode d'analyse basée sur le barcoding, permettant la détermination simultanée de l'insecte et de son cortège de pollens. Nous avons ainsi piégé 394 insectes butineurs, très majoritairement des Diptères (72%) dont 32% d'Empididae et 20% de Syrphidées, ainsi que 20% d'Hyménoptères dont 80% d'abeilles domestiques et sauvages. Les Astéracées, Apiacées et Renonculacées comptabilisent les 2/3 des visites. L'analyse des barcodes ADN des pollens a montré qu'une forte proportion des insectes capturés (87%) transportait du pollen et 45% transportaient deux à six genres botaniques différents. Les résultats mettent en évidence le rôle important des Diptères dans les prairies de montagne en tant que pollinisateurs potentiels importants à cette période de l'année. Notre étude a par ailleurs démontré que le barcoding est un outil pertinent et puissant qui sera très bientôt opérationnel.

**Mots-clés.** Prairies permanentes de montagne – Insectes butineurs – Pollinisation – Interactions plantes – Code barre ADN.

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## I – Introduction

In the mountain regions, permanent grasslands constitute the main agricultural area and the primary food supply for domestic ruminants. They represent therefore a crucial issue for these territories. The preservation of their plants diversity is bound to the stability of agricultural management but also to the pollination, natural process allowing reproduction and long-term maintenance of plants. Pollinating insects, carrying pollen from flower to flower, mainly in search of their food, are active pollen vectors and consequently important actors of the reproduction of grassland dicotyledons. However, the role and the importance of many flower-foraging insects in pollen transport are still poorly understood in grassland context especially in mountain areas. Most studies on pollination process focus indeed on crops and/or highlight the role of the grasslands as a source of vital pollinators for entomogames neighboring crops in mixed farming breeding (Rollin *et al.*, 2013). Grasslands are paradoxically poorly studied in terms of intrinsic pollination. Increase our knowledge on transport pollen by insects in grasslands is an important scientific goal that would help us to better define the issue of pollination in these types of areas. In that context, the current development of DNA analysis (DNA barcoding) presents an opportunity to assess the plant-pollinator webs. In this study, we set up field observations in mountain grasslands to explore the flower-foraging insect networks and we tested in parallel the DNA barcoding method as an alternative tool to enhance our knowledge on those interactions.

## II – Materials and methods

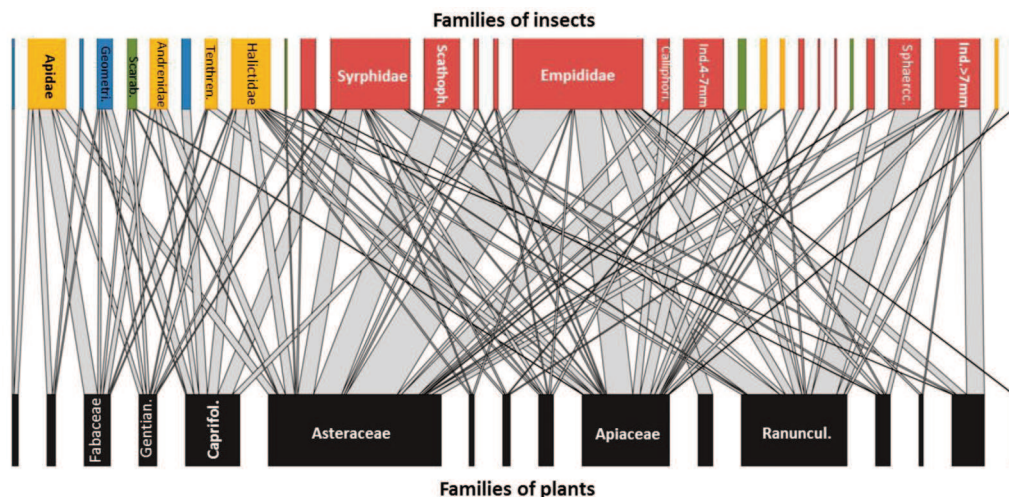
The study was implemented in the INRA experimental farm in Marcenat (Cantal, France – 45.3046N, 2.8378E, alt 1000m), located on a mountain grassy volcanic plateau. The site is characterized by a high mean annual precipitation (1205 mm, 1989-2009) and a cold mean annual temperature (7.5°C) even in summer (July: 15.4°C, August: 15.5°C). Six semi natural grasslands were sampled displaying two management regimes (mown and grazed) and three management intensities (low, medium and high level) along a floristic richness gradient. Flower-foraging insects, collecting actively pollen and/or nectar, were caught along a walking 100m-transect under clement weather conditions. Time spent per transect was 15 min gross (chronometer stopped each time an insect was caught). Three sampling periods of 2 or 3 days of trapping were done: between 7 May and 12 May (P1, 2 days), between 28 May and 2 June (P2, 3d) and between 17 and 22 June (P3, 3d). In total, 48 transect observations were done (6 plots x 8 days). Order of transects varied according the day of trapping, so that each grassland were observed in the morning, at midday or in the afternoon during a period.

Most of the insects were caught directly in a sterile vial to reduce contamination for further DNA analysis and were brought back to the laboratory. Only a few fearful insects were first caught in a clean net and then transferred directly in a sterile vial. Pollen was removed from the insect body by adding ethanol in the vial containing the insect and shaking it firmly. The insect was then removed from the vial and pinned for visual identification.

To identify insects and their pollen loads using DNA barcoding, the alcohol solution containing the pollen extracted from the insect was filtered while the 2 front legs of the insects were taken. Finally, the filter was placed in the microtube with the 2 respective legs and 30 µL of a buffer solution. Once DNA was extracted and amplified with PCR, pollen and insect were analyzed simultaneously using a small region of the Cytochrome Oxidase Subunit 1 (COI) (Hebert *et al.*, 2003) for insects and the ribosomal ITS region which includes ITS2 (Keller *et al.*, 2014) for pollens. Only the insects and their pollen of the two first collecting periods were analysed by barcoding.

### III – Results and discussion

Over the three periods, we observed and caught a total of 394 flower-foraging insects (76 in P1, 160 in P2 and 158 in P3). Abundance was higher in plots with high level of botanical diversity: average of 11 and 12 individuals trapped per transect in cut and grazed plots respectively against 6 individuals in plots cut or grazed with low level of diversity. We identified 11% of the individuals to family level, 37% to genus level and 37% to species level. Fifteen percent remained undetermined, mainly small individuals (< 7mm), and were morphotyped. Diptera represented 72% of the total catches far ahead from Hymenoptera (20%), Lepidoptera (5%) and Coleoptera (3%). Five families of Diptera counted 62% of the total catches (Empididae, Syrphidae, Scathophagidae, Sphaeroceridae, Indetermined<7mm). Empididae was the most abundant family among the total catches (23%) as among Diptera (32%), followed by the Syrphidae (14% of total catches and 20% of the Diptera). Small undetermined flies represented 21% of the Diptera. Apidae (*Apis* and *Bombus*) and Halictidae families counted each for 7% of the total catches following by the Andrenidae family (3%). All these insects were foraging on 36 plants species belonging to 16 botanical families. Most of them were found on Asteraceae (118 visits: 30%) such as *Taraxacum* (71 visits), Ranunculaceae (18%) such as *Ranunculus acris* and *Ranunculus bulbosus*, and Apiaceae (15%) such as *Conopodium majus*. These three families encompassed 63% of the total flowering species from which the insects and mainly Diptera (207 individuals) were collected. Caprifoliaceae family, represented by a single genus (*Knautia*) counted 37 visits (9%), mainly of wild bees, Syrphidae and Lepidopterae.



**Fig. 1. Flower-forager insects network obtained from visual surveys.** Rectangles represent insect families (above) and plants (below). Their widths are proportional to the sum of interactions involving them. Colours of the rectangles differ according to the insect orders.

The flower-forager network (Fig. 1) highlights that Diptera foraged 13 of the 16 plant families foraged by insects. Only the Fabaceae, the Orobanchaceae (*Rhinantus* sp.) and the Lamiaceae were not visited by flies. Empididae (Diptera) were the insect group with the largest foraging range by foraging 11 different families, mostly Asteraceae and Apiaceae. Moreover, they were the only insects to forage little flowers such as *Veronica* sp., *Viola lutea* and *Cruciata laevipes*. The Syrphidae visited also several plant families (10), mostly the Asteraceae, the Caprifoliaceae and the Ranunculaceae. Likewise, almost all of the Scathophagidae individuals were found on Asteraceae and

most of the Sphaeroceridae on Ranunculaceae. Regarding the domestic and wild bees, Apidae foraged five plant families with a preference for Fabaceae. Halictidae visited ten plant families mainly Caprifoliaceae and Asteraceae, while the Andrenidae were found only on three families. Wasps (Ichneumonidae) and sawflies (Tenthredinidae) were found nearly exclusively on Apiaceae. Although they were not abundant in our catches, the three families of Coleoptera and the four families of Lepidoptera visited a large range of families of plants (8 and 5 families of plants respectively). Our study allows pointing out that Diptera are key-flower-foraging insects at the beginning of the grazing season in our mountain grasslands. These results confirm the observations of Orford *et al.* (2015) who have recently underlined the role of these “forgotten” insects in pollination process. Like Lefebvre *et al.* (2014) in alpine meadows, we have shown that Empididae were important flower visitors. We have also observed that some small Empididae are only forager insects of three small flowers, which suggests the importance of these species in pollen dispersion for tiny blossoms. Finally, we have underlined the role of the “dung flies” (Scathophagidae) who constituted 9% of our captures of Diptera. Their reputation of effective carrier of pollen because of their plentiful pilosity has been already demonstrated by Skevington and Dang (2002). Our results corroborate the hypothesis of Pouvreau (2004) who suggests that Diptera may play an important role in pollination in regions with harsh climatic conditions such mountain areas in which pollinators like Apoidea are inactive a long period of the year. Those results allow also alerting about the very little number of taxonomists who are able to identify species from this key order beyond Syrphidea.

Regarding our DNA barcoding results, pollen was detected and identified on the body of 82% of the insects caught. The key result is that 42% of these individuals transported a mixed pollen load, composed from two different plant genera up to six genera (2% of individuals). Moreover, we have also highlighted that 3% of the insects transport pollens from non-grassland plants such as *Betula sp.*, *Quercus sp.* and *Salix sp.* However, our identifications of the insect species were less successful. We identified only 27% of the insects, most of them being Diptera (84%). Twenty four percent were not identified while the PCR produced enough reads to identify them. This result means that the individuals were not still referenced in the international databases. The barcoding of every insect is indeed in progress and represents a challenging goal in view of the huge insect species number. Another 106 individuals remained nevertheless undetermined, most of them being Hymenoptera and very small flies (< 4mm), for which the PCR produced no reads or not enough reads. PCR issue due to non-universal primers seems the main reason of Hymenoptera identification failure. Concerning the small flies, the difficulties of identification can be due to the very little quantity of DNA material we have obtained with the two tiny legs. Nevertheless, we have proved the powerfulness of the DNA barcoding for pollination study applications but we have also shown that the technique needs to be improved to be fully operational. In an immediate future, DNA barcoding will be a new tool in the taxonomists toolbox supplementing their knowledge as well as being an innovative device for non-experts who need to make identification for ecological studies (<http://www.barcodeoflife.org>).

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