

Doctoral thesis overview in Organismal Biology

**From fundamental questions to practical conservation actions:  
a study of the pollination system, population genetics and  
mycorrhizal associations in *Cypripedium calceolus* (Orchidaceae)**



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## Introduction

According to general agreement, we are currently facing the Earth's sixth mass extinction of living species (Canadell and Noble, 2001; Barnosky et al., 2011; Ceballos et al., 2017). Contrary to previous mass extinctions, the current one is caused by anthropogenic factors (Primack, 2014). Every year, destruction of natural habitat, over-exploitation of natural resources and other global change factors cause a disheartening loss of the extraordinary diversity of life (IUCN/SSC, 2008; Primack, 2014). In 2017, the IUCN Red List Assessment (<http://www.iucnredlist.org>) reported more than 13,200 animal species and 12,500 plant species falling into one of the categories of threat (IUCN, 2017). In response to this menace, the field of Conservation biology has appeared over the last 35 years with three main objectives : (i) catalogue biodiversity, (ii) study the causes of diversity decline and (iii) develop practical approaches to prevent species extinction (Primack, 2014).

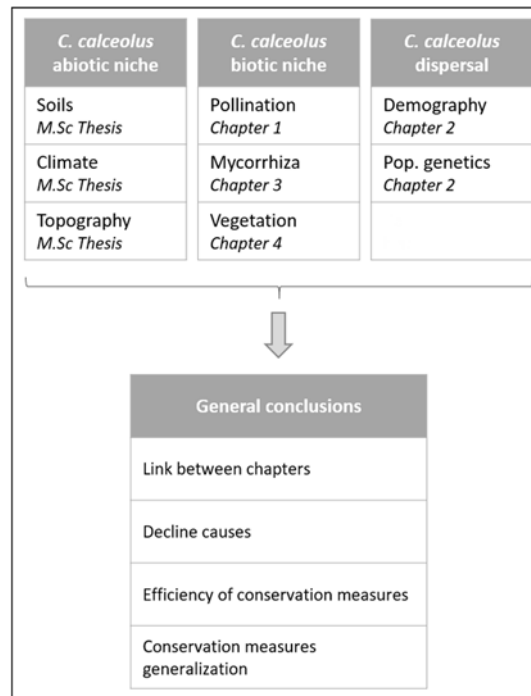
Focusing on vegetation, Swarts and Dixon (2009) argued that conservation through the reserves' system is not enough to comprehensively protect all plant species. As a matter of fact, because of loss, fragmentation and change of habitats combined with loss of species, restoration of viable populations can rarely be achieved by natural recruitment and dispersal alone (Seddon, 2010). Therefore, an effective maintenance of endangered plant species is needed, including among others (i) identification of factors threatening the populations (ii) systematic collection of baseline data on species natural history and (iii) study of population demography and genetics (Pavlik, 1994; Heywood and Iriondo, 2003).

Among flowering plants, Orchidaceae are one the most threatened family (Fay, 2018). Indeed, the complexity of their ecological interactions with pollinators and mycorrhizal fungi makes them particularly vulnerable and challenges conservationists. In order to protect them despite these difficulties, integrated conservation research has to be conducted to link all aspects of their complex ecology. In addition, translating scientific insights about orchids into practical conservation measures is urgently needed (Swarts et al., 2007; Swarts et al., 2009; Fay et al., 2015; Fay, 2016; Fay, 2018; Gale et al., 2018).

In this study, we focus on the Lady's slipper orchid *Cypripedium calceolus* L. (Orchidaceae). It is a well-known emblematic and patrimonial orchid in Europe. Indeed, it is one of the most famous plants because of its particular flowers and its exotic look (Terschuren, 1999). This Eurasian boreal species is distributed from the United Kingdom to the Pacific Ocean. This represents a large range but actual populations show a scattered distribution. Indeed, the number and size of these populations have drastically regressed and this species is rare and threatened by extinction, especially in Europe (Kull, 1999; Terschuren, 1999; Druart, 2007). As consequence, in Switzerland, numerous actions and reintroduction plans to conserve this orchid were developed, without reaching the expected outcomes. These mixed results were caused among others by a lack of ecological and biological understanding of *C. calceolus* (Storrer, 2015; Rusconi, 2017).

To develop an efficient conservation plan for this species, the aims of this thesis are to understand its (i) pollination and chemical ecology, (ii) population genetics and demography in Switzerland, (iii) relationship with mycorrhiza, and (iv) how vegetation type and structure affect its habitat use (Fig. 1 and Fig. 2).

In summary, the general target of this thesis is to develop practical conservation approaches based on fundamental knowledge about the natural history, biology, ecology demography and genetics of *C. calceolus* that could be applied and generalizable to other plant species (Fig. 1).



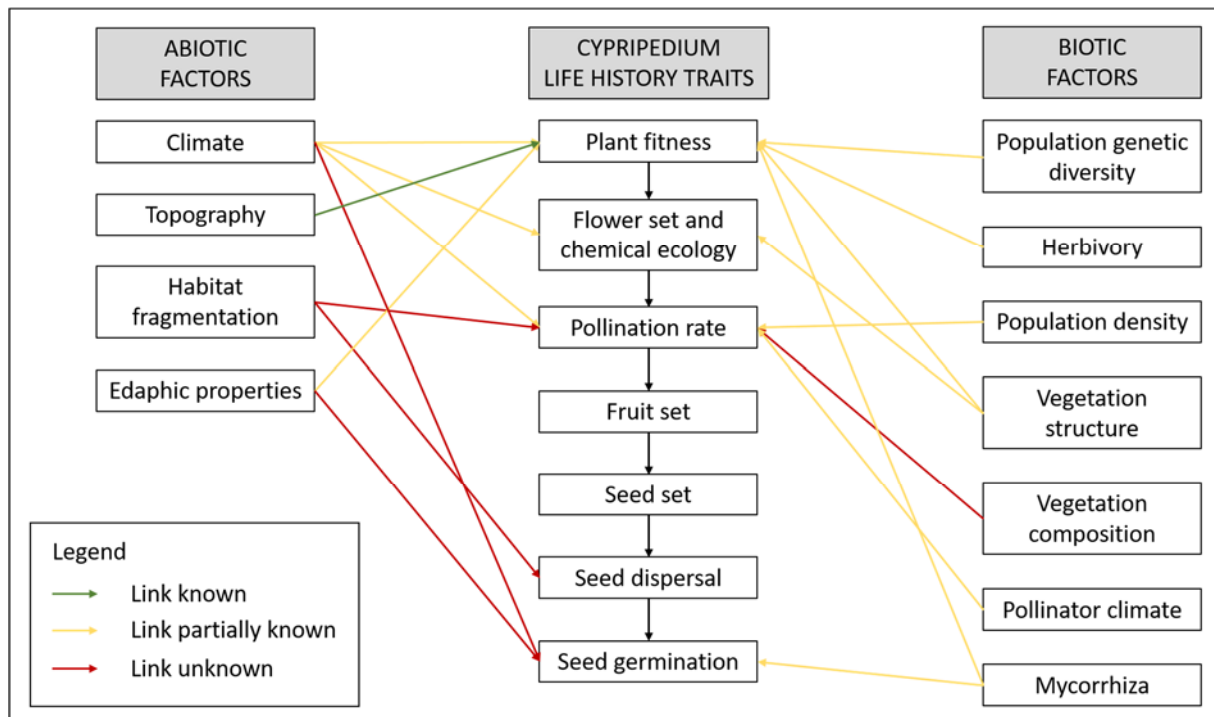
**Figure 1** : Four main field of study and their corresponding chapters in this thesis. The abiotic niche has already been studied in Rusconi (2017).

## State of art

Orchids are one of the largest family of flowering plants and are also one of the most threatened (Fay, 2018). Indeed, the complexity of their life history make them particularly sensitive and vulnerable to environmental changes (Fay and Chase, 2009b). Additional information such as (i) demography and natural history of pollinators, (ii) identification, phenology, demography and biochemistry of sympatric flora, (iii) genetic variability within orchid populations, (iv) number of genotypes that have reached flowering size vs. the number of plants that bloom annually and (v) pollination rates and fruit set vs. the impact of climatic cycles is required to monitor and conserve populations of threatened orchids (Bernhardt and Edens-Meier, 2010).

Among Eurasian orchids, the Lady's slipper orchid *Cypripedium calceolus* L. (Orchidaceae) is one of the most famous and emblematic species (Kull, 1999). In spite of its large distribution range, this species is rare and threatened with extinction (Terschuren, 1999). Hence, over years, numerous studies have focused on the complex biology of this species (e.g. Blinova, 2002; Antonelli et al., 2009; Barsberg et al., 2013). Even though knowledge about *C. calceolus* is constantly increasing, great gaps remain (Fig. 2).

Hereafter, ecological and conservational issues about orchids and *C. calceolus* that need attention for this study are concisely reviewed.



**Figure 2 :** Links between *C. calceolus* life history traits and biotic and abiotic factors. Green, yellow and red arrows highlight state of knowledge according the current understanding.

### *Pollination and chemical ecology*

Since Darwin (1862), orchid pollination has fascinated many biologists because of its complexity and ecological and evolutionary importance (Antonelli et al., 2009). More than one-third of orchids presents deceptive pollination syndrome, which means that they offer no reward to their floral visitors and instead trick them into pollination (e.g. Antonelli et al., 2009; Case and Bradford, 2009; Ackerman et al., 2011). In the most common form of deception pollination, the flower takes the appearance of a potential food source (Tremblay et al., 2005; Ackerman et al., 2011). In addition, deceptive plants can mimic insect pheromones or signals announcing nesting sites (Dafni, 1984; Ackerman, 1986; Barkman et al., 1997). However, pollinators can learn to avoid non-rewarding species, causing low visitation and fruiting rates (Case et al., 2009).

The pollination system of *C. calceolus* was initially described by Darwin (1862) and later by Nilsson (1979). Pollinators are attracted in the *Cypripedium* labellum by a sweet scent and by visual cues (Nilsson, 1979; Bergström et al., 1992; Antonelli et al., 2009). Once in the labellum, pollinators are trapped and find no reward because this orchid does not produce nectar. To leave the flower, insects have to climb through the posterior exit where pollen grains are deposited on their body (Antonelli et al., 2009; Case et al., 2009). Detailed studies trough Europe described different genera of insects as pollinators such as *Andrena*, *Apis*, *Chrysotoxum*, *Colletes*, *Erastilis*, *Halictus*, *Hoplocampa*, *Lasioglossum*, *Nomada*, *Musca*, *Pipiza*, *Platycheirus* and *Syrphus* (Erneberg and Holm, 1999; Kull, 1999; Blinova, 2002; Ishmuratova et al., 2006; Antonelli et al., 2009; Pemberton, 2013; Braunschmid et al., 2017). In most cases, pollinators are taxonomically heterogeneous females of mixed, small-medium, generalist, solitary bees (Antonelli et al., 2009; Bernhardt et al., 2010). In addition, it is important to

note that pollinator species vary according to the studied geographic area and altitude (Braunschmid et al., 2017).

While the scents emitted by *C. calceolus* to attract pollinators have been studied for over 40 years, this deceptive phenomenon is still not fully understood (Przybylowicz et al., 2012). Nilsson (1979) showed that *C. calceolus* produces acetates in addition to ordinary fragrance attractants. Those chemical compounds play an important role in *Andrena* pheromones and they may inhibit orientation of pollinators making them visiting this orchid repeatedly (Nilsson, 1979; Antonelli et al., 2009; Fay et al., 2015). However, the relevance of acetates as deliberate chemical mimicry to attract pollinators remains to be confirmed (Przybylowicz et al., 2012). More recently, Bergström (1992) found that *C. calceolus* fragrance is composed mainly of fatty acid derivatives while Braunschmid et al. (2017) detected more than 70 compounds in scents samples dominated by aliphatics, terpenoids and aromatics.

### *Population genetics and population demography*

Genetic, demographic and ecological investigations are fundamental to understand the factors contributing to plant population diversity. It is especially true for species capable of clonal reproduction such as *Cypripedium* genus (Gargiulo et al., 2018). Therefore, many papers focused on genetic diversity of orchids and more specifically on *Cypripedium* (e.g. Case, 1994; Sun and Wong, 2001; Brzosko et al., 2011). The population genetic structure of *C. calceolus* was studied in Poland (Brzosko, 2002; Brzosko et al., 2002; Brzosko et al., 2011), Estonia (Gargiulo et al., 2018) and at a broader scale through Europe (Fay et al., 2009a). Even though different techniques to characterize genetic diversity of populations were used (analyze of allozymes, chloroplast DNA and total genomic DNA), the main results concur. First, the genetic diversity within *C. calceolus* is relatively high compared with rare taxa and taxa with the same life history (Brzosko, 2002; Brzosko et al., 2002). Secondly, the genetic diversity among populations was much smaller than the genetic diversity within populations at both national and international scales (Brzosko, 2002; Brzosko et al., 2002; Fay et al., 2009a). Brzosko (2002) and Gargiulo et al. (2018) did not find a strict correlation between genetic diversity and geographical factors while Brzosko et al. (2011) found a significant pattern of isolation by distance. Moreover, signatures of a bottleneck effect and recent founder events were identified in Estonia, and genetic and genotypic diversity variables were significantly correlated with population size in Poland (Brzosko et al., 2011; Gargiulo et al., 2018). To conclude, the main factors maintaining genetic diversity in *C. calceolus* populations are longevity of genets, out-crossing breeding system and the presence of recruitment for seeds (Brzosko et al., 2002).

In populations of most animal-pollinated plant species, pollination efficiency and fruit set depend strongly on population size and local density (Fritz and Nilsson, 1994; Brys et al., 2008). Indeed, plant aggregation and co-flowering species can influence pollinator attraction (Internicola et al., 2006). In this regard, Agren (1996) showed that the positive correlation between population size and seed set is a result of insufficient pollen transfer in small populations of the self-incompatible herb *Lythrum salicaria*. Focusing on orchids, the reproductive success of rewarding the terrestrial orchid *Listera ovata* increased with increasing population size until a threshold of 30-40 flowering plants (Brys et al., 2008). For rewardless plants, on the other hand, reproductive success may rely strongly on pollinators' learning ability and consequently may be negatively correlated with population aggregation (Internicola et al., 2006). For example, the reproductive success of the rewardless orchid *Dactylorhiza sambucina* is negatively affected by its own spatial aggregation and that of co-flowering rewarding species (Internicola et al., 2006). Contrary to these trends, bees pollinating *C. calceolus* are attracted

by plants in group, particularly by those exposed to direct sunlight (Ramsay and Stewart, 1998). Additionally, there is a positive relationship between pollination rate and population size (Nilsson, 1979; Brzosko et al., 2017).

### *Mycorrhiza identification*

Orchidaceae, as numerous other green plants in different families, are partially mycoheterotrophic (Gebauer and Meyer, 2003; Bidartondo et al., 2004; Smith and Read, 2010). Mycorrhizas of the orchids types (OM) produce intracellular coils called pelotons. The distribution pattern of these structures in orchid tissues and their sufficiently distinctive appearance are the main defining attributes of OM (Smith et al., 2010). Most orchids have dust-like seeds (0.3-14 µg) with minimal nutrients reserves, making them dependent on OM for the provision of necessary resources for seeds germination (e.g. Smith et al., 2010; Sathiyadash et al., 2012). Concerning the adult phase, there is a common assumption that the photosynthesis will relieve green orchids from their fungal dependence (Smith et al., 2010; Sathiyadash et al., 2012). However, numerous studies proved the presence of OM during the adult phase, without understanding the physiological interaction between the two (e.g. Shefferson et al., 2007; Sathiyadash et al., 2012; Fay et al., 2018). In addition, Roy et al. (2009) showed that the relationships with OM can change during an orchid's lifetime with different fungi playing different roles at different life stages, at least in some species (Fay et al., 2015).

Lack of knowledge about OM makes orchid conservation even more challenging. Indeed, as mentioned above, the relationship with fungi can influence the capability of an orchid to set up and colonize new areas (De Long et al., 2012; Fay et al., 2015). In addition, the rarity of some orchid species could be linked to the sparse distribution or narrow ecological requirements of symbiotic partners (e.g. Phillips et al., 2011; Nurfadilah et al., 2013; Fay et al., 2015).

*Cypripedium* taxa and *Cypripedium calceolus* are associated almost solely with fungi from the Tulasnellaceae family (Shefferson et al., 2005; Shefferson et al., 2007). However, in *C. calceolus*, an important gap remains: it is still not known if the fungi involved in early stages of seedling development are the same as those involved in mycorrhizal relationship with adults plants (Fay et al., 2018). Shefferson (2007) observed a lack of morphological evidence of mycorrhizal colonization in some *C. calceolus* seedlings, whereas adult plants always exhibited pelotons. This is a surprising result because, as mentioned above, seedlings are more likely to need mycorrhizal nutrition than adult plants (Rasmussen, 1995; Shefferson et al., 2007). This result may be explained by the dormancy phase of some *Cypripedium* including *C. calceolus*, making them dependent on fungal carbon even during adulthood (Shefferson et al., 2007). Confirming the presence of OM in adults *C. calceolus*, Fay (2018) analyzed the enrichment of isotopes of carbon (C), nitrogen (N) and hydrogen (H) that is indicative for exchange of matter between fungi and orchids in plant leaves. All the studied individuals (native and introduced plants) were likely to have formed mycorrhizal association (Fay et al., 2018).

### *Vegetation type and structure*

The reproductive success of rewardless plants depends on the presence of co-blooming flowers that are highly attractive to pollinators and called "magnet species" (Lavery, 1992). There is a lack of studies on plants blooming concurrently with orchids (Bernhardt et al., 2010). Despite that, Johnson (2003) found that the pollination success of the deceptive orchid *Anacamptis morio* was enhanced by the presence of co-occurring rewarding magnet plants. On the other hand, Internicola (2006) showed that the pollination success of *Dactylorhiza sambucina* was negatively influenced by the presence of

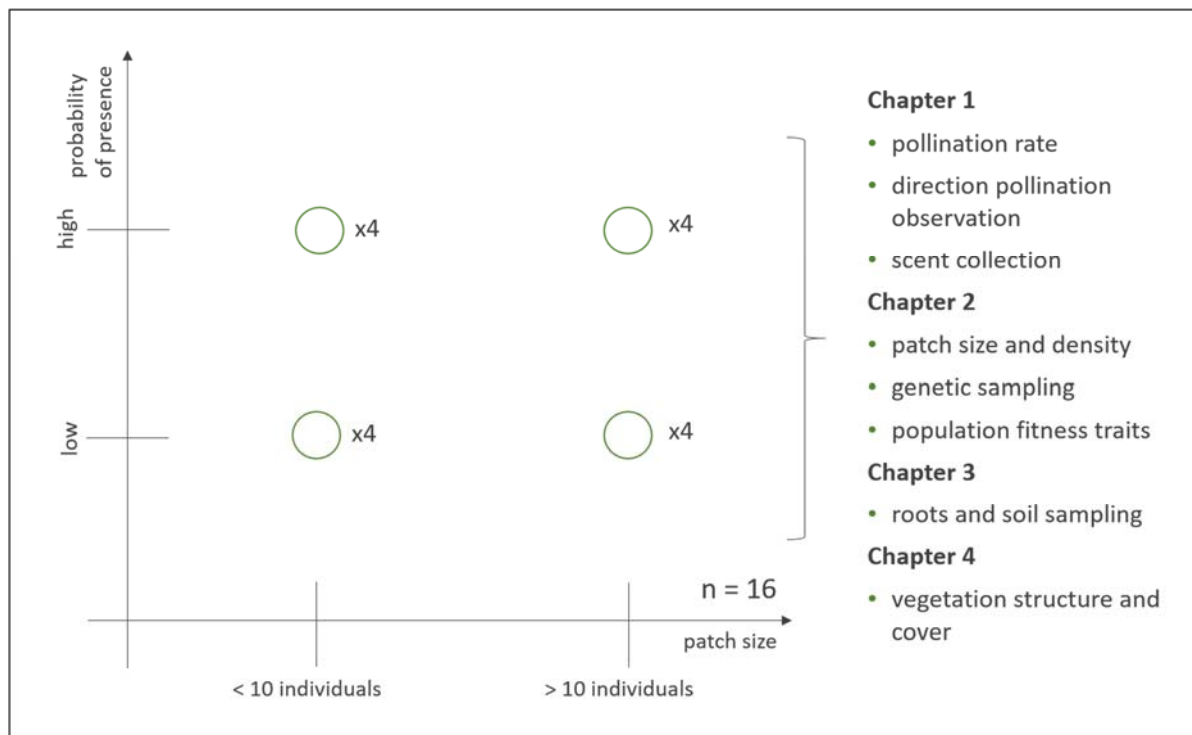
co-flowering rewarding species. Concerning *C. calceolus*, a study on 30 populations in Poland showed that the fruiting rate is positively correlated with the number of co-blooming flowers. This may indicate a magnet effect (Brzosko et al., 2017).

Plant pollination is influenced by vegetation structure, such as the absence or the closure of canopy (e.g. Bertin and Sholes, 1993; Case, 1994; Walters and Stiles, 1996). In the case of *Cypripedium parviflorum*, the forest canopy closure is correlated with a loss of pollinator visitation (Case et al., 2009). Moreover, *C. calceolus* flowering is negatively affected by shading of trees and shrubs, while shading of canopy does not influence fruit set (Brzosko et al., 2017).

## Outline of the research

In order to conduct this research, during the first year of fieldwork, 16 populations of *C. calceolus* (cantons of Vaud, Neuchâtel, Bern and Aargau, Switzerland) will be sampled (Fig. 3 **Figure 3** and Appendix I). We chose these populations according to two main criteria: (i) their size (more or less than 10 individuals) and (ii) the suitability of the habitat regarding a prediction map corresponding to the ecology of this orchid (Rusconi, 2017, Appendix I). In addition, these 16 populations cover an altitudinal gradient from 400 m to 1500 m.

Over the coming years, we will sample more *C. calceolus* populations following the same site selection strategy.



**Figure 3:** 2018 field experimental design: selection of 16 populations according to the size and probability of presence. The right column indicates the measurements that will be performed per chapter.

## *Chapter 1: Pollination and chemical ecology of *C. calceolus* in Switzerland*

### **Working hypothesis**

1. The pollinator climate varies across Switzerland and in comparison with other Eurasian countries.
2. The pollination rate is weaker in smaller populations (<10 individuals).
3. The position of a population in the landscape influences the pollination rate.
4. Pollination decreases with shading.
5. *C. calceolus* scent mimics the scent of rewarding yellow flowers.

### **Experimental design**

In each of the 16 studied *C. calceolus* populations, insects will be caught in yellow pan traps in order to draw a picture of general pollinator climate (e.g. Dötterl and Vereecken, 2010). In addition, 4 additional steps will be performed on 10 flowering individuals: (i) pollinators sampling directly in the labellum (e.g. Case et al., 2009), (ii) Quantification of the amount of sun reaching each using a sun compass (e.g. Rusconi, 2017), (iii) flower volatiles collection using adsorbent tubes (e.g. Braunschmid et al., 2017) and (iv) enumerating the number of fruits at the end of the flowering season. Furthermore, we will collect volatiles on yellow flowers co-blooming with *C. calceolus*.

Moreover, in order to study population connectivity, we will quantify habitat fragmentation and population isolation by map analysis.

## *Chapter 2: Genetics and demography of *C. calceolus* in Switzerland*

### **Working hypothesis**

1. Swiss metapopulations of *C. calceolus* are not genetically connected anymore.
2. Smaller populations of *C. calceolus* have a smaller genetic pool.
3. *C. calceolus* populations with a weaker genetic pool have a lower fitness.

### **Experimental design**

In each of the 16 studied *C. calceolus* populations, we will sample the leaves of 20 individuals (or less if there are fewer plants). We will then analyze the genetic structure of population through Switzerland with a molecular method, which is not defined yet. This molecular part will be conducted in collaboration with the Swiss federal Institute for Forests, Snow and Landscape Research (WSL).

In order to link population genetic pattern and plant vitality, we will measure population fitness traits on the 20 above-mentioned individuals. Studied traits are surface leave area (SLA), photosynthetic activity, number and size of stems, number and area of leaves and number of flowers and fruits.

Moreover, we will collect data about population surface and plant patches areas.



### *Chapter 3: C. calceolus mycorrhiza identification*

#### **Working hypothesis**

1. *C. calceolus* is involved in different symbiotic relationship with fungi over its life cycle.
2. The narrow ecological requirements of *C. calceolus* are determined by the ecological requirements of its symbiotic fungi.

#### **Experimental design**

In each of the 16 studied *C. calceolus* populations, 3 pieces of roots will be sampled on 10 adult plants and on 3 juveniles, if the conditions allow it. We will identify mycorrhiza visually (microscopy) and genetically (DNA barcoding). In addition, 10 soil samples will be collected in order to assess the diversity of symbiotic fungi present in each sites by metabarcoding.

### *Chapter 4: Vegetation type and structure of C. calceolus*

#### **Working hypothesis**

1. *C. calceolus* pollination is positively impacted by the presence of co-blooming flowers.
2. The change of forestry regime and especially the dimming of forests negatively impact *C. calceolus*.

#### **Experimental design**

In each of the 16 studied *C. calceolus* populations, we will perform vegetation inventories using the Braun-Blanquet method (Braun-Blanquet and Pavillard, 1928). In addition, we will evaluate visually the total coverage of flowering plants.

Additionally, we would like to understand the link between population regression and change in forestry regime. To this end, we will study old vegetation surveys and maps of forests through years.

## Timetable

Year	2018			2019			2020			2021		
Month	1 → 4	5 → 8	9 → 12	1 → 4	5 → 8	9 → 12	1 → 4	5 → 8	9 → 12	1 → 4	5 → 8	9 → 12
<b>Chapter 1: Pollination and chemical ecology of <i>C. calceolus</i> in Switzerland</b>												
Field sampling												
Scent analysis and pollinator identification												
Soil analysis												
Habitat fragmentation study												
Data analysis and paper writing												
<b>Chapter 2: Genetics and demography of <i>C. calceolus</i> in Switzerland</b>												
Field sampling												
DNA sequencing												
Data analysis and paper writing												
<b>Chapter 3: <i>C. calceolus</i> mycorrhiza identification</b>												
Field sampling												
Soil metabarcoding												
Plant metabarcoding												
Data analysis and paper writing												
<b>Chapter 4: Vegetation type and structure of <i>C. calceolus</i></b>												
Field sampling												
Data analysis and paper writing												
<b>Side projects</b>												
<i>C. calceolus</i> reintroduction monitoring												

**Budget**

Items	Unit price [CHF]	Quantities	2018 [CH]	2019 [CH]	2020 [CH]	2021 [CH]	TOTAL [CHF]
<b>A) Salaries</b>							
PhD (60%)	-	-	41 633.7 *	43 526.7	45 419.8	49 202.8	<b>179 783</b>
Social costs (25%)	-	-	10 408.4 *	10 881.7	11 354.9	12 300.7	<b>44 946</b>
<b>B) Mobility and publication fees</b>							
Meetings	-	-	-	2000	2000	2000	<b>6000</b>
Publications fees	-	-	-	3000	3000	3000	<b>9000</b>
<b>C) Consumables</b>							
Objectives 1 + 4 (Pollination and vegetation)							
VOCs sampling	3	16 populations * 10 plants	480				<b>480</b>
Pollinator traps	10	20	200				<b>200</b>
Soils analysis	25	16 populations * 3 samples	1200				<b>1200</b>
Travel	0.5 CHF/km	17 000	3500	2500	2500		<b>8500</b>
Objective 2 (Genetics)							
DNA sequencing	20	16 populations * 10 plants		3200	3200		<b>6400</b>
Objective 3 (Mycorrhiza)							
Soil metabarcoding	25	16 populations				400	<b>400</b>
Plant metabarcoding	25	16 populations * 10 plants				4000	<b>4000</b>
<b>TOTAL (A+B)</b>			<b>52 042.1</b>	<b>59 408.4</b>	<b>61 774.7</b>	<b>66 503.5</b>	
<b>TOTAL (C)</b>			<b>5380</b>	<b>5700</b>	<b>5700</b>	<b>4400</b>	
<b>TOTAL (A+B+C)</b>							<b>260 909</b>

\* = Funded by University of Neuchâtel

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## Appendix I

16 sampling points selected for 2018 field season.

Legend: Probability of presence according to *C. calceolus* ecology: 0 (red) means area predicted to be not adapted; 1000 (blue) means area predicted to be highly favorable.

