International Emerging Action 2019 - CNRS

Convergent evolution of deceptive pollination syndrome in *Ceropegia* and *Aristolochia*

Franco-Thai project

Centre d'Ecologie Fonctionnelle & Evolutive

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STATE OF THE ART

Although often neglected by ecologists, Diptera is one of the most important groups of pollinators for biodiversity but also for crop production (Ssymank et al. 2008). They were probably the main pollinators of early angiosperms. The long evolutionary history between Diptera and Angiosperms has produced a great diversity of pollination syndromes, which are less well known than those involving other insects such as Lepidoptera and Hymenoptera. Among these syndromes, deceptive pollination has appeared independently in various plant families, with flowers mimicking food resources, nesting sites, or mating sites.

Although deceptive pollination does not involve coevolution between plants and pollinators - since pollinators do not get the expected benefits - this type of pollination has led to a great diversification and sometimes specialization of plant lineages, as for example orchid species (Cozzolino & Widmer 2005). The genera *Ceropegia* (Gentianales: Apocynaceae) and *Aristolochia* (Piperales: Aristolochiaceae) are good examples, each with several hundred species deceiving Diptera for their pollination. Deception in both genera has long been suspected (Vogel 1961, 1978), but is still poorly understood. For example, the very nature of deception (food resource, nesting site or mating site) is generally inferred from the scent and colour of the flowers, but rarely studied in detail. The lack of knowledge on the biology of the dipteran pollinators blurs further the understanding of deceptive pollination.

Aristolochia and *Ceropegia* attract a great diversity of Dipteran families at the genus level. However, it seems increasingly evident that many species are each specialized on a few pollinator species (e.g. Ollerton et al. 2017, Heiduk et al. 2017). Among the most remarkable pollinators are flies of the families Chloropidae and Milichiidae (Figure 1), many of which are known to be kleptoparasites of predatory insects, i.e. they feed on exudates (hemolymph, for example) of injured or digested prey (external digestion by spiders, for example). Recent publications on one species of *Aristolochia* (Oelschlägel et al. 2015) and two species of *Ceropegia* (Heiduk et al. 2015, 2016) have demonstrated that these plants attract Chloropidae and/or Milichiidae by mimicking the odour of crushed insects, a strategy called kleptomyiophily. Among these smells are those of crushed bugs (Heteroptera). Some Chloropidae and Milichiidae have long been known to be attracted by these odours (Aldrich & Barros 1995), but the link with pollination has

Figure 1: Milichiid flies attracted to a flower of *Ceropegia tenuicaulis* in Thailand.

only recently been demonstrated in *Aristolochia rotunda* (Oelschlägel et al. 2015). Based on the composition of floral scents, a similar strategy is suspected in *Ceropegia* from South Africa (Heiduk et al. 2017). In addition, a very recent and unpublished study conducted in the framework of the collaboration between the two partners of this project demonstrated a similar strategy in two species of *Ceropegia* from Thailand. This highly specialized strategy is probably widespread in *Aristolochia* and *Ceropegia*, allowing study of the convergent evolution of a highly specialized pollination syndrome.

SCIENTIFIC PROJECT

Global decline of pollinators is a major concern because it threatens crop production and plant biodiversity. Most reports on pollinator decline have focused on Hymenoptera (Potts et al. 2010), and little is known about the decline of Dipteran pollinators. A great diversity of wild plants rely on pollination by Diptera and a large part of this diversity is engaged in specialized pollination, which might be particularly threatened by pollinator decline. A thorough understanding of plant-pollinator interactions is a pre-requisite to address issues in conservation of animal-pollinated plants in the wild. *Ceropegia* and *Aristolochia* are highly diversified plant genera. They are recognized for their high propensity for extreme endemism and rely largely on specialized pollination strategies with flies, making these plants particularly at risk under global pollinator decline. Little is known on the pollination biology of the several hundreds of species described in each of these genera, making risk assessment premature and conservation measures under risk of failure. In addition to potential pollinator decline, many of these species are threatened by habitat degradation and use as medicinal and ornamental plants, combined with the fact that populations are small and localized (Bagul & Yadav 2006, Murthy et al. 2012). Knowledge on pollination biology of these species might help to optimize artificial breeding, thus contributing to local market economies and preventing destructive collection in wild populations. In this project we propose to provide new keys to the understanding of pollination biology in *Ceropegia* and *Aristolochia*. We hope to draw attention to the importance to taking species interactions into account in conservation actions.

Kleptomyiophily as a pollination strategy

The first goal of this project is to estimate to what extent *Ceropegia* and *Aristolochia* rely on kleptomyiophily as a pollination strategy. For this, we will apply a four-step approach to various species in both genera.

Diversity of pollinators

The first step consists in determining the diversity of effective pollinators. As *Ceropegia* and *Aristolochia* both have pitfall trap flowers that retain pollinating flies for one or two days (probably to increase the probability of pollen transfer), we will collect and identify the insects trapped in the flowers to calculate the relative abundance of each species. Only individuals that carry the pollen of the host will be classified as effective pollinators. For each plant species we will thus record data on the diversity of fly species attracted to the flower and the diversity of effective pollinators. Determining pollen origin will require Scanning Electron Microscopy for *Aristolochia*, but can be done under the stereomicroscope for *Ceropegia* because its pollen is clumped into pollinia held in pairs by peculiar structures called the translator arms and the corpusculum to form a pollinarium (Figure 2). Both partners have access to a Scanning Electron Microscope. As many species in the families Chloropidae and Milichiidae in Thailand are not yet described, we will classify individuals into morpho-molecular taxonomic units by combining a morphological investigation with the sequencing of a DNA fragment (the mitochondrial COI marker, considered the best marker for barcoding insects). The French team is experienced in barcoding insects and has access to DNA processing facilities.

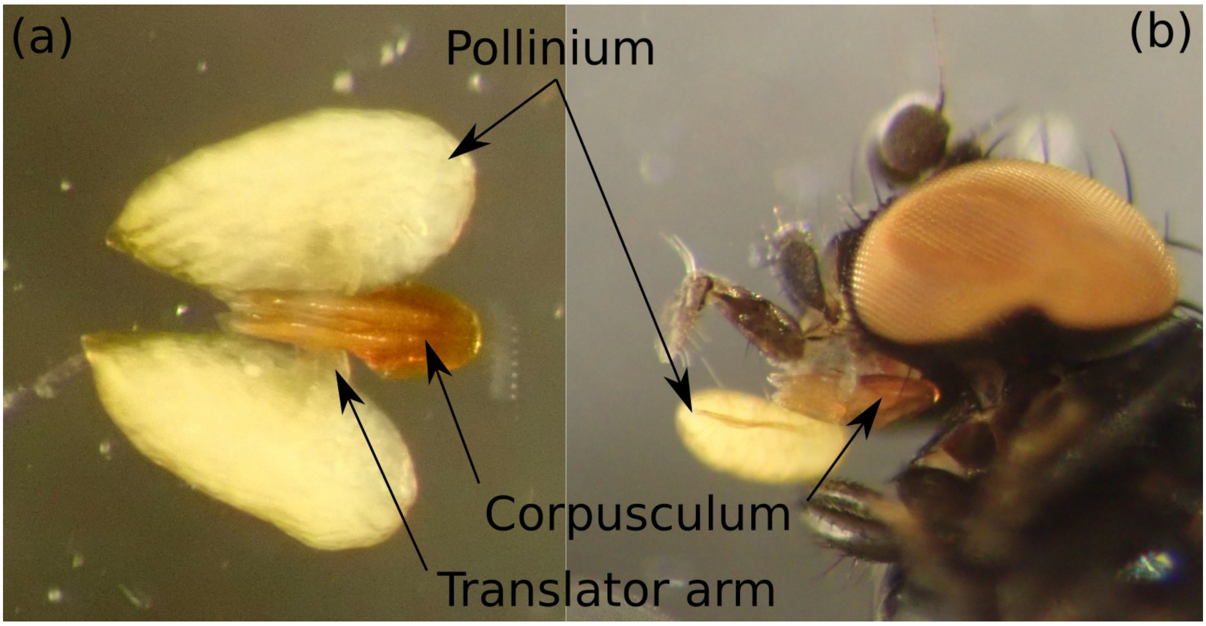

Figure 2: Pollen transfer mechanism in *Ceropegia*. (a). A pair of pollinia attached to the corpusculum, forming the unit of pollen transfer called pollinarium. (b) A pollinarium attached to the mouthparts of a Milichiid fly pollinator.

Floral scents
The second step consists in identifying the chemical composition of floral volatiles. Volatile organic compounds from each focal species will be collected *in situ* using dynamic headspace technique and identified by gas chromatography coupled with mass spectrometry. This technique is routinely used by the French team, and the Thai team is already trained in this technique. The French team has access to a well-equipped platform of chemical analysis.

Models of food source mimicked
The third step consists in identifying which kind of resource the flowers mimic (i.e. the “model”). As pollinator attraction is mediated by floral scents in most *Aristolochia* and *Ceropegia*, the model will be inferred from the nature of the volatile organic compounds emitted. We will search the literature and databases for the identified compounds to list their natural sources.

Pollinator attraction
The fourth step consists in testing the efficiency of the putative model in attracting pollinators of the focal plant species. We will compare the insects attracted to the real model and to the synthetic blend of floral scent, with the known pollinators of the focal species to validate (or not) the inferred model. Both the Thai and French teams have conducted such attraction experiments in the field (Figure 3).

This four-step approach is the best way to characterize the deceptive strategy that evolved in each focal plant species. With this information we will be able to estimate the proportion of species in each genus that rely on kleptomyiophily.

Figure 3: Attraction of the milichiid pollinators of *Ceropegia tenuicaulis* to a synthetic blend of the scent of crushed bug (*Cletus trigonus*) (left), compared to a control without scent (right).

Flower specialization in relation to pollen transfer opportunity

Although both *Ceropegia* and *Aristolochia* show a striking convergence to kleptomyiophily, the two genera strongly differ in how pollen is presented to pollinators. *Aristolochia* has free pollen that is sprinkled on pollinators of suitable size. In contrast, each *Ceropegia* flower has its pollen clumped in the form of only five pollinaria (Figure 4). This means that a single *Ceropegia* flower has only five opportunities to transfer pollen to another flower, whereas an *Aristolochia* flower can distribute its pollen to dozens of pollinator individuals. For both genera, several congeneric species are known to occur in sympatry, and even in syntopy, and could thus potentially share pollinators. Hybridization has rarely been documented in *Ceropegia*. For a *Ceropegia* flower, the cost of a pollinator moving to a flower of a different *Ceropegia* species is thus particularly high, because the pollinia would be fixed on the wrong species and pollen would be lost. This cost is lower for *Aristolochia* because each flower has many more opportunities to distribute pollen and some pollen may remain attached to the fly even after it visits other flowers. We thus expect *Ceropegia* species that occur in sympatry to be under selective pressure to each specialize on a specific set of pollinators. The second goal of this project is to test the hypothesis that sympatric *Ceropegia* species have distinct sets of pollinators, which should not necessarily be the case for sympatric *Aristolochia*.

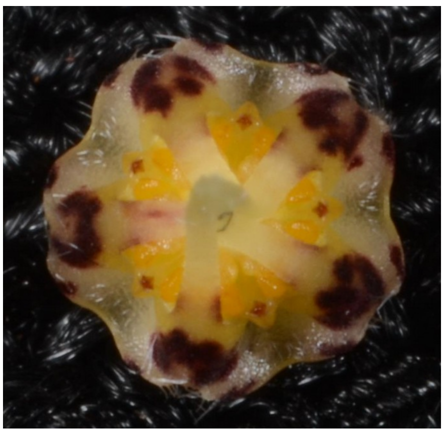

Figure 4: Photo of the inside of the flower of *Ceropegia acicularis* showing the five pollinaria (yellow parts) that constitute the only opportunities for the flower to transfer pollen to pollinators.

To test this hypothesis, we will identify pollinators (see step one in the previous section) in sympatric populations of various species in each genus. The Thai partner has already identified many localities with sympatric species of *Ceropegia*. *Aristolochia clematitis*, *A. rotunda*, *A. pistolochia* and *A. paucinervis* are common in southern France and can easily be found in sympatry. We will focus on these species. Pollinators of three of these species have already been characterized in previous studies in Spain (Berjano et al. 2009), but as there may be geographic variation (in particular according to species co-occurrence), we will identify pollinators for each species in our study sites.

QUALITY AND ORIGINALITY OF THE PROJECT

This project involves teams that are internationally recognized in their respective fields and that have the required theoretical knowledge and technical skills and facilities to address the scientific questions tackled. The project builds upon recent findings, including unpublished findings by the member teams, and addresses a hot topic in ecology: the evolution of pollination syndromes. Thus, we consider this project is of high quality because of the complementarity of the members involved and the topic investigated.

The integrative study of pollination in *Aristolochia* and *Ceropegia* is in its infancy. Most studies have considered floral morphology only, and study of the biology of pollinators has only emerged in the last few years. This project is original because it integrates various methodological approaches (chemical mediation, characterisation of floral morphological traits, classification and behavioural ecology of pollinators) to build a comprehensive understanding of pollination in these two genera and study their peculiar pollination syndromes. In addition, this project proposes to interpret the evolution of these pollination syndromes through a comparative analysis of these two distantly related plant genera. Such an approach has never been undertaken.

EXPECTED RESULTS

Scientific results:
- Identitication of pollinators in several species of *Ceropegia* and *Aristolochia*.
- Characterisation of volatile organic compounds emitted by the flowers.
- Identification of the “model” mimicked by each focal plant species.
-Determination of thelevel of specificity in pollinator attraction according to floral characteristics
- Determination of the extent of kleptomyiophily as a pollination strategy in the two genera *Ceropegia* and *Aristolochia*.
- Insights into the evolution of floral specialization in plant groups showing deceptive pollination syndromes.

Training results:
- Training of at least one French and one Thai Master students in chemical ecology and pollination biology.

Exploitation of results and technological transfer
- Results will be disseminated through scientific publications in international journals (at least two publications expected).
- Technological transfer: study of floral anatomy, classification of flies (including DNA barcoding), identification of volatile organic compounds.

PERSPECTIVES

The comparative analysis of pollination biology in *Artistolochia* and *Ceropegia* could ultimately be interpreted in a phylogenetic context to address the evolutionary trajectories leading to specialized pollination syndromes. From which pollination type(s) did kleptomyiophily evolved? Was the ancestral pollination type always the same? How did floral morphology and chemistry constrain evolutionary trajectories? Answering such questions are keys to understanding the evolution of pollination. This two-year project could serve as a proof of concept to justify and launch a bigger, long-term project.

CONTRIBUTION OF EACH PARTY AND EXPECTED BENEFITS FOR BOTH TEAMS

The study of complex pollination syndromes such as kleptomyiophily requires crossing various disciplines in ecology, in particular botany, entomology, and behavioural and chemical ecology. The French team has competences in entomology and behavioural ecology and has a sound and internationally recognized expertise in chemical ecology, in particular in chemical mediation between plants and pollinators. The Thai team is mostly specialized in botany but has also competences in chemical ecology. Moreover, the Thaï team has a thorough knowledge of the biology of *Ceropegia* (taxonomy, field ecology, etc.). Both teams include experienced field ecologists.

One of the strengths of this project is to apply the same approach to two plant genera that evolved the same pollination syndrome independently. Access to the biological material and the knowledge associated is thus a key to its success. The French and Thai teams will benefit from the access to the biological models, respectively *Ceropegia* in Thailand and *Aristolochia* in France. These two genera can be found in many countries throughout the world but the preliminary collaborative work that the two teams performed during the past two years proved that the collaboration between the two teams was effective, balanced and efficient. This is a prerequisite for such a project to be successful. In addition, the French team will benefit from the knowledge of the Thai team on floral morphology and plant systematics. The Thai team will benefit from the skills and facilities of the French team in chemical ecology and insect systematics. The contribution of each party is thus balanced, with regards to both competences and access to biological material.

This project cannot be conducted successfully at the French national level only because the competence on *Ceropegia* biology is lacking. The Thai team is one of the few in the world to have specialized on these plants and other Apocynaceae.

INVOLVEMENT OF STUDENTS AND/OR YOUNG RESEARCHERS

The French team will propose internship opportunities for Master students. The Thai team already has undergraduate and graduate students involved in the project.

ACTIVITIES AND PLANNING

Project meetings will involve participants from both countries. In addition to project coordination and data interpretation, meetings will be dedicated to establishing a fund-raising strategy in order to secure the continuation of the project and collaboration.

To optimize travelling costs, meetings will also be the opportunity for technological transfer between the two teams on topics such as study of floral anatomy, classification of flies and identification of volatile organic compounds.

The planning of field work is constrained by flowering phenology. Field work in France needs to be conducted in May because it is the peak of flowering of *Aristolochia* in southern France. Field work in Thailand needs to be conducted in July because it is the peak of flowering of *Ceropegia* in Thailand. Project meetings will be scheduled as a follow-up of field sessions in order to allow participants of both countries to contribute to field work.

Field work planned:
- May 2020, France: Collection of floral scents and pollinators of various *Aristolochia* species.
- July 2020, Thailand: Collection of floral scents and pollinators of various *Ceropegia* species.
- May 2021, France: Attraction bioassays of *Aristolochia* pollinators. Collection of *Aristolochia* pollinators in sites containing populations of several *Aristolochia* species.
- July 2021, Thailand: Attraction bioassays of *Ceropegia* pollinators. Collection of *Ceropegia* pollinators in sites containing populations of several *Ceropegia* species.

	2020												2021											
Activity	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
1. Meetings: project coordination, data interpretation, preparation of proposals for continuation of project and collaboration.					■		■										■		■					
2. Collection in the field of pollinators of *Ceropegia* (Thailand) and *Aristolochia* (France).					■		■										■		■					
3. Collection in the field of floral scents of *Ceropegia* (Thailand) and *Aristolochia* (France).					■		■																	
4. Classification of pollinators through morphological investigation (France and Thailand) and DNA sequencing (France).						■	■	■	■	■								■	■	■	■	■		
5. Identification of floral volatile organic compounds through gas chromatography and mass spectrometry (France).						■	■	■	■	■	■	■												
6. Inference on the models mimicked by flowers based on floral compounds.										■	■	■	■	■	■									
7. Pollinator attraction bioassays in the field with putative models and synthetic scents of models and flowers (Thailand for *Ceropegia*, France for *Aristolochia*).																	■		■					
8. Data analysis.									■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
9. Writing publications.																					■	■	■	■

ETHICS AND COMPLIANCE TO REGULATIONS

The biological material targeted in this project (plants and insects) is not subject to animal welfare regulations. For field work in Thailand, we will apply for research and collection permits according to Thai regulations (note that Thailand is not party to the Nagoya protocol). *Aristolochia* species targeted for field work in southern France are not subject to any protection status. To comply with the application of the Nagoya protocol in France, a declaration of access to genetic resources will be sent to the French *Ministère de la transition écologique et solidaire*. Exchange of biological material between the two countries will be subject to the signature of a Material Transfer Agreement between Chulalongkorn University and the CNRS.

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