Insect pollination in chestnut: an organized mess?

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Abstract

How are chestnuts pollinated? By wind, insects, or both? For almost 150 years, this question has been in the air. The huge production of tiny pollen grains transported by wind over long distances seems to point towards wind pollination. However, the brightly coloured flowers, the strong spermatic odour emitted by flowering trees, and the sticky pollen grains of chestnuts are characteristics of insect pollination. We present here the results of five years of research on chestnut pollination aiming at answering this question. We performed several insect exclusion experiments using insect-proof nets and two large-scale insect monitoring experiments. In netting experiments, in which access of insects to female flowers is limited, fruit set collapses. Direct observations indicate that only beetles and calyptrate flies regularly visit both male and female flowers. Wild bees, honeybees and hoverflies visit male flowers but fail to visit rewardless female flowers. The male parts of bisexual catkins play a key role in pollination: they attract walking insects on erect female flowers, increasing the probability of a contact with the tiny stigmas. Installation of beehives will not improve pollination service in chestnut orchards. Instead, the preservation of non-bee pollinators, especially calyptrate flies, is critical to the sustainable management of chestnut orchards.

Keywords: insect-pollination, wild pollinators, insect-exclusion experiments, insect monitoring, beetles, calyptrate flies, rewardless female flowers, mating facilitation

INTRODUCTION

Chestnuts are ecologically important tree species growing in temperate, Mediterranean, and subtropical regions. Some chestnut species are also economically important as they are widely cultivated for fruit production, representing several million tons of harvested fruits. The reproductive biology of this tree is intensively studied but its pollination mode remains unclear. Chestnut was initially considered wind-pollinated (Sprengel, 1811; Delpino, 1868), but since the end of the 19th century, this has been called into question. For instance, for Meehan (1879): “The male flowers of the sweet chestnut were remarkably odoriferous. A fair sized bunch in a room would give fragrance to a whole house. Where would be the use of adding this powerful odour to flowers in mere arrangements for cross-fertilization by the aid of winds?” Distinguishing between wind-pollinated and insect-pollinated plants, although often considered straightforward, can be tricky, requiring careful studies and evaluation of the evidence (Larue et al., 2021a).

In the mid-20th century, Porsch (1950) drew up a list of chestnut characteristics favouring either insect or wind pollination. He concluded that chestnuts are pollinated by both, i.e., that they are ambophilous. In several comparative studies (Deguines et al., 2014; Bastl et al., 2020; Garcia et al., 2021), chestnut has been classified as either entirely or mainly anemophilous. However, in these comparative studies, chestnut often stands out as an outlier among anemophilous species (discussed in Petit and Larue, 2022). Moreover, ambophily is theoretically very rare, because according to Stebbins (1970) plants should evolve to increase the efficiency of their main pollinating agent, and wind and insect pollination should typically select for very divergent traits. To move beyond these first observational studies and establish chestnut pollination mode on firmer grounds, it is necessary to perform insect-exclusion

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experiments. As pointed out early on by Darwin (1876), very careful experimental set ups are needed, especially if the goal is to distinguish between strict entomophily and ambophily. While establishing entomophily on such experimental grounds is necessary, it should be combined with observations of pollinating insects. The insect species involved need to be identified and evidence that they do visit both male and female flowers should be provided. Finally, a complete understanding of pollination is only achieved if the adaptive advantages of flower structure and flowering phenology for insect pollination are understood.

Before we initiated our research program on chestnut pollination, little was known on the functional aspects of chestnut pollination and on the adaptive role of its complicated inflorescences. Chestnut is a strict monoecious species, i.e., trees bear separate male and female flowers, grouped into inflorescences. Each tree has two types of inflorescence: unisexual male catkins and bisexual catkins. The numerous unisexual male catkins have only male flowers. In contrast, the less numerous bisexual catkins have a male catkin associated with one or two female inflorescences at the base. The male flowers of unisexual and bisexual catkins are similar and produce pollen of equivalent fertility (Silva et al., 2020), raising the question of their respective roles for pollination. Each female flower has six to eight styles, with a very small, crater-shaped stigma at their tip, making them unsuitable for capturing pollen highly diluted in the air. How and why do insects end up contacting the stigmas of these rewardless female flowers? So far, little information is available on the adaptive function of these complex structures for insect pollination.

Chestnut flowering phenology is also very complex: chestnut trees are duodichogamous, i.e., they have two distinct pollen emission phases during each flowering episode (Stout, 1928). The male flowers of unisexual catkins bloom first, at the end of May or early June. The tiny pollen grains are emitted in huge amounts at this time. Around 15 days later, the male part of bisexual catkins flowers in turn, resulting in a second small pollen emission phase, representing only around 3% of the total amount of pollen emitted by the tree (Larue et al., 2021a). The two pollen phases cover the long female receptivity period: this period lasts on average almost three weeks but reaches its maximum 10 to 15 days after the start of flowering (Shimura et al., 1971; Shi and Stösser, 2005). The evolutionary advantage of this unusual flowering dynamics was unclear before we started our research program.

Here we successively present a summary of the main results obtained during these five years of research on chestnut pollination. We address four successive research questions. Do insects play a role in chestnut pollination? Is chestnut purely insect-pollinated or is it partly wind-pollinated? Which insects are involved in pollination? Do the complex inflorescence structure and flowering phenology of chestnut can be interpreted as adaptations facilitating insect pollination? For the first time, we offer a complete overview of chestnut pollination mechanism, demonstrating without ambiguity the importance of non-bee pollinators for chestnut production and raising questions on the sustainable management of chestnut orchards.

CHESTNUT IS INSECT-POLLINATED

At first sight, it is difficult to identify the vector of chestnut pollen from the characteristics of the flowers. The erect catkins of chestnut inflorescences, the strong odour and the abundant nectar secretion of their highly visible flowers are characteristics of insect-pollinated plants. However, chestnut pollen can be carried by wind over kilometres (López-Orozco et al., 2020) and insect visits to unattractive female flowers seemed at first sight extremely rare. For instance, for Johnson (1988), who studied the pollination of chinkapins (*C. pumila*), "diurnal and nocturnal observations failed to detect [insects] on pistillate flowers". Giovanetti and Aronne (2011) tracked 23 honeybees visiting chestnut trees and recorded zero visit to female flowers. Zirkle (2017) studied Ozark chinkapin (*C. ozarkensis*), surveying both diurnal and nocturnal insects. He observed only a single honeybee moving from a male catkin to a female flower of the same tree. In Japan, Hasegawa et al. (2015) also noticed that in contrast to male flowers, female flowers are rarely visited by insects. Because of this apparent scarcity of pollinators, chestnut has long been considered to be mainly wind-pollinated, with insects playing a modest role at best (Klein et al., 2007).

To quantify precisely the role of insects in chestnut pollination, fruit set (i.e., the percentage of female flowers bearing a filled fruit) of control branches can be compared with that of branches covered by insect-proof nets. Open-pollinated female flowers are freely accessible to pollen transported by wind and insects, while female flowers covered with insect-proof nets are no longer accessible to insects but are still accessible to pollen transported in the air. In chestnut, fruit set is a direct indicator of pollination service quality. The female flowers are usually grouped by three in a small female inflorescence that develops into a spiny burr. If pollinated, each female flower gives a fruit with typically a single embryo (Furones-Pérez and Fernández-López, 2009; Tuğ et al., 2020), otherwise, the fruit formed is empty. Burrs have therefore 0, 1, 2 or 3 developed fruits out of three possible ones, depending on the fate of each female flower. Measuring fruit set thus provides a direct estimate of pollination success (Larue et al., 2023).

We found mentions of five prior pollinator exclusion experiments conducted on chestnuts (Clapper, 1954; Johnson, 1988; Manino et al., 1991; de Oliveira et al., 2001; Zirkle, 2017). In all these studies, control female flowers had a higher fruit set than female flowers enclosed in nets (reviewed in Larue et al., 2021a, b). We carried out a new insect exclusion experiment in 2019 on two chestnut cultivars: 'Marigoule' and 'Bouche de Bétizac'. When the insects no longer have access to female flowers, fruit set of 'Marigoule' drops by 62%, while that of 'Bouche de Bétizac' drops by 85% (Figure 1; Larue et al., 2021a), i.e., values close to those of previous experiments (for instance, average fruit set was reduced by 80% in Olivera's experiment). These studies therefore establish the preponderant role of insects in chestnut pollination.

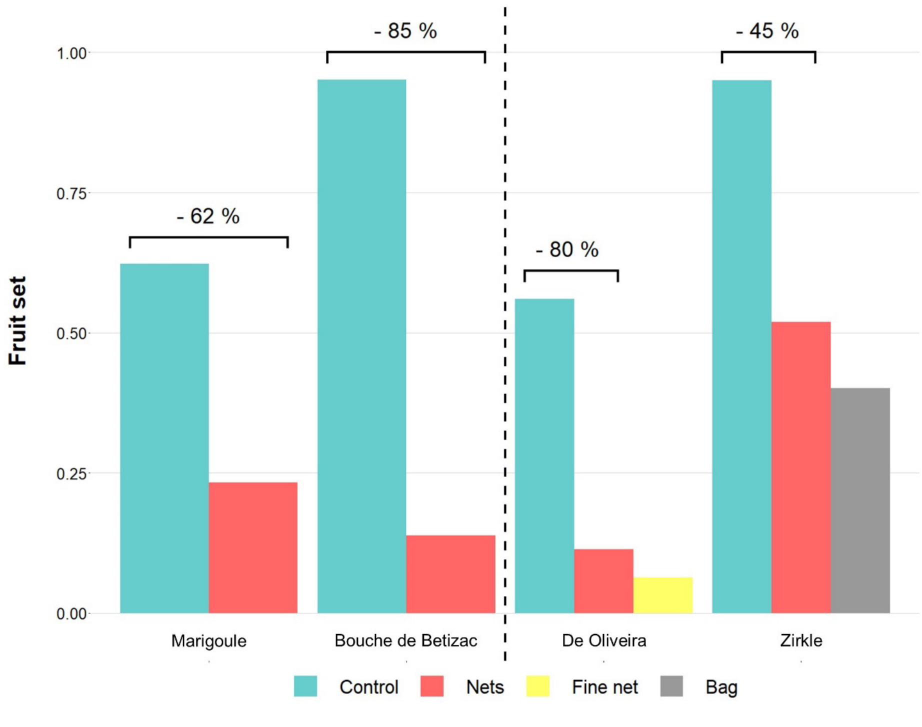

Figure 1. Comparison of pollination success in different insect exclusion experiments. reviewed in Larue et al. (2021a). On the left, results from our study on two chestnut cultivars. On the right, experiments by de Oliveira et al. (2001) and Zirkle (2017).

POLLINATION BY INSECTS ONLY

Our reassessment of chestnut pollination (Larue et al., 2021a) has challenged the long-held belief in the botanical and horticultural communities that chestnut trees are mainly wind-pollinated (Clapper, 1954; Oh and Manos, 2008; Hasegawa et al., 2015). Darwin (1876) had already performed insect exclusion experiments on various plant species using nets, stressing the need for careful experimental set ups: "if the flowers touch the net they may be cross-fertilised by bees". Indeed, we encountered this problem during our first insect-exclusion experiment: if the mesh touches the female flowers, the erect stigmas protrude from the net and insects can still pollinate them occasionally.

To clarify if chestnut is entomophilous or ambophilous, we repeated the insect exclusion experiments on several cultivars during several years, optimizing the procedure until we had a reliable protocol. We tested several mesh sizes, including special pollination nets whose air permeability had been measured by the manufacturer. Finally, we decided to combine a fine-mesh insect-proof net with a wide-mesh net to avoid any accidental contact of insects walking on the external surface of the nets with the stigmas of the female flowers. With this double net, the effect of insect exclusion could be reassessed. Our results show that, on branches covered with nets, fruit set collapses compared with that measured on controls: it is reduced on average by 94% (Figure 2; Petit and Larue, 2022). Chestnuts can therefore be formally classified as entomophilous (Rodger et al., 2021), not ambophilous. New comparative studies are now starting to consider this classification in their analyses (Qiu et al., 2023)

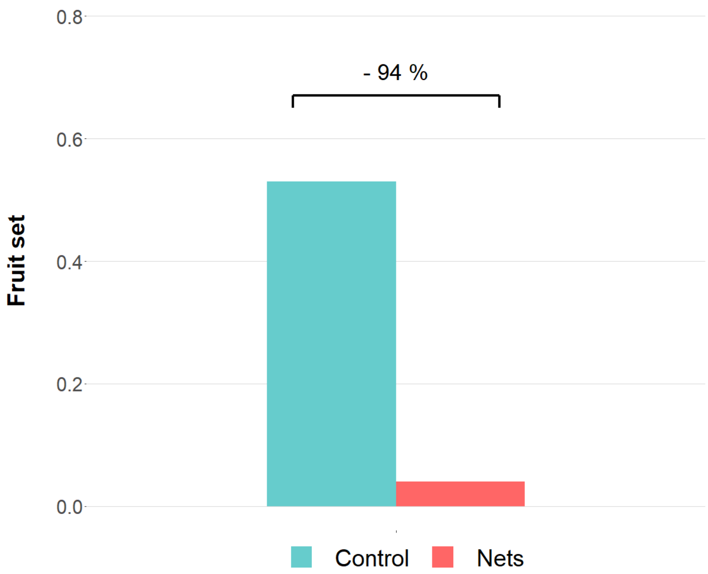

Figure 2. Fruit set difference between control branches and branches covered with double nets.

WHICH INSECTS ARE INVOLVED IN CHESTNUT POLLINATION?

The apparently low frequency of insect visits to female flowers mentioned in the introduction has probably discouraged researchers to carry out insect pollinators monitoring: we could find no formal studies of insect pollination on female chestnut flowers in the literature. To identify the pollinators involved, we decided to carry out a large-scale insect monitoring in the INRAE chestnut germplasm collection in Villenave d'Ornon during two years in 2019 and 2021 (Larue et al., 2021b). We relied on photographic monitoring: during each survey, we photographed all insects visiting each studied tree. From these photographs, we identified insects using identification keys. We classified as pollinators only those insect species regularly visiting both male and female chestnut flowers.

In 2019, we inventoried 4203 arthropods on 16 chestnut trees and noticed 66 interactions with female flowers in nearly 67 h of observations (Figure 3; Larue et al., 2021a). Beetles, mainly red soldier beetles, and Diptera, mainly calyptrate flies, visit male and female flowers and can therefore be considered as chestnut pollinators. Bees, on the other hand, visit male but not female chestnut flowers, so they are visitors, not pollinators. In this first experiment, we noticed that interactions with female flowers take place preferentially during the second pollen emission phase, when the unisexual male catkins have faded and the bisexual catkins are in full bloom.

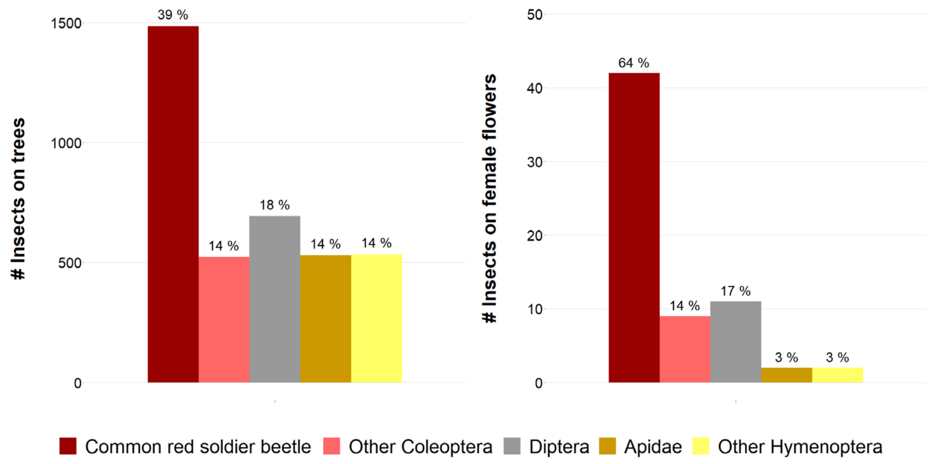

Figure 3. Abundance of insects on chestnut trees versus on female flowers measured during our first insect monitoring study in 2019 (Larue et al., 2021a).

To confirm these results, we performed another insect monitoring study in 2021, accurately recording the phenology of unisexual and bisexual catkins for each insect visit. This made it possible to test whether interactions with female flowers occur during the first or second phase of pollen emission. This time, we identified 4051 arthropods, 239 of which interacted with female flowers in around 35 h of monitoring (Figure 4; Pauly et al., 2023). This study confirmed our earlier results. Beetles (especially red soldier beetles) and calyptrate flies are the most abundant insects on chestnut trees, the most frequent visitors of female flowers and therefore the most important chestnut pollinators candidates. In contrast, hoverflies and bees – both honeybees and wild bees – only rarely visit chestnut female flowers and hence do not contribute to its pollination.

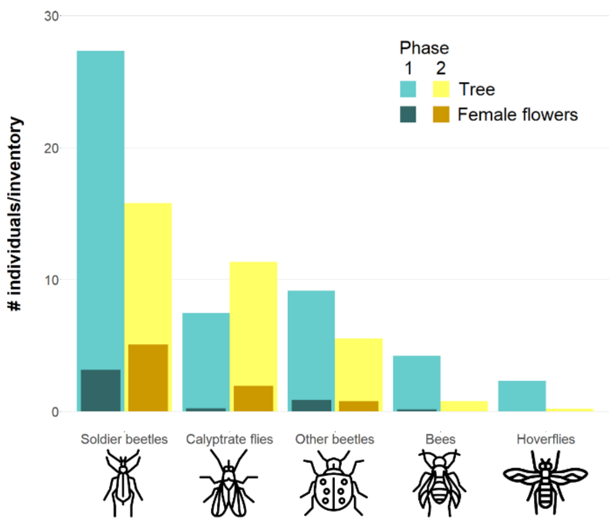

Figure 4. Number of insects per inventory observed on chestnut trees and twice the number of insects per inventory observed on female flowers during the first and second staminate phases in the study of 2021.

Most interactions with female flowers took place during the second phase of pollen emission. Even if visitation rates of red soldier beetles and calyptrate flies are low, our ongoing modelling analyses indicate that nearly all female flowers of the studied trees are visited at least once by these insects and typically several times, thanks to the long receptivity period of female flowers. Importantly, visits to female flowers by calyptrate flies alone are sufficient to ensure pollination of almost all flowers of the studied trees.

CHESTNUT POLLINATION, AN ORGANIZED MESS?

The adaptive functions of chestnut flowering phenology and inflorescence architecture seem at first sight obscure. In particular, duodichogamy is very rare in plants and its adaptive function was until recently unclear. In chestnut, we have shown that the male part of bisexual catkins actually plays a key role in insect pollination: it attracts insects close to female flowers, increasing their chances of being visited and fertilized (Pauly et al., 2023). The insects involved – beetles and calyptrate flies – frequently walk on twigs and climb on female inflorescences. The numerous and tiny pollen grains present on their legs or bodies is deposited on the tiny stigmas at the tips of female flower styles. This pollination mode is sometimes called "mess and soil" due to the assumed erratic behaviour of the insects and their lack of precision in pollination (Faegri and Van Der Pijl, 1979). However, in the case of chestnut, the orientation of female inflorescences plays a role in pollination: our preliminary results suggest that those female inflorescence facing upwards are visited more frequently by beetles and flies than those facing downwards.

Unisexual male catkins are much more numerous than bisexual catkins (Larue et al., 2021a). Only male flowers produce rewards for insects: nectar, and in the case of male-fertile trees, pollen. Unlike female flowers, the base of male flowers, which secretes nectar, fluoresces in response to UV radiation, making it easily perceptible by insects (Figure 5). The hypothesis of intersexual Bakerian mimicry we proposed in Larue et al. (2021a) was based on an apparent similarity between male catkins, covered by long stamens, and female inflorescences, covered by long styles . Even if male and female inflorescences look similar to us, they look different to insects due to differences in UV reflection, so the hypothesis of auto-mimicry is not upheld. Moreover, female flowers are physically closer to male catkins from bisexual inflorescences than to male catkins from unisexual inflorescences, which should limit self-pollination due to the delayed release and reduced amount of pollen coming from bisexual catkins. This is important because self-pollination can have strong negative effects on fruit production (Larue et al., 2023). Self-fertilized ovules abort due to late-acting self-incompatibility mechanisms, resulting in ovule usurpation, i.e., loss of cross-fertilization opportunities when ovules are disabled by self-pollination (Xiong et al., 2019; Larue et al., 2023).

The spermatic odour of chestnut flowers is due to nitrogen compounds, in particular 1-pyrroline, known to attract flies (Zhang et al., 2019). Similarly, chestnut nectar, produced on the disc at the base of male flowers, contains a low proportion of sucrose (less than 15%; Kim et al., 2014), a value characteristic of fly-pollinated plants (Abrahamczyk et al., 2017). Along with our finding that inflorescences are adapted to pollination by walking insects, this fits well with the hypothesis that chestnuts are adapted to fly pollination, especially calyptrate flies. The hypothesis of fly pollination is further supported by the abundance of flies and the scarcity of beetles on flowering chestnut trees in forests (Larue et al., 2021a). Calyptrate flies appear to be particularly efficient pollinators as they take-off more frequently than beetles, which tend to stay a very long time on the same tree. By potentially visiting a greater number of trees, calyptrate flies should therefore carry a more diversified pollen mix and better ensure cross-fertilization (Larue et al., 2021a). Chestnut inflorescences are therefore characterized by a suite of adaptations to pollination by (calyptrate) flies, which is likely more reliable and effective than pollination by beetles.

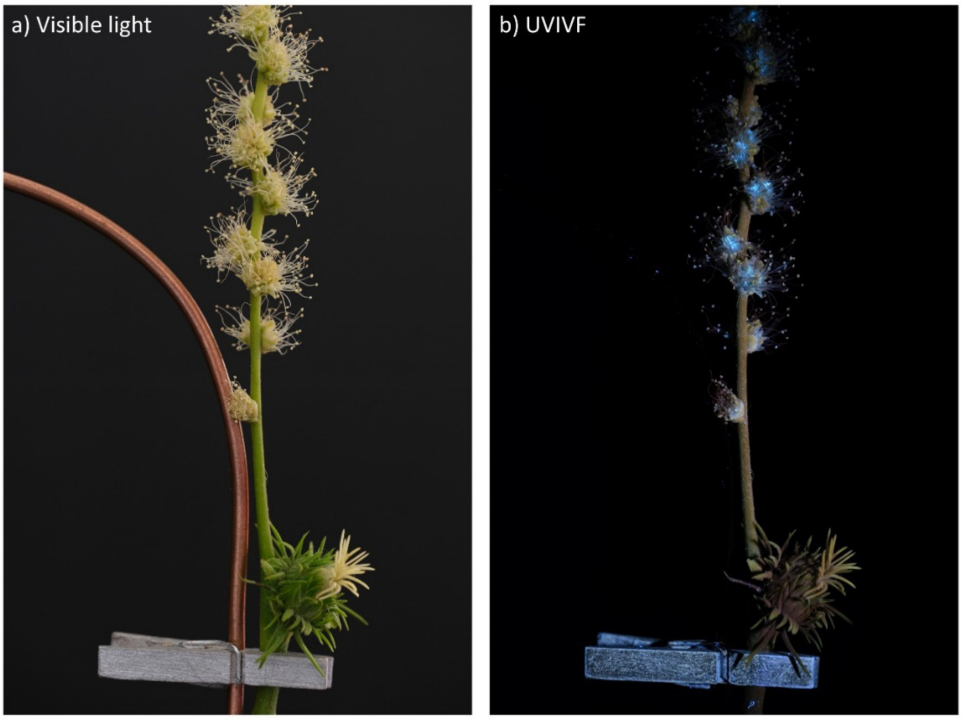

Figure 5. Photographs of a bisexual catkin. a) In visible light, male and female chestnut flowers appear yellow; b) Under ultraviolet light, a wavelength which is perceived by insects, the male flowers and in particular their nectaries at the base of flowers fluoresce and appear brightly coloured. They are thus very visible to insects. Instead, female flowers do not fluoresce.

CONCLUSIONS

We believe that our study puts an end to a debate on chestnut pollination mode that had lasted for almost 200 years. We have shown that chestnut is neither wind-pollinated nor ambophilous. Instead, it is entirely insect-pollinated. The insects involved are beetles and especially calyptrate flies, not bees. Hence, planting pollen donor trees upwind or using honeybee hives in chestnut orchards will not improve pollination and fruit production. At first glance, chestnut pollination syndrome seemed obscure but we are starting to clarify the main mechanisms. Chestnut pollinators collect pollen by foraging on male catkins. They subsequently deposit this pollen while climbing on female flowers, matching with the description for "mess and soil" pollination (Faegri and Van Der Pijl, 1979). Although female flowers are rewardless, pollinators are attracted to them by nearby nectar-producing male catkins (Pauly et al., 2023). The apparent rarity of insect interactions with female flowers reported in the literature is largely due to observers checking flowers at a too early phase. Moreover, the long receptivity period of female flowers compensates for the low frequency of insect visits. Eventually, pollination rate is high enough to ensure that all female flowers are visited several times by pollinators.

The idea that chestnut pollination is still under evolution has been repeatedly stressed. For instance, Porsch (1950) argues that chestnut is an ancient beetle-pollinated tree shifting towards wind pollination. However, recent phylogenomic research in *Fagaceae* do not support this view. Instead, chestnut and other clades of the castaneoid lineage are all insect-pollinated. Insect pollination corresponds to the ancestral character, whereas wind pollination in the oaks, chestnuts' close cousins, evolved from an insect-pollinated ancestor around 56 million years ago (Zhou et al., 2022).

Chestnut appears to be well adapted to pollination by calyptrate flies. Unfortunately, the needs of these pollinators are still poorly understood. This is a serious problem when trying to optimise pollination in chestnut orchards. What is clear, however, is that agro-environmental measures adapted to bees (whose habitats and regimes are much better known than those of flies) will not suffice. The needs of larvae and adults are different in diptera and may even vary between sexes at the adult stage (Davis et al., 2023). In addition to floral management schemes, pollinator conservation strategies should consider habitats beneficial to larvae, such as wet organic materials and dung.

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