

First report on the pollination of *Neotinea maculata* (Orchidaceae) by minor worker ants of the *Temnothorax exilis* group (Hymenoptera: Formicidae)

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Abstract. Ants commonly visit flowers seeking for food resources such as nectar. However, only eight species of orchids are known for having an effective pollinaria removal by ants. In this study, the visit of the orchid *Neotinea maculata* (Desf.) Stearn by ants is described in one of the three known populations from the province of Valladolid (Spain). Through direct observation, six minor worker ants of *Temnothorax exilis* (Emery, 1869) group (Hymenoptera: Formicidae) were found visiting flowers of six plants of *N. maculata*, and four of them were carrying pollinaria on their heads. Other insects were not observed with pollinaria or visiting the flowers. Nevertheless, despite ants observed in the field only promoted cross-pollination between flowers of the same individual (geitonogamy), there is a possibility that the ants would visit another plant's inflorescence. Thus, ants could be considered not only as visitors but also as true pollinators of *N. maculata*.

Keywords. Pollinaria removal; Ant-pollination; Formicidae; *Neotinea maculata*; Orchidaceae; Pollination; *Temnothorax exilis*.

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Introduction

Along their evolutionary history, orchids have developed many pollination mechanisms, such as attracting pollinators offering nectar as reward or deceiving pollinators through food and sexual deceptions (Delforge, 2006; Gaskett, 2011; Johnson *et al.*, 2013). However, despite the wide range of pollinators on orchid species, ants have been traditionally considered inefficient pollinators (Faegri & van der Pijl, 1979; Peakall & Beattie, 1989, 1991; de Vega & Herrera, 2012; de Vega *et al.*, 2014). Worldwide, only eight orchid species (*Chamorchis alpina* (L.) Rich., *Chenorchis singchii* Z.J.Liu, K.W.Liu & L.J.Chen, *Dactylorhiza viridis* (L.) R.M. Bateman, Pridgeon & M.W. Chase, *Epipactis palustris* (L.) Crantz, *E. thunbergii* A. Gray, *Leporella fimbriata* (Lindl.) A.S. George, *Microtis parviflora* R. Br. and *Neottia listeroides* Lindl.) are pollinated by them (Jones, 1975; Nilsson, 1978; Brantjes, 1981; Peakall *et al.*, 1987; Peakall, 1989; Peakall & Beattie, 1989, 1991; Sugiura *et al.*, 2006; Wang, 2008; Zhongjian *et al.*, 2008; Schiestl & Glaser, 2012; Claessens & Seifert, 2018).

The European orchid *Neotinea maculata* (Desf.) Stearn is a widely distributed species that has small flowers with short spurs (0.8–2 mm) and narrow labella.

The flowers are compactly placed facing the same side on a narrow spike, varying their colour from pink to white. In the Mediterranean region, the flowering period for this species is typically from March to June (López, 2005). Although it is known that *N. maculata* is incidentally pollinated by tiny beetles from the genus *Spermophagus* (Berger, 2003; Delforge, 2006; Wilcox, 2014; Claessens & Kleynen, 2016, 2018), this plant species is mainly self-pollinated by autogamy, sometimes even cleistogamy (Delforge, 2006; Duffy *et al.*, 2009). Pollination by Lepidoptera, Diptera and Hymenoptera (the main insect orders that include effective pollinators) has not been previously reported for *N. maculata* (Claessens & Kleynen, 2018; Schatz *et al.*, 2020). Furthermore, autogamy produces low levels of heterozygosity within populations of *N. maculata* along its distribution range (Duffy *et al.*, 2009) and therefore, it is of a great importance to study if a real diversity of pollinators exists. This will help to understand the patterns and mechanisms that may favor cross-pollination in this orchid.

In this work, we report for the first time the occasional visits of *Neotinea maculata* by minor worker ants of the *Temnothorax exilis* (Emery, 1869) group (Hymenoptera: Formicidae) with an effective pollinaria removal. The behavioral pattern of these ants during their visit to the flowers of *N. maculata* is described.

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Materials and methods

The study was carried out during the spring 2018 in Laguna de Duero (latitude 41°34' N, longitude 4°44' W), one of the three known localities with this orchid in the province of Valladolid in Spain (Figure

1A) (García-Gila, 2019; García-Gila & Fernández-Alonso, 2019). This orchid population grows under a *Pinus pinea* L. woodland mixed with *Quercus ilex* subsp. *ballota* (Desf.) Samp., and a scrubland composed of *Halimium umbellatum* subsp. *viscosum* (Willk.) O. Bolòs & Vigo.

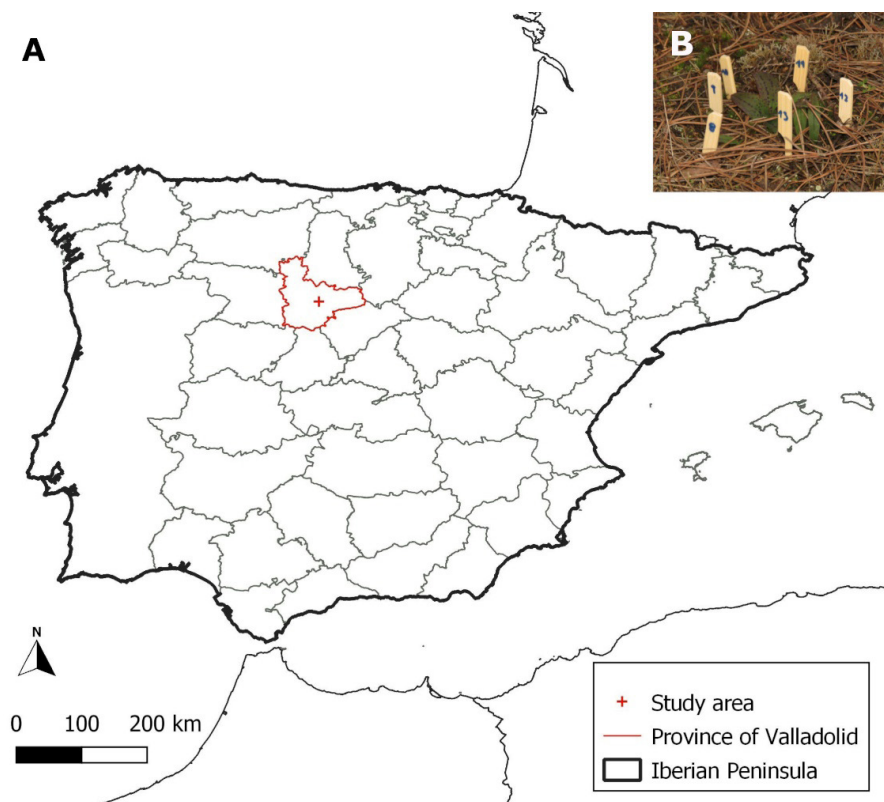


Figure 1. A, Study area; B, Individuals of *Neotinea maculata* marked with wooden tags to follow their flowering over time.

We tagged every individual (Figure 1B) to be able to follow their entire flowering period and complete fruit development. On March 20th, the individuals present in the population were counted for the first time, and in mid-June, each dehiscent individual was counted for the last time. Moreover, the number of flowers, the number of developed and dehiscent fruits per inflorescence were counted during their life cycle.

We spent a total of 44 hours of sampling through 11 days, from April to May (4 hours per day). The number of flowers (Figure 2A) per plant was counted, and the number of pollinaria removed was recorded flower by flower throughout the entire flowering period. Flowers without pollinaria (Figure 2B) were considered as visited. On April 22nd, when most of the flowers were open, the behaviour and foraging patterns of the ants on the inflorescences and flowers of *Neotinea maculata* were observed on the field between 10:30 and 14:30 hrs. This period is generally used to sample Mediterranean insects for covering their hours of maximum activity, especially in Hymenoptera (Willmer, 1983; Cerdá & Retana, 1989; Fellers, 1989; Cerdá *et al.*, 1991; Redolfi *et al.*, 2002–2003). The time spent by every ant on its visit to inflorescences and flowers was also recorded. Five ant individuals were collected for later identification with a binocular microscope, using an identification key

of Iberian ants Collingwood (1976) and photographs available on AntWeb.

Map of the study area was made using QGIS software (QGIS Development Team, 2019).

Results

At the end of March, the population of *Neotinea maculata* was composed by 53 individuals in vegetative stage. During April, we reported that only 25 individuals (47.1%) developed inflorescences with between 13 to 17 flowers per inflorescence (383 flowers in total). The number of individuals decreased due to herbivory by hares (*Lepus granatensis*) and roe deers (*Capreolus capreolus*). At the end of May, when the flowering period finished, 18 individuals with 288 flowers were counted. We observed that 96 flowers (33.3%) did not have pollinaria (Figure 2B). It corresponds to 27.5%–36.3% flowers per inflorescence without pollinaria. We found that 92.7 % of all the flowers developed fruits, with 153 fruits in total (165 flowers in 10 inflorescences). At the end of the fructification period (mid-June), there were only 7 individuals found and 91.9 % of all the flowers per inflorescence set dehiscent fruits, with a total of 125 dehiscent fruits (136 flowers in 7 inflorescences).

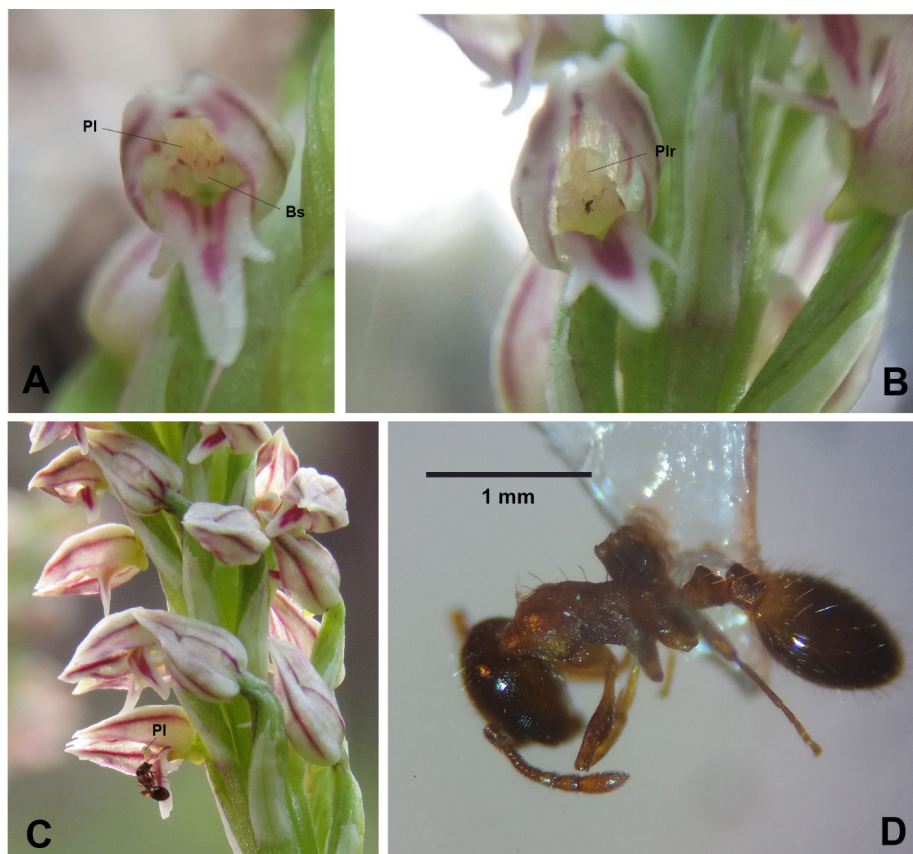


Figure 2. Flowers of *Neotinea maculata* and minor worker ant of *Temnothorax exilis* group. A, Front view of a flower with pollinaria; B, Front view of a flower with pollinaria removed; C, An ant carrying pollinaria of *N. maculata* on its head; D, Detail of the ant collected in the field. Abbreviations: PI, Pollinaria; Plr, Pollinaria removed; Bs, Bursicle.

Ants that were observed as visitors of *Neotinea maculata* (Figure 2C) were collected in the field and they were identified as minor workers of the *Temnothorax exilis* group (Figure 2D). A total of six worker ants visited 19 flowers and four of them were carrying one pollinaria on their heads (Figure 2C, Table 1). During our observation period, each ant only visited one inflorescence and

between 1 to 6 flowers per inflorescence (Table 1). They altogether made a total of 24 flower entries, in average 4 entries per ant. The ants spent between 12.9 and 30.7 seconds working per flower (19 flowers in total) and between 139.5 and 209.1 seconds per inflorescence (6 inflorescences in total). Other insects were not observed with pollinaria or visiting the flowers.

Table 1. Pollinaria carried over, entries to flowers and time spent per flower and per inflorescence by *Temnothorax exilis* specimens during their visits to *Neotinea maculata*.

Ant	Pollinaria carryover	Entries	Visited flowers	Mean time (s) inside flowers (mean \pm sd)	Total time (s) per inflorescence
1	Yes	3	2	28.5 \pm 8.50	122
2	No	7	3	12 \pm 1.15	193
3	Yes	5	3	31 \pm 0.57	332
4	Yes	2	1	24 \pm 0.0	87
5	Yes	5	4	29 \pm 8.21	150
6	No	2	6	14.67 \pm 1.31	162
Mean per ant		4 \pm 0.81	3.2 \pm 0.7	21.8 \pm 8.9	174.3 \pm 34.8

Discussion

During our sampling effort, the population of *Neotinea maculata* in Laguna de Duero was only visited by minor worker ants of the *Temnothorax exilis* group. We

observed four ants with an effective pollinaria removal. However, whereas the pollinaria were removed in 33.3% of all flowers, each ant was observed to remove only four pollinaria. These observations could indicate two possible hypotheses. First, that the total number

of visits by ants was higher than the number of visits observed on the field, with these visits by ants occurring in hours outside of the sampling schedule (de Vega *et al.*, 2009). And second, that other pollinators such as the previously reported *Spermophagus* species or moths could have visited flowers of *N. maculata* along the afternoon or during the night, respectively. Overall, ants were considered as true pollinators of this plant species. Despite they were not observed carrying out the cross-pollination between different individuals, they were still promoting cross-pollination between flowers of the same plant. This type of self-pollination is known as geitonogamy (de Jong *et al.*, 1992) and it has been reported in another European orchid species, *Neottia nidus-avis* (L.) Rich. (Van der Cingel, 2001).

Although Duffy *et al.* (2009) showed the presence of sugars in the labellum, it is still not clear whether *Neotinea maculata* has “true” nectariferous spurs (Claessens & Kleynen, 2018). We have observed that ants spent on average more than 20 seconds visiting each flower. This may indicate that they have a foraging pattern inside the flowers and therefore, it is probable that: 1) *N. maculata* offers an inconspicuous quantity of nectar never measured before; or 2) the sugars discovered by Duffy *et al.* (2009) are responsible for this attraction in the ants. On the other hand, Schiestl & Glaser (2012) reported that some ant species (e.g. *Formica exsecta* Nylander, 1846 or *Myrmica lonae* Finzi, 1923) can also be attracted by floral scents in *Chamorchis alpina* (L.) Rich). Thus, the vanilla scent emitted by *N. maculata* (Claessens & Kleynen, 2018) might be also playing an important role attracting the ants. Notwithstanding, more studies are needed to know whether this plant really has “true” nectariferous spurs and to elucidate what are the real factors conditioning the behaviour of *Temnothorax exilis* ants.

The percentages of both developed (92.7%) and dehiscent (91.9%) fruits are higher than the actual percentage of flowers without pollinaria (33.3%). Considering insect-pollination as an infrequent reproductive strategy of this orchid (Berger, 2003; Delforge, 2006; Wilcox, 2014; Claessens & Kleynen, 2016, 2018), the high reproductive success of this population might have been facilitated by the autogamy. However, our data are insufficient to support this statement since we did not investigate the percentage of autogamous fruits. In the field experiment carried out on this orchid species, Duffy *et al.* (2009) reported that there were no differences in fruit and seed production between self and outcrossed plants. In addition, they suggested that homozygosity had no negative effects on its reproduction. Consequently, we believe that *Neotinea maculata* may have had a low insect-pollination specialization along its life history that could have favoured autogamy as the main reproductive strategy.

Nevertheless, further studies are needed to know whether the interaction observed during our study is actually frequent and if it occurs in other populations of *Neotinea maculata*. Besides, the mechanisms through which this orchid is generating an attraction in the ants are still waiting to be uncovered and more

studies focused on measuring the levels of nectar and floral fragrances would help to untangle this question. Finally, the reproductive biology in populations of this species should be studied in long-term, especially from a viewpoint of the life cycle stages and the herbivory occurring within this orchid.

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