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Citation

Claessens, J., Achterberg, C. van, Haas, E. de, Claessens-Jansen, M., & Gravendeel, B. (2025). Identification of pollinators of lesser twayblade *neottia cordata* with DNA barcoding reveals strong links with pine forest-related fauna. *Nordic Journal Of Botany*, 2025(3). doi:10.1111/njb.04396

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Note: To cite this publication please use the final published version (if applicable).

NORDIC JOURNAL OF BOTANY

Research article

Identification of pollinators of lesser twayblade *Neottia cordata* with DNA barcoding reveals strong links with pine forest-related fauna

Jean Claessens¹✉, Cees van Achterberg¹, Emma de Haas¹, Marijke Claessens-Janssen² and Barbara Gravendeel¹

¹Naturalis Biodiversity Center-Evolutionary Ecology, Leiden, the Netherlands

²Geulle, the Netherlands

Correspondence: Jean Claessens (jean.claessens@naturalis.nl)

Nordic Journal of Botany

2025: e04396

doi: 10.1111/njb.04396

Subject Editor: Magne Friberg

Editor-in-Chief: Sara Cousins

Accepted 14 October 2024

Published 3 December 2024



www.nordicbotany.org

Many European terrestrial orchids are in decline. To curb this negative trend and preserve remaining populations, more ecological knowledge is needed. Surprisingly little is known yet about the identity and efficiency of pollinators of lesser twayblade *Neottia cordata*, a small terrestrial orchid species associated with pine trees through joint mycorrhizae. We identified its small and inconspicuous pollinators with DNA barcoding and assessed its fruit set with the help of observations submitted to various nature platforms. We caught pollinators on Terschelling in the Netherlands during the flowering season of 2013 and 2014. Insects were identified with 28S and COI sequences obtained from both fresh and museum-preserved material identified by specialists. Several pollinators were detected, belonging to either parasitoid wasps (Braconidae) and spider wasps (Pompilidae), active during sunny periods, or fungus gnats (Mycetophilidae and Sciaridae), active during overcast conditions. Combined pollinator efforts resulted in a continuous average fruit set above 70% in Europe over the past 135 years. The parasitoid wasps were identified as *Bracon pineti* and *Blacus* sp., which strongly depend on pine trees for their prey. The fungus gnats were identified as *Austrosiara hyalipennis*, *Trichosia lengersdorfi*, *Allodia lugens* and *Phronia forcipata*. All four species are known to deposit their eggs in the vicinity of fruiting bodies of cone caps *Strobilurus stephanocystis*, mushrooms growing on pine cones, as their larvae feed on the fungi. *Priocnemis pertubator* and *Anoplius viaticus* (Pompilidae) are also important pollinators. Results obtained show that lesser twayblade is even more intricately linked to pine forest ecosystems than previously thought. Management of growth sites of *N. cordata* should be tailored towards preserving pine tree forests with plots that are constantly being rejuvenated to generate young pine trees and prevent succession towards birch–oak forests. Such forests not only provide mycorrhizal fungi and layers of needle litter needed for germination and development of the seeds of this orchid but also pinewood-decaying fungi that provide egg deposition sites and food for its pollinators.

Keywords: Braconidae, fungus gnats, Ichneumonidae, Mycetophilidae, orchids, parasitoid wasps, Pompilidae, Sciaridae

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Introduction

With an estimated 28 000 species, the orchid family (Orchidaceae) is diverse (Cribb et al. 2003, Tsai et al. 2017, Fay 2018), and distributed throughout the world, except in the polar and desert regions. In their evolution, orchids have shown a rapid diversification, due to several factors: different pollination strategies, aggregation of pollen in larger units, and the production of dust-like seeds (Tremblay et al. 2005, Bretkopf et al. 2015, Givnish et al. 2015). Other hypotheses include the evolution of epiphytism and whole genome duplication (Gravendeel et al. 2004, Moriyama and Koshida-Takeuchi 2018).

In an orchid's life history, there are two major bottlenecks: pollination and seed germination (Tremblay and Otero 2009). Most orchids are dependent on pollinators for pollen transfer, and on the access to mycorrhizal fungi for germination and seed development (Jersáková et al. 2006, Micheneau et al. 2009, Rasmussen et al. 2015). Seed germination is limited by the access to mycorrhizal fungi for germination and seedling development. Orchids are renowned for the amount and complexity of pollination strategies (Schiestl et al. 1999, van der Cingel 2001, Jersáková et al. 2006). Orchids can provide some kind of reward: nectar, pollen, resin, fragrances, oil or wax (Dressler 1981); among European orchids, nectar is the most common reward (Neiland and Wilcock 1998, Johnson et al. 2004, Jersáková et al. 2008). Approximately 30–40% of all European orchids are deceptive species, relying primarily on food deception or sexual deception (Schiestl et al. 1999, Cozzolino and Widmer 2005, Schiestl 2005, Jersáková et al. 2006, Brzosko et al. 2021).

The genetic material of orchids is aggregated in pollinia (coherent masses of pollen grains that are transferred as a unit during pollination), and the attraction of potential pollinators and the precise placement of the pollinia on the insect's body is essential for successful fertilization. Precise adaptation of the flower structure to the insect's morphology ensures an increased precision of pollen transfer, thereby influencing plant fitness (Potts et al. 2010). This close match between flower and pollinator is a result of phenotypic selection (Moré et al. 2012, Trunschke et al. 2020). However, a very strong adaptation to a specific pollinator or a class of pollinators can also be a threat to the survival of the species, as the disappearance of the insect could reduce seed- and/or fruit set. Phenological mismatches between pollinators and orchids have already been reported (Hutchings et al. 2018). The early spider orchid *Ophrys sphegodes* relies on male bees for pollination. The males emerge before the females and pollinate the orchid as they search for a mate, but researchers have found that climate change is causing the females to emerge before the orchid's peak flowering time, reducing the orchid's reproductive success (Robbirt et al. 2011, Hutchings et al. 2018). However, not all orchid species have specialized pollination systems (Tremblay 1992, Jersáková 2006). Orchids with a wide pollinator spectrum can suffer severe pollen losses due to inefficient pollen transfer by non-specific vectors but can benefit from a high visitation rate.

Despite their omnipresence, many orchids are highly threatened (Fay 2018). In Europe, habitat loss is the main reason for the decline of orchid populations (Kull and Hutchings 2006, Kolanowska and Jakubska-Busse 2020, Štípková and Kindlmann 2021). One example of a threatened European orchid species is lesser twayblade, *Neottia cordata* (Arbeitskreise Heimische Orchideen 2005, Kotlínek et al. 2018, Tsiftsis et al. 2019). It has a circumpolar distribution, occurring in the boreal-temperate zone and the foothills of the arctic zone in Europe, Asia and large parts of North America. Its southernmost boundary is formed by the mountainous regions in the south (Pyrenees, Alps, Caucasus). It is widespread in the northern parts of its range (Scotland, Scandinavian countries), reaching as far as Greenland and Iceland (Hultén and Fries 1986, Tsiftsis et al. 2019). In the Netherlands, it is very rare and now only grows on the Wadden Islands (NDFD 2021). The first mention of *N. cordata* on the Wadden Islands dates back to 1949 (Weijer 1949), since then *N. cordata* spread to all the Wadden Islands, except Texel. It is a Red List species, protected by law (van der Meijden et al. 2000).

In most European orchids, it takes four to six weeks after pollination for the seed to mature. However, in *N. cordata* fruit set occurs very quickly: within two weeks after pollination, the seed is ripe and the capsules open. Regularly we found plants that had swollen capsules in the lower part, whereas the uppermost flowers were newly opened (Löjtnant and Jacobsen 1977). *Neottia cordata* is often mistaken for long-flowering, because the perianth stays green after pollination, whereas in most orchids the perianth dries out and turns brown. Ziegenespeck (1936) supposes this is to support the supply of nutrients. Pollination data for this orchid are almost exclusively available from North American localities (Ackerman and Mesler 1979). In North America, fungus gnats (Sciaridae and Mycetophilidae) are the most important and abundant pollinators; pollination efficiency is high (61–78% of all studied flowers from different stations set fruit (Ackerman and Mesler 1979). In Europe, the species shows a strong decline, mainly due to changes in its biotope or habitat loss (Arbeitskreise Heimische Orchideen 2005, Kotlínek et al. 2018, Tsiftsis et al. 2019).

The aims of this study were to 1) identify the pollinator spectrum of *N. cordata* in the Netherlands, and 2) investigate if photos showing fruit set of *N. cordata*, obtained from data uploaded to various publicly accessible nature observation platforms in Europe gathered by citizen scientists, could be used for computing fruit set. By unravelling the relationships between orchid, pollinators and biotope, we hope to contribute to the conservation of this rare orchid species in the Netherlands and beyond.

Material and methods

Study site

Within the Terschelling dune system there are some cultivated coniferous woodlands with *Pinus nigra* J.F.Arnold,

planted at the beginning of the 20th century to reduce the effects of strong sand drifts on the island. In such pine forests, we can distinguish several stages of succession. At first, there is no vegetation at all, the vegetation is dominated by terrestrial growing lichens of the genera *Cladonia* and *Cladina*; this type of forest is known as lichen forest or Cladonio–Pinetum (Westhoff 1959, Zumkehr 2011, 2012). As the forest ages, it enters a next phase called a moss forest or Leucobryo–Pinetum, characterised by e.g. *Leucobryum glaucum* Hedwig, *Rhytidiadelphus loreus* (Hedw.) Warnst. and *Lepidozia reptans* Dumort. (van Tooren et al. 2002). Gradually phanerogams appear, and after about 25 years the biotope is suitable for orchids, including *N. cordata* and *Goodyera repens* R.Br. (Westhoff 1959, Weeda et al. 1994). *Neottia cordata* preferably grows in a thick layer of needle litter, providing the needed moist environment; it is not found in a dry pine forest (Vermeulen 1958). As the moss layer thickens, the forest reaches its final stage, the birch–oak forest, Betulo–Quercetum roboris. Herbs and shrubs develop, the canopy is less dense, the forest gets drier, the humidity decreases and the growing conditions are no longer suitable for *N. cordata* (Zumkehr 2011).

Study species

Neottia cordata is a boreal–montane species; in Europe it occurs in wet heathlands, bogs, mires and in coniferous forests. It prefers very acidic to acidic substrates, nutrient-poor humus or peat (Harrap and Harrap 2005, Kotlínek et al. 2018). *Neottia cordata* reproduces by means of long roots that do not penetrate deep into the substrate (Kotlínek et al. 2018). *Neottia cordata* is mainly associated with nonectomycorrhizal Sebacinale Clade B (Těšitelová et al. 2015). Less frequent are rhizoctonias from Ceratobasidiaceae and Tulasnellaceae, ectomycorrhizal fungi from Russulaceae, Atheliaceae (Tylospora), Thelephoraceae, and numerous presumably endophytic ascomycetes and basidiomycetes (Yagame et al. 2016, Schiebold et al. 2018).

Neottia cordata has two opposite, heart-shaped leaves; the stem is hairy, green to reddish purple, carrying generally 6–12, up to 20 yellowish-green to reddish-purple flowers (Fig. 1A). Sepals and petals form a loose hood. The lip splits and forms two elongated lobes; in its centre is a median nectar-secreting groove (Fig. 1B). At the lip base is another nectar-secreting zone, similar to its sister species *Neottia ovata* Bluff & Fingerh. (Claessens and Kleynen 2011). The rostellum has sensitive hairs at its apex, acting as a lever that enables the extrusion of viscid fluid when touched, thus gluing the pollinia to a visiting insect. *Neottia cordata* has foetid-smelling nectar (Brackley 1985), which is often an indication that flies oviposit on the plant, but such behaviour has never been observed (Ackerman and Mesler 1979, Hoy 2002). Observations of pollinators of *N. cordata* in North America included fungus gnats of the genera *Mycetophila* (Mycetophilidae), *Sciara* and *Corynoptera* (Sciaridae), other occasional pollinators were crane flies (Tipulidae) and parasitoid wasps (Braconidae and Ichneumonidae) (Ackerman and Mesler 1979). In Europe,



Figure 1. (A) *Neottia cordata*, flowering plant. Hoorn (the Netherlands), 4 May 2013, (B) *Neottia cordata*, flower. Hoorn (the Netherlands), 7 May 2013. Photographs by Jean Claessens.

fungus gnats and occasionally beetles have been observed (Summerhayes 1968, Claessens and Kleynen 2011, 2016). The nectar is freely accessible, and there is no adaptation to a specific group of pollinators. Visiting insects can land in different positions on the flower and eventually move toward the column, guided by the nectar trail on the lip. Darwin (1877) described the pollination mechanism of *Neottia* as highly specialized, but with unspecialized pollinators, which was later confirmed by (Nilsson 1981).

The species is self-compatible, usually not self-pollinating (Kirchner 1922); the coherence of the tetrads in the pollinia is much higher than in other *Neottia* species (Ackerman and Williams 1980).

Study site

The study site was located on Terschelling in Hoornse bos, 53°24'19"N, 5°22'7"E. It belongs to the cushion moss–pine forest community (Leucobryo–Pinetum) (Schaminée et al. 2010). The dominant tree species is *Pinus nigra* with little undergrowth of *Sorbus aucuparia*, *Prunus serotina* Ehrh., *Dryopteris dilatata* (Hoffm.) A.Gray, *Polypodium vulgare*, *Betula pendula* Roth., *Ilex aquifolium*, *Lonicera perichyenum*, *Goodyera repens*, *Hypnum jutlandicum* Holmen & E.Warncke, *Hypnum cupressiforme* Hedw. and *Lophocolea bidentata* Dumort. *Neottia cordata* is the most widespread species with several thousands of individuals. The study site is part of a Natura 2000 reserve (Natura 2000 2021) and is specially protected because of the presence of this orchid species. On Terschelling, 95% of the total occurrence of *N. cordata* in the Netherlands is found, of which 40% occurs on the study site.

Floral visitors

Observations of visitors were made on Terschelling in 2013 and 2014. In 2013 observations were made between 11:30

and 16:30 h for five days and between 19:30 and 22:00 h for two days. Several large plant clusters were checked for the presence of insect visitors by patrolling along a transect; visitors and pollinators were collected and preserved in alcohol. In 2014 we observed for 7 h, totalling 42 h of observation. Fresh insect material was collected on 5–10 May 2013 and on 24 April 2014 and afterwards identified using both morphology and DNA barcoding. For morphological comparison, we used the specimens deposited in the entomology collection of Naturalis Biodiversity Center.

DNA barcoding of pollinators

For extraction of total genomic DNA from the insects studied, the Dneasy Blood & Tissue Kit (Qiagen, Valencia, California, USA) was used. Two modifications in the protocol were made. First of all, instead of cutting the insect into small parts previous to extraction, as indicated in the original protocol, the entire insect was submerged in 180 µl ATL buffer + 20 µl Proteinase K, followed by 3 h of incubation at 57°C. The Diptera collected in 2013 were incubated overnight at 57°C. This modification was done so that the specimens would be available for resampling. The insects remained intact and could therefore be added to the entomology collection of Naturalis Biodiversity Center after drying. DNA elution was done twice with 50 µl AE buffer. Between each pipetting step, the column was centrifuged for 1 min at 8000 rpm. Different DNA barcoding regions were chosen based on earlier studies: 16S, 28S and COI (Kambhampati et al. 2000, Shi et al. 2005, Zaldivar-Riverón et al. 2006). As both fresh and museum-preserved material were analysed, we used both long (600–660 bp) regions as well as minimalistic barcode regions (100–130 bp). Primers for the latter region were designed during this study. Amplifications were done in a Bio-Rad S1000 Thermal Cycler under the following conditions:

COI: denaturation for 3 min at 94° followed by 40 cycles of 15 s at 94°, 30 s at 45° and 40 s at 72°, and a final extension of 5 min at 72°. 16S: denaturation for 3 min at 94° followed by 35 cycles of 15 s at 94°, 30 s at 48° and 1 min at 72°, and a final extension of 7 min at 72°. 28S: denaturation for 3 min at 94° followed by 45 cycles of 15 s at 94°, 30 s at 55° and 1 min at 72°, and a final extension of 8 min at 72°.

MiniCOI: denaturation for 3 min at 94° followed by 40 cycles of 15 s at 94°, 30 s at 45° and 40 s at 72°, and a final extension of 5 min at 72°. Amplification was performed in a 25 µl reaction volume consisting of 18.8 µl Ultrapure mQ, 2.5 µl Qiagen PCR buffer CL 10x concentration, 1 µl Forward primer, 1 µl 10 pmol µl⁻¹ reverse primer, 0.5 µl 2.5 mM dNTPs, 0.25 µl 5 U µl⁻¹ Taq and 1 µl DNA template.

For a second COI amplification and COI mini barcodes the following 25 µl reaction volume was used: 17.8 µl Ultrapure mQ, 2.5 µl Qiagen PCR buffer CL 10x concentration, 0.5 µl 25 mM MgCl₂, 0.5 100 mM BSA, 1 µl Forward primer, 1 µl 10 pmol µl⁻¹ reverse primer, 0.5 µl 2.5 mM dNTP, 0.25 µl 5 U µl⁻¹ Taq and 1 µl DNA template. The resulting amplicons were Sanger sequenced on an ABI 3730 at Macrogen, Amsterdam. Electropherograms were analyzed with Sequencher ver. 4.10.1 and DNA sequences were blasted against NCBI Genbank and the BOLD database (Supporting information). Identifications were considered correct only for similarity hits of 99% or higher.

Pollen export and import

Pollinia removal and pollen deposition (the male and female fitness component) of 33 plants with 330 flowers was recorded in Schluderbach (Italy) in June 2006 and Hoornse bos, Terschelling (the Netherlands) in May 2013. Pollinia removal or deposition was observed with a 10x-magnifying lens and a powerful torch (Table 1, Supporting information). Each flower was inspected and recorded as pollinated if pollen was deposited on the stigma. Pollinia removal and deposition did not always occur simultaneously, in some flowers the pollinia were still in the anther while the pollen was already deposited on the stigma.

Fruit set

Fruiting success was calculated as the number of fruits divided by the number of flowers (Table 2, Supporting information). Fruiting plants of *N. cordata* are easy to identify because the fruits are spherical (Fig. 3). During seed ripening the ovary almost doubles in size. A spherical fruit is a characteristic found in only two other European species, *Neottia ovata* and *Goodyera repens*. *Neottia ovata* has much larger leaves and is a much larger plant. In *G. repens*, the fruits are angled upwards, close together and on one side of the inflorescence, whereas in *N. cordata* the fruits are horizontal and arranged around the stem in a very loose inflorescence. Also, the fruits of *G. repens* do not appear until much later in the season, when the plants of *N. cordata* have already disappeared. This makes confusion with other species in the field very unlikely. The fruits are delicate and easily compressed, so that they remain attached to the stem when dried. Due to the large difference in shape between fertilized and unfertilized fruiting ovaries, it was possible to determine fruit set even in herbarium specimens, with only intact and undamaged specimens included in the count. Data on fruit

Table 1. Pollen export and import of *Neottia cordata* in Hoornse bos (the Netherlands) and Schluderbach (Italy).

	Pollen export and import of <i>N. cordata</i> (n = 33)					
	A	B	C	D	C + D	B + D
	Pollinia present, not pollinated	Pollinia present, pollinated	Pollinia absent, not pollinated	Pollinia absent, pollinated	Total pollinia removal	Total pollinia receipt
Hoornse bos	69	64	18	146	164	210
Schluderbach	0	3	0	30	30	33

Table 2. Fruit set of *Neottia cordata* in the Netherlands and in Europe.

Location	Date	Fruit set <i>Neottia cordata</i>			
		Plants	Flowers	Pollinated	Not pollinated
Garmisch-Partenkirchen (D)	August 2004	47	382	347 (90.8%)	35 (9.2%)
Scuol (CH)	July 2012	22	167	143 (85.6%)	24 (14.4%)
Terschelling (NL)	May 2014	40	435	414 (95.2%)	21 (4.8%)
Chichiliane (F)	June 2015	21	191	162 (84.8%)	29 (15.2%)
Gresse-en-Vercors (F)	June 2015	24	208	153 (73.6%)	55 (26.4%)
Garmisch-Partenkirchen (D)	July 2021	12	100	91 (91%)	9 (9%)

set were obtained with three different methods. Firstly, during visits to different sites in Germany, France, Switzerland and the Netherlands in 2004, 2012, 2014, 2015 and 2021, the total number of flowers and swollen, pollinated fruits was counted (Table 2). The second method was the use of herbarium specimens. To assess whether a swollen ovary was equivalent to pollination, the inflorescence of one dried herbarium specimen of *N. cordata* (deposited at the herbarium of Naturalis Biodiversity Center) was boiled up and two flowers were examined. In both cases, remains of pollinia could be observed on the stigma. We therefore concluded that a swollen ovary was equivalent to pollination. This allowed us to use available herbarium vouchers (94 vouchers of plants collected in the period 1887–1978 throughout Europe and deposited at Naturalis Biodiversity Center) to calculate fruit set. An herbarium search of European vouchers from 1887 until 1930 (Herbarium Renz) yielded an additional 51 usable vouchers. In a third method, we used photos of 141 fruiting *N. cordata* plants from the Wadden Islands Terschelling, Schiermonnikoog and Vlieland, made in the period 2006–2022 and posted on the publicly available internet repositories [Waarneming.nl](https://www.waarneming.nl) (2003), [Observation.org](https://www.observation.org) (2003) and [GBIF](https://www.gbif.org) (2003). These websites are widely used for uploading nature observations. To investigate if these photos could be

used for counting fruit set, we examined a population of 12 fruiting *N. cordata* in the Bavarian Alps, near Garmisch-Partenkirchen (Germany). We numbered each plant, noted the fruit set of each plant and also took photographs of all plants. Afterwards, we counted the fruit set of the numbered and photographed plants and compared the results with our counts in situ. Our counts in the field matched the counts from the photos. Having established that photographs of pollinated orchids could be used to determine pollinator efficiency, we examined 1416 photographs in the databases of [Waarneming.nl](https://www.waarneming.nl), [Observation.org](https://www.observation.org) and [GBIF](https://www.gbif.org), which are all

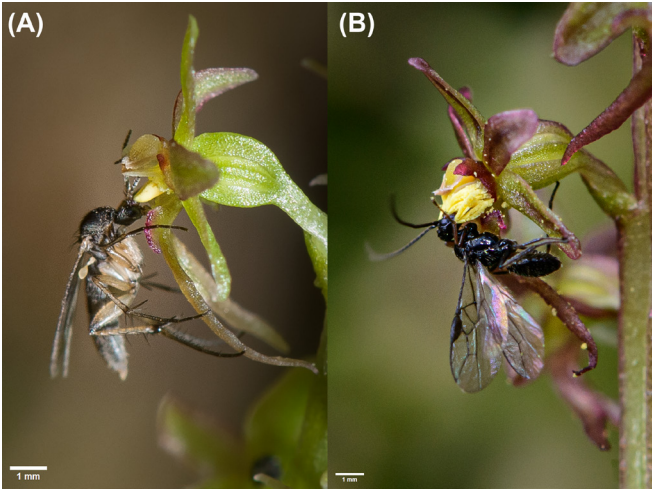


Figure 2. (A) Mycetophilidae pollinating *Neottia cordata*. Pramaran (CH), 16 June 2012, (B) Braconidae pollinating *Neottia cordata*. Hoorn (NL), 8 May 2013. A bunch of pollinia, collected by the insect, sticks to the stigma and prevents the parasitoid wasp from getting away. Photographs by Jean Claessens.



Figure 3. *Neottia cordata*, fruiting plant, inflorescence with swollen capsules. Photograph by Jean Claessens.

Table 3. Pollinators and visitors of *Neottia cordata* recorded in this study.

Order	Family	Species	Sex	No. of individuals	Pollinator/visitor	Locality	Date
Hymenoptera	Braconidae	?	unknown	2	pollinator	Pramaran (CH)	18 June 2012
Hymenoptera	Braconidae	?	male	abundant	pollinator	Terschelling (NL)	9 May 2013
Hymenoptera	Braconidae	<i>Blacus</i> sp.	unknown	1	pollinator	Terschelling (NL)	24 Apr. 2014
Hymenoptera	Braconidae	<i>Bracon pineti</i> Thomson	unknown	1	pollinator	Terschelling (NL)	8 May 2013
Hymenoptera	Formicidae	<i>Formica fusca</i> L.	unknown	3	visitor	Terschelling (NL)	4 May 2013
Hymenoptera	Formicidae	<i>Myrmica rubra</i> L.	unknown	8	visitor	Terschelling (NL)	5 May 2013
Hymenoptera	Pompilidae	<i>Anoplius viaticus</i> L.	unknown	1	pollinator	Terschelling (NL)	24 Apr. 2014
Hymenoptera	Pompilidae	<i>Priocnemis perturbator</i> Harris	female	26	pollinator	Terschelling (NL)	5 June 2013, 24 Apr. 2014
Diptera	Bolitophilidae	<i>Bolithophila (Cliopisa) aperta</i> Lundström	male	2	pollinator	Pramaran (CH)	15 June 2012
Diptera	Empididae	?	unknown	1	visitor	Pramaran (CH)	15 June 2012
Diptera	Mycetophilidae	?	unknown	9	pollinator	Pramaran (CH)	16 June 2012, 26 June 2014
Diptera	Mycetophilidae	<i>Mycetophila trinotata</i> Staeger	female	1	pollinator	Pramaran (CH)	15 June 2012
Diptera	Mycetophilidae	<i>Phronia</i> sp.	female	1	pollinator	Pramaran (CH)	15 June 2012
Diptera	Lonchopteridae	<i>Lonchoptera lutea</i> Panzer	unknown	1	visitor	Terschelling (NL)	24 Apr. 2014
Diptera	Mycetophilidae	<i>Allodia lugens</i> Wiedemann	unknown	various	pollinator	Terschelling (NL)	24 Apr. 2014
Diptera	Mycetophilidae	<i>Phronia forcipata</i> Winnertz	male/female	4	pollinator	Terschelling (NL)	24 Apr. 2014
Diptera	Sciaridae	<i>Austrosclara hyalipennis</i> Meigen	male	1	pollinator	Terschelling (NL)	9 May 2013
Diptera	Sciaridae	<i>Trichosia lengersdorfi</i> Heller, Köhler & Menzel	male	2	pollinator	Terschelling (NL)	24 Apr. 2014
Araneae	Tetragnathidae	<i>Metellina</i> sp.	unknown	8	visitor	Terschelling (NL)	7 May 2013

checked for correct species identifications by validators. A search of the photos in [Waarneming.nl](https://www.waarneming.nl) retrieved 141 utilizable records, searches of [GBIF](https://www.gbif.org) and [Observation.org](https://www.observations.org) revealed another 158 and 90 records, respectively. Only photos showing the entire inflorescence with sufficient detail were used. In total, 389 photos (27.5%) out of 1416 were used for counting fruit set (Supporting information)

Results

Visitors and pollinators

All visiting insects, with one exception, belonged to the order Diptera or Hymenoptera, spread over seven families. Insects of five families were pollinators; in most cases we observed one or several pollinators per family, but in three families we observed larger numbers of pollinators (Table 3). Insects were defined as pollinator if they had pollinaria attached to their body and visited several flowers, leaving and/or depositing pollinaria. Species identifications were obtained by matching DNA sequences with reference data in NCBI GenBank and BOLD. Species from eight families were pollinators; Sciaridae and Mycetophilidae were most abundant, followed by Braconidae, Tipulidae, Anthomyiidae, Pompilidae, and one observation of Bolitophilidae and Ichneumonidae (Table 3, Supporting information). Sciaridae and Mycetophilidae (Fig. 2A) alighted on the inflorescence

and actively searched for nectar, visiting and revisiting most flowers, thereby removing and depositing pollinia. They were active during overcast conditions and showed an alternation between inspecting behaviour and immobile periods, which lasted from 25 s to two minutes.

Braconidae (Fig. 2B) were found predominantly when it was not too windy. They were alternating between moving on the plant for a short time, then checking the flower and licking the lip. Then they stayed immobile for one to 18 minutes, after which they repeated the movement and inspection behaviour. Of the individuals observed, 94% (79 out of 84 individuals) carried and deposited pollinia. In total, they stayed on one plant for a long time, up to almost 1 h. In some cases, we observed that an insect was glued to the stigmatic surface.

The spider wasp *Priocnemis perturbator* Harris was an important pollinator, constantly patrolling, mostly active in spots that were in full sun. After landing on a plant, it crawled up and down the plant several times, inspected several flowers and then flew to the next plant. Meanwhile, it deposited already attached pollinia on the stigma and got new pollinia glued to its clypeus. It was also observed in windy conditions, but only if the plant was in direct sunlight.

Ants (*Formica fusca* and *Myrmica rubra*) inspected the flowers; in one case an ant removed pollinia but was able to rub them off. Spiders (*Metellina* sp.) were regularly seen waiting on the flower spike or in their web for prey. Some carried pollinia, but they are not considered pollinators because we did not observe them depositing any pollinia on a stigma.

Pollen export/import and fruit set

In Hoornse bos (the Netherlands) ($n=28$) pollen export was 63.2% and pollen import 68.4%; in Schluderbach (Italy) ($n=5$) pollen export was 90.9% and pollen import 100%. In Hoornse bos, in 20.8% of all flowers pollen was already deposited onto the stigma while the pollinia were still in the anther (Table 1, Supporting information). No autogamous pollination was observed.

The mean fruit set in European populations (without the Netherlands) was $83.8 \pm 15.3\%$. The mean fruit set of the Wadden Islands was $73.5 \pm 28.1\%$ (Table 2, Supporting information).

Discussion

Pollinators of lesser twayblade in the Netherlands are all part of a pine tree community

By applying a combination of DNA barcoding and matching specimens with reference collections in Naturalis Biodiversity Center, the pollinators of *N. cordata* collected in Hoornse bos, Terschelling could all be identified to species level. This adds more details to previous findings by Ackerman and Mesler (1979) for North America, who also found that the pollinators were parasitoid wasps and fungus gnats. *Neottia cordata* is not specifically adapted to pollination by one particular insect group. The easily accessible nectar, secreted in minute quantities on the lip and the lip base is accessible to a wide range of insects with small mouthparts and does not require adaptation to a specific body shape. The floral architecture facilitates pollination by fungus gnats. The sister species, *Neottia ovata* Bluff & Fingerh. is also pollinated by a wide range of insects from different families (Claessens and Kleynen 2011, Kotlínek et al. 2015). The small amount of nectar produced may serve to filter out certain potential pollinators: insects with high nectar requirements are more likely to avoid the plant because their needs are not met. This makes the nectar supply especially suitable for insects for whom a small amount of nectar is enough to meet their nectar needs. There is a strong relationship between fungus gnats and plant habitat: fungus gnat pollination is mainly found in habitats where other pollinators are absent (Mochizuki and Kawakita 2018). In continuously moist environments fungus gnats are present throughout the year and can act as reliable pollinators, while other pollinators such as bees are rare in these conditions. In fact, in Hoornse Bos on Terschelling, in addition to fungus gnats, we only observed parasitoid wasps, ants and spiders during our fieldwork.

The foul-smelling nectar may be an adaptation to sapromyophily, but ovipositing behaviour was never observed by Ackerman and Mesler (1979) and us. Lemoine (2018) described the floral visiting behaviour of Mycetophilidae, observed in Norway. The insects started at the top of the inflorescence, descended upside down and probed the nectar of all flowers, from time to time remaining motionless. This is

in line with our observations on Terschelling, where only nectar-feeding behaviour was observed. Nectar and scent seem to act as attractants for insect visitors (Ackerman and Mesler 1979, Claessens and Kleynen 2011, 2016). Irrespective of the position in which the insects land, they eventually turn towards the stigma, guided by the nectar trail on the lip and attracted by the second nectar secretion zone at the lip base. We inspected 33 plants with 243 flowers, but never found any signs of autogamous pollination (Table 1), nor did we find any mention of autogamy in literature. *Neottia cordata* apparently relies on insect vectors for pollination.

The main fungus gnat pollinators found on Terschelling belong to Mycetophilidae and Sciaridae. *Trichosia lengersdorfi* Heller, Köhler & Menzel, *Phronia forcipate* Winnertz, *Allodia lugens* Wiedemann and *Austrosciara hyalipennis* Meigen are all fungus gnats. Their larvae feed on fungi, fungal mycelia in rotten wood and decaying plant material (Mochizuki and Kawakita 2018). They are generally considered to be inefficient pollen vectors due to their small body size and poor flight ability (Proctor et al. 1996). However, their ubiquity compensates for this (Mesler et al. 1980, Mochizuki and Kawakita 2018). Sciaridae have a worldwide distribution and can survive even in extreme habitats such as subantarctic islands or high altitudes. In Europe, more than 600 species are known (Mohrig 2003).

Next to fungus gnats, fruiting bodies of the fungus *Strobilurus stephanocystis* (Kühner & Romagn. ex Hora) Singer were found by us in large numbers on Terschelling at the same locality as the orchids during their main flowering season. This fungus grows on decaying cones of pine and spruce trees and has been found on the cones of *Pinus nigra*. The larvae of Mycetophilidae and Sciaridae, regular pollinators of *N. cordata*, primarily feed on fungi and decaying organic matter (Mead and Fasulo 2001, Jakovlev 2011, Pöldmaa et al. 2015), providing an additional link between this orchid and pine forest ecosystems.

Parasitoid wasps observed were *Bracon pineti* Thomson, *Blacus* sp. and unidentified Braconidae. They are known for laying their eggs in caterpillars of microlepidoptera or Coleoptera larvae, living in decaying wood, pine cones or under the bark of the trees (van Achterberg and Altenhofer 1997) or on aphids feeding on pine needles (Žikić et al. 2012). They use the nectar of *N. cordata* as a food source, because no other flowering plants are present at the time of the orchid's flowering.

In addition to parasitoid wasps, *Priocnemis perturbator* Harris and *Anoplius viaticus*, both spider wasps (Pompilidae), were very active in sunny conditions, searching for spider prey. Spiders were observed using the flowering *N. cordata* plants to capture prey by spinning webs or ambushing on the plant. *Priocnemis perturbator* and *Anoplius viaticus* are species of sandy soils and open forests (Peeters et al. 2004). Above all *P. perturbator* was an important pollinator, which was observed in various weather conditions. Its fast-searching behaviour allowed it to visit and pollinate many plants in a short time.

Neottia cordata is a relatively recently arrived species in floristic observations of the Netherlands, having been recorded

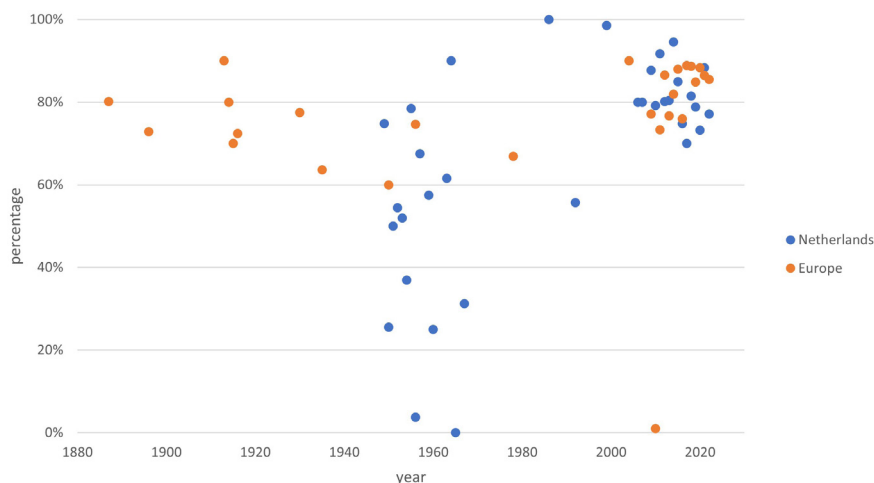


Figure 4. Fruiting percentage of *Neottia cordata* in the Netherlands from 1949 onwards and in Europe from 1887 onwards.

from 1949 onwards on Ameland, one of the Wadden islands (Westhoff 1959). In Hoornse bos it was found in 1955, about 25 years after the planting of non-native *Pinus nigra*. By that time the mycorrhiza associated with *Pinus* were well established in the soil, enabling the germination of seeds of *N. cordata*. Orchid seeds lack a food reserve in the form of endosperm and rely on a mycorrhizal relationship with a fungus for development (Li et al. 2021). When the *Pinus nigra* forest enters the Leucobryo–Pinetum phase, it is suitable for orchids. The pines form a dense canopy that provides sufficient shade for the drought-sensitive orchid and maintains a sufficiently high moisture level. Another advantage of the relatively dense canopy is that few other plants can develop. As more herbs and shrubs appear, the forest enters the Betulo–Quercetum roboris phase and conditions are no longer suitable for the non-competitive orchid (Zumkehr 2011, 2012). Those familiar with *N. cordata* in the Alps will be surprised to find the orchid in such a seemingly dry habitat. However, humidity is high due to the proximity of the North Sea. The groundwater level is high enough to provide the moisture conditions required by the orchid.

The fungus gnats are dependent on the fungi associated with *P. nigra*, as are Braconidae and Pompilidae for their prey living on or around the pine trees or associated fungi. The existence of such an interdependent community stresses the importance of conserving not just a single species, but protecting all members of the community, all of which depend on the continued existence of a *Pinus nigra* habitat.

Continuous high fruit set of lesser twayblade due to its dual pollination strategy

In orchids, fruit set is generally more pollinator than resource-limited (Nilsson 1992). High fruit and seed set result in high recruitment rates (Jacquemyn and Brys 2010) and is therefore important for the ability of a population to survive. In the Netherlands, *N. ovata* shows a continuous high fruit set (Fig. 4). Apparently, in this interdependent system where

few other flowering plants grow, unceasing visits from insect visitors are guaranteed. Although fungus gnats are considered inefficient pollen vectors, their numbers and their attachment to this particular biotope, which provides food and prey, ensure a continuous high pollination rate. Nectar is produced in minute quantities, forcing the insects to return again and again to satisfy their energy needs. The fungi that grow on decaying *P. nigra* cones are important for oviposition and food for larvae. Pollination of *N. cordata* involves a dual strategy: on the one hand, the orchid is pollinated by insects attracted by smell, colour and nectar present. On the other hand, additional pollination takes place because insects in the biotope of *N. cordata* are looking for prey, and in the absence of other nectar sources rely on the orchid to meet their nectar needs. It seems that this dual strategy ensures a continuous high fruit set.

Conclusions

We discovered a mutual dependence of both *Neottia cordata* and its pollinators on a specific biotope: pine forests at the Leucobryo–Pinetum stage of development. The pine forests of the Dutch Wadden Islands were all planted at the same time and all trees are therefore equally old. If natural succession is allowed to continue, these forests will become more open due to the death of mature pine trees and gradual transition towards the Betulo–Quercetum roboris stage with birch and oak trees. As a result, the needle litter and pine cones on the forest floor will disappear, and cover of the underlying herb layer will increase, ultimately causing the disappearance of these orchids and their pollinators. We therefore recommend to apply a cyclic management, where plots of pine forest are constantly being rejuvenated (Zumkehr 2011, 2012). The result of such a cyclic management is that there will always be pine forest plots that are in a developmental phase favourable for these orchids to germinate, develop into seedlings and ultimately plants with flowers and fruits that release new seeds.

Acknowledgements – We would like to thank Bertie Joan van Heuven, Mark Smeets and Frank Stokvis for their help in the laboratory. Piet Zumkehr gave us permission to use his reports, for which we are grateful. Jan Bunnik (Staatsbosbeheer) allowed us to carry out fieldwork on Terschelling and Frederique Bakker provided access to the entomology collection of Naturalis Biodiversity Center.

Funding – The author(s) received no financial support for the research and authorship of this article.

Author contributions

Jean Claessens: Conceptualization (supporting); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Project administration (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (lead). **Cees van Achterberg:** Investigation (supporting); Resources (supporting). **Emma de Haas:** Investigation (supporting); Resources (supporting). **Marijke Claessens-Janssen:** Investigation (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Barbara Gravendeel:** Conceptualization (lead); Formal analysis (supporting); Investigation (supporting); Methodology (supporting); Supervision (lead); Writing – original draft (supporting); Writing – review and editing (supporting);

Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.rxdbrvk7> (Claessens et al. 2024).

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