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MOLECULAR ECOLOGY

Molecular Ecology (2015) **24**, 3269–3280 doi: 10.1111/mec.13236

Mycorrhizal diversity, seed germination and long-term changes in population size across nine populations of the terrestrial orchid *Neottia ovata*

HANS JACQUEMYN,* MICHAEL WAUD,*† VINCENT S. F. T. MERCKX,‡ BART LIEVENS† and REIN BRYS*

*KU Leuven, Department of Biology, Plant Conservation and Population Biology, B-3001 Leuven, Belgium, †KU Leuven, Campus De Nayer, Department of Microbial and Molecular Systems (M^2S), Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), B-2860 Sint-Katelijne-Waver, Belgium, ‡Naturalis Biodiversity Center, Leiden University, Leiden, the Netherlands

Abstract

In plant species that rely on mycorrhizal symbioses for germination and seedling establishment, seedling recruitment and temporal changes in abundance can be expected to depend on fungal community composition and local environmental conditions. However, disentangling the precise factors that determine recruitment success in species that critically rely on mycorrhizal fungi represents a major challenge. In this study, we used seed germination experiments, 454 amplicon pyrosequencing and assessment of soil conditions to investigate the factors driving changes in local abundance in 28 populations of the orchid *Neottia ovata*. Comparison of population sizes measured in 2003 and 2013 showed that nearly 60% of the studied populations had declined in size (average growth rate across all populations: −0.01). Investigation of the mycorrhizal fungi in both the roots and soil revealed a total of 68 species of putatively mycorrhizal fungi, 21 of which occurred exclusively in roots, 25 that occurred solely in soil and 22 that were observed in both the soil and roots. Seed germination was limited and significantly and positively related to soil moisture content and soil pH, but not to fungal community composition. Large populations or populations with high population growth rates showed significantly higher germination than small populations or populations declining in size, but no significant relationships were found between population size or growth and mycorrhizal diversity. Overall, these results indicate that temporal changes in abundance were related to the ability of seeds to germinate, but at the same time they provided limited evidence that variation in fungal communities played an important role in determining population dynamics.

Keywords: extinction debt, mycorrhizal fungi, orchids, population dynamics, seed germination

Received 12 November 2014; revision received 24 April 2015; accepted 7 May 2015

Introduction

Recruitment from seeds represents a major factor determining the distribution and abundance of plant populations and therefore represents a key component in the life history of plants (Clark *et al.* 2007). In general, two major factors determine whether and where recruitment will take place. If plants do not produce a sufficient number of viable seeds or seeds are unable to reach vacant sites, recruitment will be limited by seed availability. If, on the other hand, the availability of suitable environmental conditions is limited, seedling recruitment will be limited due to microsite limitation (Eriksson & Ehrlén 1992). In plant species that rely on mycorrhizal symbioses for germination and seedling establishment, the availability of suitable mycorrhizal fungi may constitute an important part of microsite lim-

Correspondence: Hans Jacquemyn, Fax: +32 16321968;
E-mail: hans.jacquemyn@bio.kuleuven.be

itation. This may be particularly true for orchids, as their dust-like seeds lack an endosperm and completely rely on fungal colonization for successful germination and growth into an underground heterotrophic achlorophyllous stage called a protocorm (Rasmussen 1995; Smith & Read 2008). As such, mycorrhizal fungi inevitably form an essential part of the orchid life cycle and the diversity of mycorrhizal fungi with which an orchid associates may be an important factor affecting orchid distribution and abundance (Swarts *et al.* 2010; Graham & Dearnaley 2012).

Despite these straightforward predictions, there is little evidence that supports this hypothesis and detailed analyses of spatial distribution patterns of orchids and assessments of mycorrhizal diversity across large geographic scales suggest that both are not necessarily related to each other (McCormick & Jacquemyn 2014). For example, the narrowly endemic orchid *Piperia yadonii* associates with a wide diversity of fungi (Pandey *et al.* 2013), whereas the widespread *Cypripedium calceolus* associates with only a limited number of fungi (Shefferson *et al.* 2007). Similarly, Phillips *et al.* (2011) showed that in several *Drakaea* species, rarity was not related to mycorrhizal diversity. Bailarote *et al.* (2012) compared mycorrhizal diversity between a rapidly declining orchid species and a species showing no notable changes in distribution area and concluded that decline was also not related to mycorrhizal diversity, suggesting that other factors than mycorrhizal diversity may be more important in governing orchid rarity and decline.

Apart from interspecific differences in mycorrhizal diversity, recent studies have also shown that fungal community composition can differ largely between populations of a single species (Ramsay *et al.* 1987; Jacquemyn *et al.* 2012; Pandey *et al.* 2013), suggesting that regional variation in mycorrhizal communities may be a natural feature of orchid species associating with a large diversity of mycorrhzial fungi. Assuming that different fungal strains may vary in nutrient uptake efficiency (van der Heijden *et al.* 2003) or stimulation of germination, differences in fungal community composition can also be expected to impact on orchid population dynamics and abundance. However, at present, little is known about the factors driving differences in fungal community composition among populations and how these differences shape the abundance and dynamics of orchid populations. Comparing mycorrhizal communities, seed germination and changes in local abundances across multiple populations can therefore be expected to provide some novel insights into how differences in mycorrhizal community composition affect orchid population dynamics and abundance (McCormick *et al.* 2009, 2012). Additionally, comparison of mycorrhizal communities between root and soil samples can provide indirect clues about the overall diversity of mycorrhizal fungi at a given site and the role mycorrhizal fungi play in affecting orchid population dynamics. The absence of key fungi may explain why seed germination is limited, or why populations decrease in size. However, despite the increasing number of studies that have investigated mycorrhizal associations in established plants and germinating seeds and protocorms, the number of studies that have related seed germination and orchid population dynamics to fungal community composition is still limited.

Here, we investigated changes in local abundance in 28 populations of the orchid *Neottia ovata* over a 10-year study period in eastern Belgium. *Neottia ovata* is a long-lived rhizomatous orchid species that occurs predominantly in forest habitats, but sometimes it can be found in meadows as well (Delforge 2006). At present, little is known about the mycorrhizal fungi associating with *N. ovata* (but see Oja *et al.* (2015) and Těšitelová *et al.* (2015) for recent analyses). More specifically, for each study population, we measured its population size in 2003 and compared it with the population size in 2013. To get an overview of the mycorrhizal communities associating with *N. ovata* and to test the hypothesis that the mycorrhizal communities were related to local environmental conditions, variation in mycorrhizal communities was assessed in both the roots and the soil for a subsample of nine populations using 454 amplicon pyrosequencing. We further tested the hypothesis that small populations or populations with negative growth rates had significantly different mycorrhizal diversity and community composition than large populations or populations with positive growth rates. Further, seed germination experiments were performed *in situ* to test the hypothesis that long-term changes in abundance were related to the ability of seeds to germinate *in situ*. Finally, we tested the hypothesis that seed germination was significantly related to mycorrhizal diversity and community composition or to local soil conditions.

Material and methods

Study species

Neottia (*Listera*) *ovata* is a perennial, rhizomatous orchid that can be regularly found in forests and to a lesser extent in meadows (Delforge 2006). It is one of the most common orchids in western Europe, and its geographical range reaches eastern Siberia in Asia (Delforge 2006). The species flowers from mid-May until the beginning of June. Flowers of *N. ovata* secrete large amounts of nectar onto the labellum surface and have a distinct and somewhat sweet scent that attracts many

insect species (Brys *et al.* 2008). Although flowers of *N. ovata* are specifically adapted to ichneumons for successful pollination (*Hymenoptera parasitica*, Ichneumonidae) (Müller 1878; Nilsson 1981), our own observations indicate that it is mainly pollinated by a large suite of generalist pollinators (Brys *et al.* 2008). Fruit production varies between populations and is strongly related to the number of flowering plants (Brys *et al.* 2008; Jacquemyn *et al.* 2009). *Neottia ovata* is a long-lived orchid species with an estimated half-life of 80 years (Tamm 1972).

The seeds germinate in spring and the young protocorm rests during the summer (Fuchs & Ziegenspeck 1926; Rasmussen 1995). In autumn, a highly mycotrophic, short thick root develops below the apical bud. In the second and third year, similarly shaped roots develop that are a bit longer and more slender than the previous ones. Only after the fourth spring, the first leafy shoot develops. From this stage onwards, the rhizome further develops. Roots generally continue to grow and have a lifespan of about 10 years. Little is known about the mycorrhizal fungi associating with *N. ovata*. Rasmussen (1995) mentioned that adult roots are generally devoid of mycorrhizal fungi and that they are easily invaded by a fortuitous infection that is not associated with germination. However, recent analyses by Oja *et al.* (2015) and Těšitelová *et al.* (2015) showed that the predominant mycobionts of *N. ovata* belonged to the Sebacinales.

Study area and temporal changes in spatial distribution and abundance

To study temporal changes in distribution and local abundance of *N. ovata* populations, in spring 2003, the exact location of all populations present was determined within a 100-km² study area in the eastern part of Belgium (Voeren). This area consists of a patchwork of agricultural fields, grasslands, urban land and houses, and forests. Most forests are old-growth forests, which are situated on gentle slopes with an altitude in the range 150–250 m above sea level (Jacquemyn *et al.* 2006). As a result, soil moisture content and texture differ between the lower and higher parts of the studied forest fragments, and tree species composition changes accordingly. On the lowest parts of the investigated forests, soils are relatively wet and loamy. Here, ash (*Fraxinus excelsior*) and poplar (*Populus x canadensis*) are the dominant tree species. On the drier slopes, a mixed forest type consisting of pedunculate oak (*Quercus robur*), wild cherry (*Prunus avium*), sycamore (*Acer pseudoplatanus*), hornbeam (*Carpinus betulus*) and beech (*Fagus sylvatica*) is found. On the highest parts, soils are more sandy and dry. Here, silver birch (*Betula pendula*) and pedunculate oak make up the dominant species of the tree layer. In general, there is sharp transition between forests and the neighbouring land, which mostly consists of intensively managed grasslands or arable fields.

The exact location of each population was determined with GPS, and for each population, all individuals were counted in spring 2003. In spring 2013, the whole study area was screened again to detect new populations arising through colonization. Additionally, all known populations were revisited, and for each population, the number of plants was counted again. Based on population sizes in 2003 and 2013, we calculated the relative population growth rate as $(\log(X_1) - \log(X_2))/t$, where X is the size of the population and t is the duration of the sampling period.

Sampling of mycorrhizal communities

To assess variation in orchid mycorrhizal communities across populations, nine populations that differed in size were selected within the study area. In spring 2014, young roots of five plants were collected in the most central part of each of the study populations to determine patterns of mycorrhizal associations. Roots were surface sterilized (30-s submergence in 1% sodium hypochlorite, followed by three 30-s rinse steps in sterile distilled water) and microscopically checked for mycorrhizal colonization. Subsequently, DNA was extracted from 0.5 g mycorrhizal root fragments using the UltraClean Plant DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories Inc., Solana Beach, CA, USA). Additionally, the soil was sampled to see whether mycorrhizal communities in the soil varied between populations. In each population, three 1 × 1 m plots were established in close proximity to the plants from which roots were sampled. In each plot, 10 topsoil samples were randomly taken with a 2.5-cm-diameter soil auger to a depth of 5 cm below the litter layer, bulked and returned to the laboratory. After collection, soil samples were refrigerated (4 °C) until processing. All samples were processed within 24 h. Approximately 5 g of soil was dried for 24 h and homogenized, and afterwards, large material (sticks, rocks, roots, etc.) was removed. For each composite, soil sample DNA was extracted from two separate 0.5 g soil subsamples using the PowerSoil DNA Isolation Kit as described by the manufacturer (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Each pair of DNA extracts was then pooled, resulting in 27 DNA samples from soil, next to 45 DNA extracts from plants. Next, amplicon libraries were created using the broad-spectrum basidiomycete internal transcribed spacer (ITS) primers ITS1OF-C (5′-AACTCG GCCATTTAGAGGAAGT-3′)/ITS1OF-T (5′-AACTTGGT CATTTAGAGGAAGT-3′) and ITS4OF (5′-GTTACTAG

GGGAATCCTTGTT-3′) (Taylor & McCormick 2008). All samples were assigned unique MID (multiplex identifier) barcode sequences according to the guidelines for 454 GS-FLX PLUS Lib-L sequencing (Table S1, Supporting Information). Polymerase chain reaction (PCR) amplification was performed in duplicate in a 25 μL reaction volume containing 0.15 mM of each dNTP, 0.5 μM of each primer, 1 U Titanium Taq DNA polymerase, 1X Titanium Taq PCR buffer (Clontech Laboratories, Palo Alto, CA, USA) and 1 μL of a 10 times diluted DNA extract. PCR conditions were as follows: initial denaturation of 2 min at 94 °C followed by 30 cycles of 45 s at 94 °C, 45 s at 59 °C and 45 s at 72 °C. After resolving the amplicons by agarose gel electrophoresis, amplicons within the appropriate size range (~750–1000 bp) were cut from the gel and purified using the Qiaquick gel extraction kit (Qiagen, Hamburg, Germany). Purified dsDNA amplicons were quantified using the Qubit fluorometer (Invitrogen) and pooled in equimolar quantities of 1.00E+10 molecules per sample, resulting in two amplicon libraries, each representing one of the two PCR replicates. The quality of the amplicon libraries was assessed using an Agilent Bioanalyzer 2100 and high-sensitivity DNA chip (Agilent Technologies, Waldbronn, Germany). Each amplicon library was loaded onto 1/8th of a 454 Pico Titer Plate (PTP). Pyrosequencing was performed using the Roche GS-FLX PLUS instrument and Titanium chemistry according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany).

Soil analyses

The same soil samples as for mycorrhizal analyses were further used for more detailed soil chemical analyses. After collection, samples were stored in a watertight bag in a refrigerator at 5 °C for a maximum of 1 month, and thoroughly homogenized just prior to analysis. Soil organic content was determined by percentage weight lost after combustion in a muffle oven, and soil pH was determined using a glass electrode. Soil extractable N was determined using a 1 M KCl-extraction of $NH4^+$ and $NO3^-$ and subsequent colorimetrical analysis using a segmented autoflow analyser (Robertson *et al.* 1999; Skalar, Breda, The Netherlands). Finally, soil extractable P was determined using Olson-P extraction and the extracts were colorimetrically analysed using the molybdenum blue method (Lajtha *et al.* 1999). Percentage soil moisture was determined using a hand-held Hydrosense Soil Water Content Measure System in exactly the same plots where the soil samples were taken. For each plot, 10 replicates were taken at the same time when samples for mycorrhizal analysis were taken. Given that spatial patterns of soil moisture content are known to be fairly constant through time (Diez 2007), we considered one measure to be sufficient for the purpose of this study.

Seed germination experiment

To investigate whether seed germination varied among populations, a seed germination experiment was established in the same subset of nine populations using the modified seed package method of Rasmussen & Whigham (1993). When seeds were ripe (beginning of June 2013), fruits were harvested from the biggest population in the study area to exclude potential population effects that may create differences in seed quality. After harvesting, all seeds were merged, and subsamples from this seed pool were immediately buried in seed packages. Per seed package, approximately 250 seeds were placed within a square of 53-μm mesh phytoplankton netting, enclosed within a Polaroid slide mount. Packages were buried into the soil at each corner of the same 1-m^2 plots where roots and soil samples were taken. At each corner, four seed packets were placed vertically in the ground, leading to a total of $16 \times 9 = 144$ seed packages that were left in the ground for about 1 year.

In May 2014, seed packages were retrieved, gently washed and maintained moist in paper towel for 1 day until examination. Packages were then rinsed with tap water, opened with a mini-knife and rigorously checked under a dissecting microscope for germination. As orchid seed germination stages can be variable (Ramsay *et al.* 1986), germination was considered to have occurred when clear signs of mycorrhiza formation were present and the leaf primordia had developed (stage 3 sensu Ramsay *et al.* (1986)). For each package, seed germination was determined by inspecting successful development of protocorms.

Data analysis

Fungal diversity and community composition. Sequences obtained from the 454 pyrosequencing run were assigned to the appropriate sample based on both barcode and primer sequences, allowing zero discrepancies, and were subsequently trimmed from the barcodes and primers using CUTADAPT 1.0 (Martin 2011). Sequences were trimmed based on a minimum Phred score of 30 (base call accuracy of 99.9%) averaged over a 50-bp moving window and sequences with ambiguous base calls or homopolymers longer than eight nucleotides were rejected, as were chimeric sequences detected by the UCHIME chimera detection program (*de novo* algorithm) (Edgar *et al.* 2011). Sequences which passed all quality control procedures were used as the

basis for all further analyses. Minimum and maximum sequence lengths were set to 200 and 500 nucleotides, respectively. For further analysis, sequence data obtained for both PCR replicates were combined for each sample.

Operational taxonomic units (OTUs) were determined using UPARSE (Edgar 2013), wherein sequences exceeding 97% sequence homology were clustered into the same OTU. OTUs representing only one sequence in the whole data set (global singletons) were removed from further analysis as it has been shown that this improves the accuracy of diversity estimates (Ihrmark *et al.* 2012; Waud *et al.* 2014). The remaining OTUs were assigned taxonomic identities to the highest taxonomic rank possible/family level based on BLAST (Altschul *et al.* 1990) results of representative sequences (as indicated by UPARSE) using GenBank (Benson *et al.* 2008), including uncultured/environmental entries. Finally, OTUs were manually screened for possible orchid-associating mycorrhizal families based on the data provided in Table 12.1 in Dearnaley *et al.* (2012). Only OTUs corresponding to known orchid-associating mycorrhizal families were retained for further analysis.

A phylogenetic analysis was performed with the OTUs that were assigned to Sebacinales to determine their identity to either Sebacinales clade A (or Sebacinaceae sensu *stricto*) or Sebacinales clade B (or Serendipitaceae) (Weiß *et al.* 2004). ITS sequence data of representatives of both clades and Auriculariaceae outgroup taxa were downloaded from GenBank and aligned with the Sebacinales OTUs using the MAFFT v.6.814b alignment tool (Katoh *et al.* 2002) implemented in GENEIOUS PRO v5.5.6 (Biomatters, New Zealand). Poorly aligned regions were trimmed using the heuristic 'automated1' method implemented in TRIMAL v1.3 (Capella-Gutierrez *et al.* 2009). The GTR+I+G substitution model was selected to best fit the data with JMODETEST 2.1.5 (Darriba *et al.* 2012) using the Akaike information criterion. Phylogenetic analysis was performed under the maximum likelihood optimality criterion with RAXML v7.2.8 (Stamatakis 2006). Clade support was estimated by nonparametric bootstrap analyses on 500 pseudo-replicate data sets.

Mycorrhizal diversity, seed germination and population growth. To assess whether fungal diversity differed between root and soil samples and between populations, a generalized linear model with a Poisson distribution and a log-link function was used. The number of putative orchid mycorrhizal OTUs per sample was used as the dependent variable and population, sample type (root vs. soil) and their interaction as fixed factor. We used a related-samples Wilcoxon signed rank test to investigate whether the average similarity (calculated as Jaccard's similarity index) in mycorrhizal communities across populations differed between root and soil samples. A Mantel test (Mantel 1967) was used to test the hypothesis that similarity in mycorrhizal communities among populations decreased with increasing distance between populations. Nonmetric multidimensional scaling (NMDS) using the VEGAN package (Oksanen *et al.* 2013) in R (R Core Development Team 2013) was used to visualize differences in mycorrhizal communities across populations, and between soil and root samples. Partial canonical correspondence analysis (pCCA) using the ordistep function in the VEGAN package (Oksanen *et al.* 2013) was used to see which of the soil variables significantly explained variation in mycorrhizal communities. Significance of the final model was tested using the ANOVA.CCA function in VEGAN. Finally, we related population size, population growth rate and percentage seed germination to fungal community composition (scores of the first and second NMDS axis) using Spearman's rank correlation analysis. The same analysis was used to see whether percentage seed germination was affected by any of the measured soil variables. Permutational multivariate analysis of variance (PERMANOVA) using the adonis function in the VEGAN package (Oksanen *et al.* 2013) was used to test the hypothesis that fungal community composition differed significantly between populations with and without seed germination.

Results

Changes in population size

Within the study area, 28 populations were found in spring 2003 (Fig. S1, Supporting Information). *Neottia ovata* occurred predominantly in low-altitude, species-rich mixed forests, in which ash (*Fraxinus excelsior*), poplar (*Populus canadensis*), wild cherry (*Prunus avium*), sycamore (*Acer pseudoplatanus*), hornbeam (*Carpinus betulus*) and beech (*Fagus sylvatica*) were the most common tree species. No populations were found at higher altitudes or in neighbouring grasslands. At the sites in which *N. ovata* occurred, the soils were relatively wet, loamy and calcareous. Populations varied greatly in size, ranging from one single plant to more than 2500 plants in the largest population. Sixteen (57.1%) populations had decreased in size between 2003 and 2013, one of which went extinct. Eleven (39.3%) populations had expanded their size, and one (3.6%) population did not change in size (Fig. 1). The average growth rate was −0.01 (±0.04), indicating that across all populations within the study area, the species had decreased in size by 1%. No new populations were discovered during the 2013 survey.

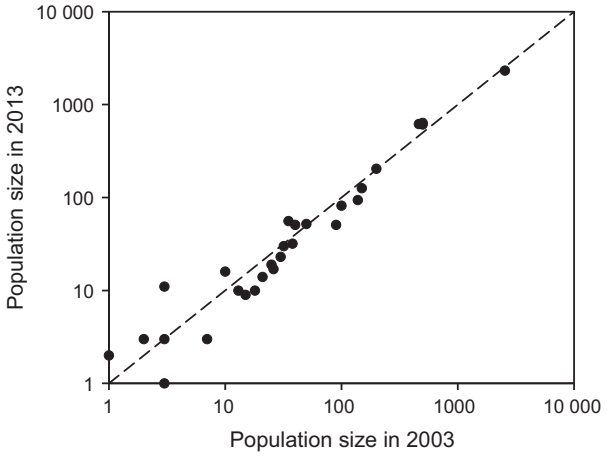

Fig. 1 Changes in population size between 2003 and 2013 in 28 *Neottia ovata* populations in eastern Belgium. Points above the dashed line represent populations with increased population sizes, whereas points under this line represent populations that have decreased in size. One population went extinct and was not depicted on the graph.

Mycorrhizal diversity

The quality-filtered pyrosequencing data set containing both the root and soil samples comprised 512 OTUs (74568 sequences), of which 68 (44001 sequences – 59.01%) could be assigned to putatively orchid mycorrhizal OTUs (Table S1, Supporting Information). Most orchid mycorrhizal sequences were related to members of the Sebacinales, comprising members of both clade A (13 OTUs – 5380 sequences) and B (11 OTUs – 23702 sequences) (Weiß *et al.* 2004; Fig. S2, Supporting Information), Tulasnellaceae (four OTUs – 4902 sequences), Ceratobasidiaceae (five OTUs – 3899 sequences) and Thelephoraceae (13 OTUs – 3737 sequences). Besides, there were some representatives of the genera *Mycena* (three OTUs – 1638 sequences), *Clavulina* (one OTU, 371 sequences), *Hymenogaster* (four OTUs – 86 sequences), *Psathyrella* (four OTUs – 129 sequences) and *Inocybe* (six OTUs – 157 sequences). Representative sequences for each mycorrhizal OTU found in this study were submitted in GenBank under the Accession Numbers KR082155-KR082222. Twenty-one orchid mycorrhizal OTUs occurred exclusively in roots, 25 OTUs occurred solely in soil, and 22 were observed in both the soil and roots, indicating that the mycorrhizal communities in the roots shared 32% of the total number of species with those in the soil. The nonmycorrhizal OTUs were mostly Basidiomycota and Ascomycota, but occasionally, representatives of Glomeromycota (three OTUs) and Chytridiomycota (two OTUs) were also observed (data not shown). These fungi were, however, not further considered in subsequent analyses. Although the total number of OTUs observed in soil and root samples did not differ substantially (368 and 345 OTUs, respectively), fungal diversity per individual sample was significantly higher ($\chi^2 = 72.51$, $P < 0.001$) in soil than that in root samples (average number of OTUs per sample: 47.0 and 25.4, respectively).

Variation in mycorrhizal communities across populations

Across all root samples and populations, members of the Sebacinales, Thelephoraceae, Ceratobasidiaceae and Tulasnellaceae comprised 16, 8, 5 and 4 OTUs, respectively. Sequences related to members of Sebacinales and Thelephoraceae were most prevalent in root samples (Fig. 2a, Fig. S3a, Supporting Information). All other fungi were only sporadically observed in the roots. Representatives of Sebacinales were present in all populations (Fig. 3), whereas members of Ceratobasidiaceae, Thelephoraceae and Tulasnellaceae were absent in four, one and one populations, respectively. Within the Sebacinales, members of clade B were retrieved in the roots in each population, whereas members of clade A were only found in four populations (Fig. S3a, Supporting Information). The number of orchid mycorrhizal OTUs

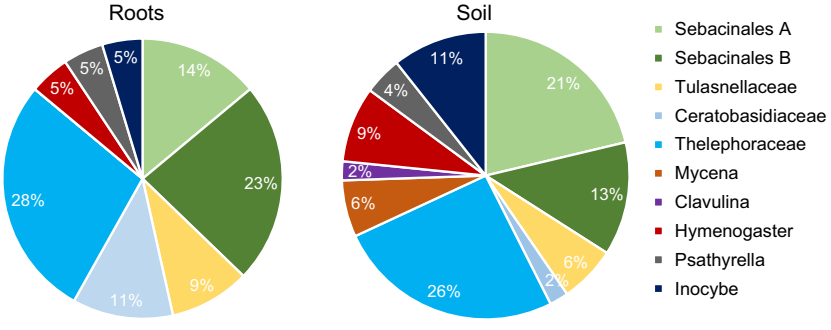

Fig. 2 Incidence of putative orchid mycorrhizal fungi in both roots and soil samples collected in nine populations of the forest orchid *Neottia ovata* in eastern Belgium.

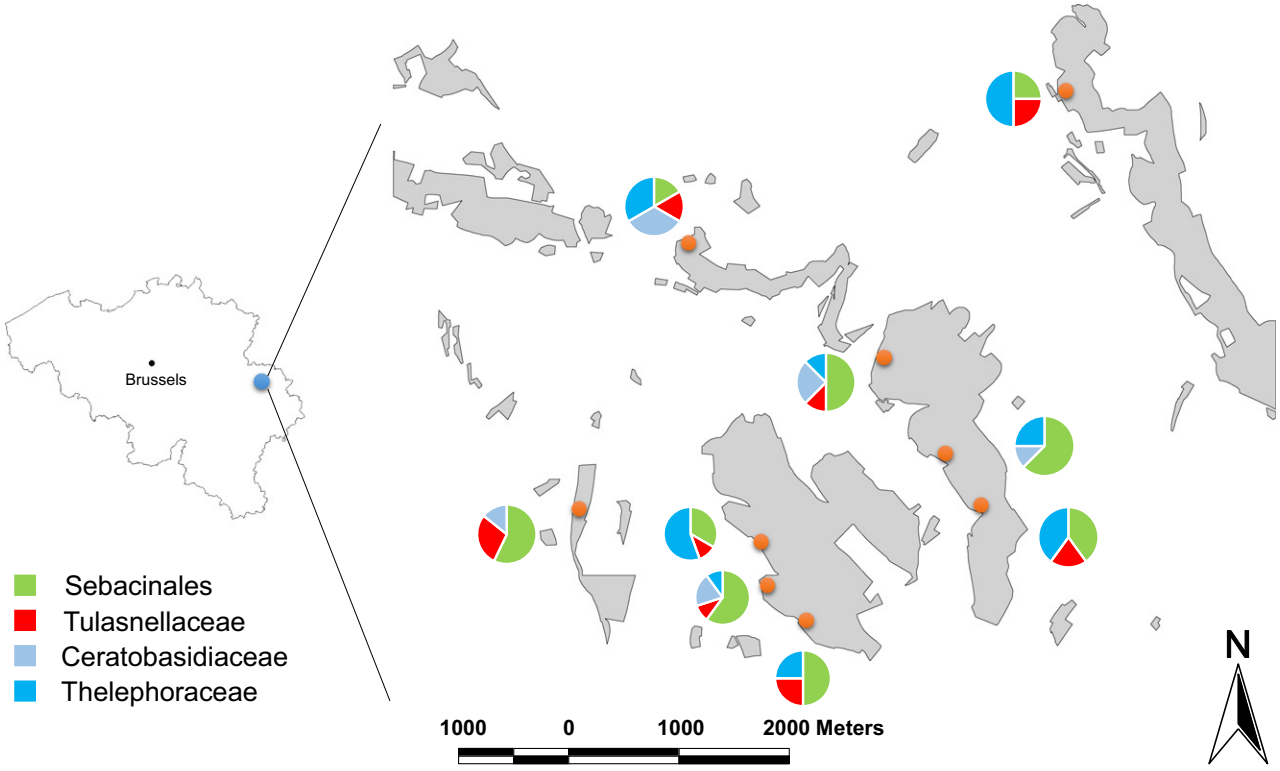

Fig. 3 Frequency distribution of the four orchid mycorrhizal families (Tulasnellaceae, Ceratobasidiaceae, Sebacinaceae and Thelephoraceae) obtained from the roots of 45 individuals collected in nine populations (five individuals per population) of *Neottia ovata* in eastern Belgium. Forest habitat is presented in grey, whereas agricultural land and urban zones are represented in white.

per individual plant varied between one and seven (average: 2.84) and most plants associated with fungi of several families simultaneously (data not shown).

Individual soil samples contained a significantly ($t = -3.71$, $P < 0.001$) higher number of orchid mycorrhizal OTUs than root samples (average number of orchid mycorrhizal OTUs: 4.46 (range: 1–8) and 2.84 (range: 1–7), respectively), but the total number of orchid mycorrhizal OTUs that were identified in each population did not differ significantly between root and soil samples ($t = 1.35$, $P > 0.05$). In the soil samples, members of the Sebacinales were present in each population, but in contrast to root samples, where Sebacinales of clade B were predominant, Sebacinales fungi of clade A showed the highest diversity in the soil (Fig. 2b). Members of the Thelephoraceae and Tulasnellaceae were recovered in six and three populations, respectively, whereas members of the Ceratobasidiaceae were virtually undetected in soil samples (Fig. 2b, Fig. S3b, Supporting Information).

The overall similarity in mycorrhizal communities between populations was significantly higher (Wilcoxon signed rank test statistic: −3.83, $P < 0.001$) for root than for soil samples (average Jaccard index: 0.172 and 0.047, respectively), indicating strong differences in local community structure in the soil. There was also no significant ($P > 0.05$) relationship between pairwise similarity in mycorrhizal communities and geographic distances ($r_M = 0.071$ and 0.067 for root and soil samples, respectively), indicating that nearby populations not necessarily harboured similar mycorrhizal communities. Nonetheless, the results of the nonmetric multidimensional scaling ordination analysis showed that for most populations, except for population 27, there was some concordance in mycorrhizal community composition between root and soil samples (Fig. S4, Supporting Information). Population size and growth were also not significantly ($P > 0.05$) related to mycorrhizal diversity ($r_s = 0.15$ and 0.37, respectively).

Mycorrhizal communities and soil properties

Soil pH in individual plots varied between 5.74 and 7.54 (average ± SD: 7.01 ± 0.41). Soil extractable nutrients varied considerably across populations, constituting a gradient in soil N ($NH4^+ + NO3^-$) ranging from 41.99 mg N/kg soil to 194.2 mg N/kg soil (average 81.9 mg N/kg soil, SD 32.2), whereas soil P varied

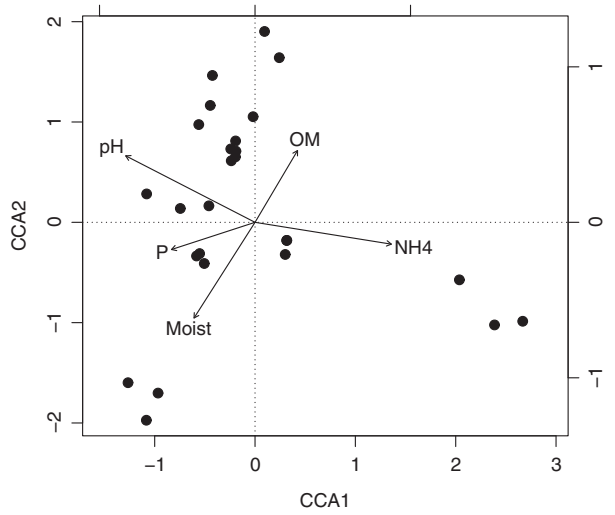

Fig. 4 Relation between mycorrhizal communities and soil variables (soil moisture content, organic matter (OM), pH, phosphorus (P), nitrate (NO_3) and ammonium (NH_4) content) in 27 soil samples collected in nine populations (three samples per population) of *Neottia ovata*.

between 3.1 mg P/kg soil and 31.6 mg P/kg soil (average 10.9 mg P/kg soil, SD 7.36). Percentage soil moisture varied between 15.9% and 36.5%, whereas percentage organic content varied between 8.8% and 28.6%. Partial CCA analysis showed that mycorrhizal communities were significantly (pseudo-$F = 2.16$, $P < 0.001$) related to soil moisture content, pH, $NH4^+$, P and organic matter, but not to $NO3^-$ (Fig. 4).

Seed germination

The percentage of seed packages containing protocorms varied between 0 and 33.3 (average: 8.7%). In five populations, no germination was observed. Seed germination was significantly ($P < 0.01$) related to population size and relative population growth rate ($r_s = 0.86$ and 0.84, respectively) (Fig. 5a,b), but not to fungal diversity ($r_s = 0.19$, $P > 0.05$) or fungal community composition ($r_s = 0.11$ and -0.37, $P > 0.05$). We also found no significant difference in fungal community composition between populations with and without seed germination (pseudo-$F = 0.73$; $P > 0.05$), but seed germination significantly increased with increasing soil moisture content ($r_s = 0.79$, $P = 0.01$) and soil pH ($r_s = 0.77$, $P = 0.02$) (Fig. 5c,d).

Discussion

Mycorrhizal communities associating with Neottia ovata

In this study, we applied 454 amplicon pyrosequencing to assess the mycorrhizal communities associating with the terrestrial forest herb *N. ovata*. Our results clearly illustrate that the dominant fungi associating with *N. ovata* were members of the Sebacinales, although associations with members of the Tulasnellaceae and Ceratobasidiaceae were also observed. These results strongly agree with the observations of Oja *et al.* (2015) and Těšitelová *et al.* (2015), who also found that Sebaci-

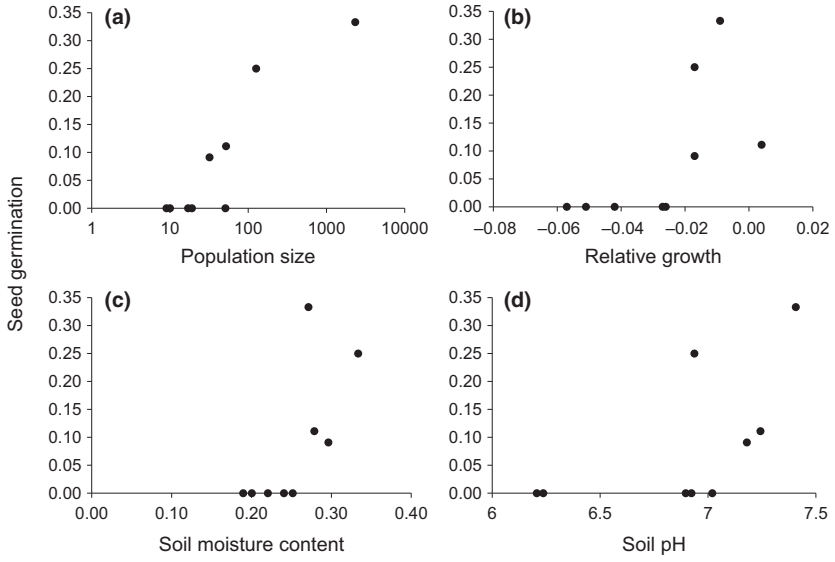

Fig. 5 Relationships between seed germination, population characteristics and soil variables in nine populations of the forest orchid *Neottia ovata*. Seed germination was determined as the proportion of seed packages containing protocorms. Population characteristics were (a) population size and (b) population growth rates. The population growth rate was calculated as the difference in population size in 2003 and 2013 divided by the time interval. Significant soil variables were (c) soil moisture content and (d) soil pH.

nales were the prime associates of *N. ovata*. Sampling four different locations in Estonia, Oja *et al.* (2015) found 15, five and six unique OTUs belonging to the Sebacinales, Ceratobasidiaceae and Tulasnellaceae, respectively. These results match almost perfectly with the number of OTUs observed here (16, 5 and 4 OTUs). Těšitelová *et al.* (2015) sampled 28 populations of *N. ovata* across Europe and found 28 Sebacinales OTUs, nine Tulasnellaceae OTUs and three Ceratobasidiaceae OTUs. Bidartondo *et al.* (2004) showed that Sebacinales were the major fungi associating with the related achlorophyllous *Neottia nidus-avis*. Similarly, Selosse *et al.* (2002) and McKendrick *et al.* (2002) found that sebacinoid basidiomycetes were the dominant fungi associating with *N. nidus-avis*. These results thus indicate that *N. ovata*, which is a photosynthetic orchid, associates with fungi also associate with its nonphotosynthetic congener *N. nidus-avis*. Interestingly, the diversity of Sebacinales fungi of clade B was highest in the roots, whereas Sebacinales fungi of clade A showed the highest diversity in the soil. These results match with the ecology of both clades as fungi of clade B have been shown to be mycorrhizal fungi in Ericaceae, green orchids and liverworts, whereas fungi of clade A are mainly ectomycorrhizal fungi that associate with trees and mycoheterotrophic orchids (Selosse *et al.* 2007; Weiß *et al.* 2011).

Besides members of the Sebacinales, we found a large number of ectomycorrhizal fungi of the Thelephoraceae, in both root and soil samples. These fungi have previously been shown to associate with other orchids that typically occur in forests. For example, McCormick *et al.* (2009) showed that the major fungi associating with the mycoheterotrophic *Corallorhiza odonthorhiza* were *Tomentella* fungi. Bidartondo & Read (2008) further showed that in the forest orchids *Cephalanthera damasonium* and *C. longifolia,* several members of the Thelephoraceae, including *Tomentella* and *Pseudotomentella*, were found in germinating seeds. Besides, several *Inocybe* and *Hymenogaster* fungi were found associating with established *N. ovata* plants. Overall, these results indicate that *N. ovata* associates with a wide diversity of fungi that are found in other orchids that typically occur in forest habitats. The presence of ectomycorrhizal fungi has been recorded before in some partially mycoheterotrophic orchids (Bidartondo *et al.* 2004; Abadie *et al.* 2006; Bidartondo & Read 2008), suggesting that these fungi contribute to the nutrient budget of these plants. Whether they contribute of the nutrient budget of *N. ovata*, however, warrants further investigation. Previous studies using stable isotope analyses have shown that carbon nutrition of *N. ovata* may be partly or fully autotrophic depending on the study site (Gebauer & Meyer 2003; Těšitelová *et al.* 2015).

Partitioning of mycorrhizal diversity across populations

We further showed that orchid mycorrhizal communities varied substantially across populations and that similarity in mycorrhizal communities was higher in the roots than that in the soil, suggesting that the orchid tends to associate with a subset of the available potentially mycorrhizal fungi. Nonetheless, the overall similarity index was low, although adjacent populations within the same forest complex tended to have higher similarities in mycorrhizal communities. Mantel tests further showed that there was no significant relationship between pairwise similarity in mycorrhizal communities and geographic distances. These results are in line with those of Pandey *et al.* (2013), who also found large variation in mycorrhizal communities between populations in the terrestrial orchid *Piperia yadonii*. Similarly, Ramsay *et al.* (1987), using morphological observations, reported pronounced variation in mycorrhizal associations across populations and habitats in several photosynthetic orchids in Australia. Jacquemyn *et al.* (2014) showed that in highly diverse Mediterranean grassland, orchid mycorrhizal communities differed substantially among sites, suggesting that local environmental conditions may impact on orchid mycorrhizal communities and that fungi may have a patchy distribution across the landscape. However, these results contrast with findings of Selosse *et al.* (2002), who showed that populations of the achlorophyllous *N. nidus-avis* often shared the same or very closely related fungi. Nonetheless, members of the Sebacinaceae were present at each site, indicating that these fungi are most likely the prime associates of *N. ovata*. What determines mycorrhizal variation remains unclear at the moment and needs further exploration, but it is reasonable to assume that differences in tree composition largely determine ectomycorrhizal communities. Whereas some sites, especially those on wetter soils, were dominated by ash, wild cherry and poplar, sites occurring on drier soils were mainly dominated by beech, hornbeam and sycamore.

Population decline and seed germination

The majority of the *N. ovata* populations studied here showed a slow and gradual decline in population size across the sampling period (2003–2013), and one population even went extinct. Only a small number of populations were able to expand in size, but increases in population size were in most cases limited. These results correspond with general observations that the species is slowly declining in the rest of Flanders (Meeuwis 2006). Interestingly, our results showed that variation in population size was not related to fungal

community composition. Moreover, our seed germination experiments further revealed that recruitment from seeds was limited, particularly in small populations or populations showing a decline in size. These results corroborate earlier findings of Tamm (1972) and Rasmussen (1995), who also noted that recruitment from seeds is very limited in *N. ovata*. Low germination rates have also been reported in several species of the genus *Epipactis* (Těšitelová *et al.* 2012). McKendrick *et al.* (2002), on the other hand, found high germination rates in *N. nidus-avis*, particularly at locations where the species was abundantly present.

Interestingly, we found no relationship between seed germination and fungal community composition. The lack of a relationship between fungal community composition and seed germination does not necessarily provide direct evidence that other factors than the presence of suitable mycorrhizal fungi had a more important impact on seed germination in the studied populations. For example, it might be that some crucial fungi were limiting in some soils, but occurred at frequencies too low to be detected by our sampling. However, the strong relationships between soil variables and seed germination suggest the opposite and support the results of earlier studies that have shown strong relationships between abiotic conditions and seed germination. Diez (2007), for example, showed that seed germination in the forest orchid *Goodyera pubescens* was significantly affected by distance to adult plants and abiotic soil conditions. In particular, high seed germination was observed at places with high soil moisture content, higher organic content and lower pH. In *N. ovata*, most populations that lacked seed germination occurred on drier soils with low pH, whereas populations where seed germination was observed mostly occurred in wetter soils with higher pH. The significant relationship between percentage seed germination and soil moisture content and soil pH supports these findings. These results further suggest that with increasing acidification and desiccation of the soil, seedling recruitment will become more and more unlikely in future, making long-term survival of most of these existing populations questionable. Moreover, patchy distributions of mycorrhizal fungi and distance-dependent seed germination may further restrict seed germination, particularly in small populations.

Lack of recruitment may be further explained by the consistently low fruit set in small populations of the studied *N. ovata* populations (Brys *et al.* 2008; Jacquemyn *et al.* 2009). Populations that contained <50 flowering plants produced on average less than five fruits per plant (average fruit set: <0.2). Low fruit and seed set combined with low seed germination rates resulting from deteriorating environmental conditions are therefore the most likely reasons why most small populations decreased in size during the last 10 years. However, only one of the investigated populations went extinct during the period of monitoring, and this population consisted of one single plant in 2003. The low extinction rates can be explained by the fact that death rates in *N. ovata* are extremely low and that individuals can survive during long periods of time (Tamm 1972). Long-term demographic monitoring of several plots in Sweden has shown that most individuals of this species observed for the first time in 1944 were still present in 1972, indicating that individuals can easily reach an age of 28 and more. Because temporal changes in abundance were related to the ability of seeds to germinate and the long lifespan allows the species to survive for long periods of time, the observed overall decline in population size is therefore mainly attributable to the lack of establishment of new recruits and sporadic disappearance of established plants. With time, however, these populations, especially the very small ones (i.e. populations containing <10 plants), are bound to go extinct, illustrating why a so-called extinction debt may persist for many years (Lindborg & Eriksson 2004; Vellend *et al.* 2006).

To conclude, our results showed that desiccation and deterioration of local habitat conditions rather than differences in fungal community composition were the most likely forces driving population decline and that mycorrhizal symbioses most likely did not play an important role in determining the population dynamics and long-term viability of this long-lived orchid species.

Acknowledgements

RB and MW were supported by the European Research Council (ERC starting grant 260601 – MYCASOR awarded to HJ). We would like to thank four anonymous reviewers and the Subject Editor for detailed comments and suggestions that significantly improved the quality of the manuscript.

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H.J. and R.B. designed the study and performed the field work. MW performed the molecular analyses. H.J., V.S.F.T.M. and M.W. analyzed the data. H.J. wrote the first draft of the paper. All authors read, edited and approved the final manuscript.

Data accessibility

Raw sequences were deposited on the NCBI database (BioProject ID: PRJNA280573 – Biosamples: SUB895222). Representative sequences for each of the orchid mycorrhizal OTUs identified in this study were submitted to GenBank under the Accession nos KR082155-KR082222. Coordinates and population sizes of all studied *Neottia ovata* populations as well as data on seed germination and soil conditions were deposited on the Dryad databank (doi:10.5061/dryad.6tj39). The Sebacinales alignment and the resulting tree file were also deposited.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Location of the study area and overview of the studied populations within the study area.

Fig. S2 Highest likelihood tree obtained with the Maximum Likelihood analyses of the Sebacinales data set.

Fig. S3 Overview of the number of orchid mycorrhizal OTUs retrieved in (a) the roots and (b) the soil across nine populations of *Neottia ovata* in Belgium.

Fig. S4 **N**onmetric multidimensional scaling (NMDS) plot of mycorrhizal fungi detected in root and soil samples across nine populations of the forest orchid *Neottia ovata*.

Table S1 List of operational taxonomic units (OTUs) corresponding to orchid-associating mycorrhizal families discovered in this study.