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Abundance and distribution of *Corallorhiza odontorhiza* reflect variations in climate and ectomycorrhizae

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Abstract. The abundance and reproductive activity of orchids have been linked to variations in weather conditions, but few investigators have examined the relationships between orchid flowering dynamics and the distribution and abundance of mycorrhizal fungi. We quantified the abundance of flowering individuals of *Corallorhiza odontorhiza*, a mycoheterotrophic orchid, over a 14-year period and mapped the distribution of individuals in six of the 14 years. For two seasons, we conducted intensive and extensive studies of the mycorrhizal fungi that were associated with *C. odontorhiza*. The annual abundance of flowering plants was statistically related to growing-season precipitation and winter temperature, and the distribution of individuals within the study plot was related to the abundance and distribution of appropriate host fungi. We used DNA sequencing to identify ectomycorrhizal root tips that hosted *Tomentella* fungi that could potentially support *C. odontorhiza*. We found that *Tomentella* spp. were distributed throughout the study plot and on all ectomycorrhizal tree species, including in areas that have historically supported few or no orchids. However, there were fewer ectomycorrhizal trees, total ectomycorrhizal root tips, and root tips hosting *Tomentella* spp. in areas with few or no orchids compared to areas with abundant orchids. Furthermore, one *Tomentella* taxon dominated in *C. odontorhiza* rhizomes but was never found except immediately adjacent to *C. odontorhiza* plants. This suggests that abundance of flowering *C. odontorhiza* reflects both the presence of "preferred" taxa and abundance of appropriate host fungi associated with ectomycorrhizal roots. Results of this research provide the first indication that the relationship between plants and mycorrhizal fungi may be influenced by both the relative abundance of fungi that are sufficient to support orchid growth and by the presence of particular fungal types that are especially good at supporting orchid growth.

Key words: Corallorhiza odontorhiza; *ECM; ectomycorrhiza; mycoheterotrophic; orchid; Orchidaceae; Smithsonian Environmental Research Center;* Tomentella.

Introduction

One of the central goals of ecology is to understand the abundance, diversity, and distribution of organisms and to relate those to interactions among organisms and their environment (Agrawal et al. 2007). For any plant species, it is widely understood that abundance and distribution are determined by a combination of abiotic and biotic factors. Abiotic factors such as winter temperatures and rainfall have been found to have strong effects on plant survival (e.g., Pfeifer et al. 2006), flowering (e.g., Augspurger 1980, Primack and Stacy 1998, Pfeifer et al. 2006), and dormancy (e.g., Shefferson et al. 2001). Biotic factors such as herbivores and pathogens can also have substantial effects on plant performance (e.g., Whigham 2004). Both abiotic and biotic factors undoubtedly influence distribution at the scale of species ranges.

Several studies have identified relationships between environmental stress and the dynamics of orchid populations. The relationship between flowering and climatological variables has received particular attention (e.g., Wells and Cox 1991, Janečková and Kindlmann 2002, Gorchakovskii and Igosheva 2003, Pfeifer et al. 2006, Reddoch and Reddoch 2007), and investigators have examined the relationships between climatological conditions and dormancy in terrestrial orchids (e.g., Shefferson et al. 2001, Tali 2002, Kéry et al. 2005, Pfeifer et al. 2006). The general pattern that has been observed is that environmental stress affects plant performance and population dynamics through short- and long-term effects on mortality, dormancy, and reproduction.

Plant performance and distribution are often intertwined. Plants rarely persist in locations where they consistently perform poorly. Flowering and subsequent

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seed set are aspects of performance that clearly impact plant distribution through seed rain, but factors affecting performance can also affect apparent plant distribution. In some plants, especially mycoheterotrophic plants and geophytes that experience whole-plant dormancy, a phenomenon in which plants do not emerge above ground in one or more years (Lesica and Steele 1994, Shefferson et al. 2003), plant distribution can be most reliably observed when the plant is flowering. Dormancy is used here, in accordance with recent ecological literature, in reference to plants that are difficult to see in a population survey. It does not purport any physiological meaning such as cessation of growth or reduced metabolism. When vegetative, these plants may not emerge above ground, so their distribution cannot be easily noted. Similarly, little is known about seedling establishment in these plants, as seedlings may not be visible until they flower. The phenomenon of whole-plant dormancy is especially common in the Orchidaceae (Kull 2002, Lesica and Crone 2007) and is brought to the extreme in many mycoheterotrophic plants that only emerge above ground when flowering. In these plants, factors affecting flowering appear inseparable from factors affecting plant distribution. Disentangling these two factors requires examining both over many years or exhaustive, often prohibitively disruptive, belowground surveys. Perhaps because of this, factors affecting seedling establishment and resulting plant distribution within orchid populations are poorly understood (e.g., Jacquemyn et al. 2007).

Spatially heterogeneous abiotic factors such as soil composition and fertility have clear effects on plant distribution within populations. However, soil microbes, both influenced by soil composition and independent of it, can be at least as important as abiotic and macroscopic biotic factors in determining plant distribution (e.g., van der Heijden et al. 1998, Klironomos 2002). For example, the distribution of pathogens (Van der Putten et al. 1993, Packer and Clay 2000, Klironomos 2002, Reynolds et al. 2003) and mycorrhizal fungi (e.g., Bever 1994, van der Heijden et al. 1998) in the soil have been shown to have strong effects on plant community composition. Nara (2006) found that the distribution of ectomycorrhizal fungi, as a group, and their host shrubs affected the distribution of a community of ectomycorrhizal shrubs in a primary successional habitat. However, only pathogen distribution (Packer and Clay 2000) has been linked to the distribution of particular plant species. Although Diez (2007) and Jacquemyn et al. (2007) noted that seed germination is highest near parent plants, implying but not demonstrating an effect of fungal distribution on seed germination, the distribution of particular mycorrhizal fungi has not previously been related to plant distribution.

Microbes are increasingly being recognized as important drivers of plant distribution as researchers apply molecular techniques to the study of plant community ecology (Reynolds et al. 2003). The distribution and abundance of mycorrhizal fungi should be especially important for terrestrial orchids, as all species have obligate relationships with mycorrhizal fungi, which they digest for nutrients, at one or more life history stages (Rasmussen 1995). Orchid protocorms, the life history stage between seed germination and a seedling, are obligate mycoheterotrophs (Leake 1994, Whigham et al. 2008), meaning they obtain most, if not all, carbon heterotrophically from associated fungi, and isotopic analyses have shown that many studied orchids obtain at least some carbon and other nutrients from fungi throughout their lives (Gebauer and Meyer 2003, Julou et al. 2005, Girlanda et al. 2006, Zimmer et al. 2007). Furthermore, many orchids require specific mycorrhizal fungi, sometimes a single species, to germinate and grow (e.g., McCormick et al. 2004, Bidartondo and Read 2008). This is especially true of mycoheterotrophic orchids, of which all studied species have been found to have specific fungal requirements (reviewed in Taylor et al. 2002). Many green plants also have greater specificity in their use of mycorrhizal fungi than previously appreciated and may have an ecological requirement for specific fungi in some environments or communities (e.g., Van der Putten et al. 1993, Bever et al. 1997, De Deyn et al. 2003), but with both specific fungal requirements and mycorrhizal associations that are obligate for the plants but not for their fungi, orchids may be extremely likely to have distributions that are driven, in part, by the distribution of the fungi on which they depend.

In this paper we explore factors that influence the flowering, performance, and distribution of *Corallorhiza odontorhiza* var. *odontorhiza*, a widely distributed mycoheterotrophic terrestrial orchid. We have three objectives. First, we use data from a long-term permanent study plot to test the hypothesis that climatological factors controlled the annual abundance of flowering individuals. Second, we mapped the distribution of flowering plants in the permanent plot over a six-year period to test the hypothesis that the distribution of individuals was nonrandom. Third, because *C. odontorhiza* associates with fungi belonging to a single genus (*Tomentella*), we tested the hypothesis that orchid distribution within the permanent plot was limited by the distribution of appropriate fungi and possibly specific ectomycorrhizal (ECM) hosts.

Methods

Study system

Corallorhiza odontorhiza var. *odontorhiza* (Willd.) Nutt. (hereafter referred to as *C. odontorhiza*) is a mycoheterotrophic perennial that remains completely below ground until the autumn, when an inflorescence emerges from a swollen pseudobulb-like structure that is part of an underground coralloid rhizome. Most flowers are cleistogamous and self-pollinated, but some inflorescences terminate in a few chasmogamous flowers (Freudenstein 1997). Ovaries begin to swell as soon as the inflorescence appears (Catling 1983), and seeds are

shed after the first frost (M. K. McCormick, *personal observation*).

As a mycoheterotrophic species, *C. odontorhiza* does not have a two-way exchange of nutrients. *C. odontorhiza* is strictly dependent on the fungi with which it associates, and all mycoheterotrophic orchids studied so far associate with basidiomycete or ascomycete fungi that form ectomycorrhizae with trees (e.g., Taylor and Bruns 1997, Selosse et al. 2002, Bidartondo et al. 2004, Bidartondo 2005). Like other mycoheterotrophic orchids, *C. odontorhiza* associates only with specific mycorrhizal fungi, being limited to members of a single fungal genus (McKendrick et al. 2000*b*, Taylor et al. 2002).

C. odontorhiza (Appendix A) is a common orchid throughout mixed hardwood and occasionally conifer forests of the eastern United States and is distributed from southern Minnesota and southern Maine in the north to Oklahoma and northern Florida in the south (Freudenstein 1997). For 14 years (1994–2007) we have monitored the only extensive population of *C. odontorhiza* at the Smithsonian Environmental Research Center (SERC) in Edgewater, Maryland, USA. The forest canopy in this part of the mixed-hardwood forest is composed of a mixture of *Liriodendron tulipifera* L., *Fagus grandifolia* Ehrh., *Quercus* spp., and *Carya* spp. (in order of dominance; G. Parker, *unpublished data*) with few understory trees, shrubs, and herbs (Appendix B). The tree composition and soil characteristics (Appendix B) of this site do not distinguish it from other SERC forest stands, but while flowering individuals of *C. odontorhiza* occur sporadically in some years in other areas of SERC forests, this is the only location that consistently supports a substantial population of *C. odontorhiza*.

Inflorescences, reproductive effort, and survival

During each of the 14 years of monitoring, we tallied all emergent inflorescences and counted the number of flowers and fruits (capsules) on each inflorescence in a permanent 50×80 m study plot that was divided into 5×5 m subplots. We used the annual abundance of flowering plants as a surrogate for actual population size, as it is not possible to determine the number of underground coralloid rhizomes. As we will demonstrate and as others have shown (e.g., Kéry et al. 2005, Shefferson et al. 2005), the abundance of flowering individuals is an underestimate of actual population size, as many nonflowering individuals remain undetected below ground.

We used stepwise regression techniques (Systat, Version 10.2, Chicago, Illinois, USA), with variables with significance $<\alpha = 0.15$ removed at each step, and Bonferroni corrections for multiple comparisons, to examine the relationship between climatological factors, the annual abundance of flowering individuals, and fruiting success. Climatological factors we considered were growing-season (April–September) rainfall, winter (October–March) rainfall, annual (October of the previous year to September of the flowering year) rainfall, mean growing-season temperature, and mean winter temperature. We considered the annual abundance of inflorescences both as the total number of plants emerging and also as a proportion of plants emerging the previous year. Similarly, we considered fruiting success both as the number of fruits per plant and as the proportion of flowers producing mature fruits. Precipitation data were obtained from long-term records compiled at the nearby SERC weather station. Monthly mean air temperatures measured at the Baltimore–Washington Airport were obtained from the National Climatic Data Center (NCDC; *available online*).[6] We compared precipitation and temperature during the winter and during the growing season with the number of individuals flowering in a given year and with the number of flowers produced per plant and fruit set per flower. Flowering and climatic data are given in Appendix C. Data presented in the text are given as means ± SE.

Twice during the 14 years we monitored survival of 100 randomly chosen plants in the study plot to determine the extent of among-year correlation that could be due to reflowering of individual plants. For 100 individuals from the 1996 flowering cohort, we monitored survival on a monthly basis from November 1996 to September 1997 by carefully digging around the flowering stalk of each plant to examine rhizomes. For the 2004 cohort, we monitored rhizomes twice, in November 2004 and July 2005. The flowering of surviving marked plants was noted for both cohorts in November.

Distribution of flowering plants

For six consecutive years, we mapped all flowering individuals to the nearest 2 cm by measuring their distance from the two nearest grid markers within each of the 5×5 m subplots. We used this mapping to determine how consistent plant distribution was among years. We used O-ring statistics (Programita; Wiegand and Maloney 2004) to examine the spatial pattern of plant flowering and its consistency within the study plot. O-ring statistics were calculated among pairs of adjacent years for the years during which plant distribution was mapped (1999–2004), with year 1 fixed and year 2 random, consistent with testing whether plant distribution in year 2 was attracted or repelled relative to the distribution of year-1 plants, and taking into account the background pattern of plant distribution (Wiegand and Maloney 2004).

After mapping flowering individuals, we identified 5×5 m subplots that consistently had dense aggregations of *C. odontorhiza* and others that consistently had few or none. We used this disparity of distribution to determine whether several abiotic variables (soil bulk density, soil

[6] ⟨http://www.ncdc.noaa.gov/⟩

pH, soil moisture, light) might be driving plant distribution within the study plot. During summer 2007 each abiotic variable was measured at 40 points within the main study plot. Twenty points were within 5 × 5 m subplots with historically and currently dense *C. odontorhiza*, and 20 were within subplots with few or no plants. Points in dense subplots were located randomly within the area of the subplot with abundant flowering *C. odontorhiza* (i.e., sample points were not specifically located adjacent to a flowering plant but were within an area with dense inflorescences). Points in sparse subplots were randomly located within the subplot. At each point we collected one 2.5 cm diameter, 5 cm deep soil core. Each soil core was weighed to determine bulk density, and the soil was homogenized, passed through a 2-mm sieve, and 5.00 g ± 0.01 g of soil was placed in a paper cup. We added 10 mL H_2O to each cup, mixed for 5 s, and allowed it to sit for 10 minutes before the pH was measured using a pH electrode (pHTestr 3+ Double Junction, Oakton Instruments, Vernon Hills, Illinois, USA). Approximately 10 g of the remaining soil was transferred to a preweighed and dried paper coin envelope and dried in a convection oven at 60°C for 48 h. Dried soils were then reweighed to determine soil moisture. At the same locations we also measured photosynthetically active radiation (PAR) at midday using mean photon flux density in the 400–700 nm waveband over a 1 m long ceptometer (Sunfleck, Model SF-80, Decagon Devices, Pullman, Washington, USA).

Ectomycorrhizal trees, root tips, and mycorrhizal fungi

We hypothesized that *C. odontorhiza* distribution would be governed by the availability of appropriate ectomycorrhizal (ECM) fungi and/or particular tree hosts. ECM fungi are known to be patchily distributed as a result of host preference (e.g., Ishida et al. 2007) or particular soil conditions (e.g., Tedersoo et al. 2003), and we also hypothesized that *Tomentella* spp., the host fungi for *C. odontorhiza*, might be affected by the distribution and identity of ectomycorrhizal root tips.

To test the hypothesis that *C. odontorhiza* distribution was affected by the distribution of appropriate fungi, we needed to find out where appropriate fungi were located and with which trees they were associated. To locate appropriate fungi we sampled ECM tree roots in two ways. During the summer of 2005, we sampled *C. odontorhiza* rhizomes and ECM roots with the goal of determining (1) the range of host fungi used by *C. odontorhiza*; (2) whether host fungi were associated with particular tree species; and (3) whether orchids in dense and sparse parts of the study plot associated with different fungi and/or different tree species. To determine the range of fungi that formed mycorrhizal associations with *C. odontorhiza* in this study plot, we used DNA sequencing to identify the fungal associates of 43 *C. odontorhiza* plants within the 50 × 80 m study plot for reference (GenBank accession numbers EU625803–EU625845). These orchids were randomly chosen from orchids that had flowered in 2004 and for which we were able to locate rhizomes. In summer 2005, we removed one soil core 5 cm deep × 5 cm diameter from 28 locations adjacent to marked plants that had flowered in 2004. Fourteen locations were in four groups of three to five in dense areas of the study plot, and 14 were in four groups of three to five in sparse areas (governed by plant distribution). We removed all ECM root tips from each of these cores by washing the soil gently through a 1-mm sieve and sorted the roots into morphotypes using a dissecting microscope (Brundrett et al. 1996).

During fall 2005 we used a different sampling scheme to address a slightly different but related question. Our goal with this sampling was to determine whether potential host fungi were distributed differently in dense and sparse parts of the study plot. We chose four (5 × 5 m) subplots in which to sample ECM roots, two of which had dense *C. odontorhiza*, and two had sparse or no historic or current *C. odontorhiza*. In each subplot we removed eight 2.5 cm diameter × 5 cm deep soil cores adjacent to randomly chosen plants or, in plots with no plants, in randomly chosen locations in each subplot. We removed all ECM root tips from each soil core and freeze dried and ground the remaining soil for DNA analysis. We also mapped all ECM trees in each target subplot and the surrounding eight subplots (an area 15 × 15 m) and measured their diameter at breast height. For trees <2 m tall we measured trunk diameter 5 cm above the soil surface. We limited measurements to trees with a trunk diameter >5 mm at 5 cm above the soil surface.

All ECM root tips were divided into morphotypes and counted. We then randomly chose two tips of each morphotype to identify both the fungus and the tree using a direct polymerase chain reaction (PCR) technique similar to that used by Iotti and Zambonelli (2006). We compared sequences for identical morphotypes obtained from different samples to determine whether morphotypes adequately separated different species. For each analyzed root tip, we cut the tip into four approximately 0.5-μm^3 sections using a sterile scalpel under a dissecting microscope and placed each section in a PCR tube with 9.5 μL of water. PCR amplification was performed directly on each section of root tip using 1.25 μL of each primer, 0.1 μL bovine serum albumin (BSA), and 12.5 μL of PCR Mastermix (Red Mix Plus PCR Mastermix, PGC Scientifics, Frederick, Maryland, USA). All samples were centrifuged ~10 s at ~10 000 rpm prior to amplification. PCR cycles were as follows: 1 min initial denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. This technique allowed us to process a greater number of samples at a lower cost than would have been possible without access to high-throughput DNA extraction facilities. Two sections of each tip were subjected to PCR amplification with the tomentelloid-selective PCR primer pair SSU1318-Tom (Taylor and McCormick 2008)/LSU-Tom2-AACTCGACTCTTT-

GAGAGCG, and two were amplified with a plant-specific primer pair ITS1-P (TTATCATTTAGAGGAAGGAG, developed by T. D. Bruns)/ITS4 (White et al. 1990) to allow us to identify the tree forming each ECM that included appropriate fungi. This approach allowed us to determine how many root tips were occupied by fungi appropriate for supporting orchid growth and which trees hosted them. We compared the total number of *Tomentella* root tips using ANOVA as for total number of ECM root tips.

We analyzed the abundance of ECM root tips in each sample group (consisting of three to five subsamples for summer and eight samples per subplot for fall sampling) using ANOVA with *C. odontorhiza* density as a fixed effect and sample time (summer vs. fall) and sample group (nested within density × time) as random effects. We analyzed the total trunk area ($\pi \cdot dbh^2/4$) of ECM trees in the 36 subplots using ANOVA with *C. odontorhiza* density as a fixed effect. Data presented in the text are mean ± SE.

Tomentella *spp. in soil*

Fungal abundance on ECM root tips does not necessarily reflect hyphal abundance in the soil or availability for orchid colonization. So, in addition to testing whether appropriate fungi formed ECM only in dense parts of the study plot, we examined the distribution of appropriate fungi in the soil. After removing the ECM roots from each soil core in fall 2005, we freeze dried the remaining soil and ground it to ensure that it was homogeneous. Then we extracted DNA from 0.4 g of the dried, ground soil using FastDNA for Soil kits (Qbiogene, Irvine, California, USA). We used several *Tomentella*-specific PCR primer pairs to ensure that the greatest variety of *Tomentella* spp. were identified. Some primer pairs (SSU1318-Tom/LSU-Tom2, SSU1318-Tom/ITS-Tom4) yielded a single amplicon from each soil sample while others (SSU1318-Tom/ITS4, ITS1-F/LSU-Tom2) often produced amplicons of multiple sizes (Appendix D). Several samples with single amplicons were sequenced from each primer pair, and a subset of the samples with multiple amplicons were selected for analysis of multiple taxa using cloning. Two soil samples (one from a dense and one from a sparse area) were amplified with both primer pairs that produced multiple amplicons to determine how the taxa amplified by the two primer pairs differed. Cloning was performed using TopoTA chemically competent cells (Invitrogen, Carlsbad, California, USA) as per manufacturer's instructions. We then chose 10 colonies with appropriate-sized inserts from each sample to amplify using PCR with the vector primers M13f/M13r. Each single-amplicon sample or clone was sequenced using Big Dye version 3.1 chemistry and run on an ABI 3100 sequencer (ABI, Foster City, California, USA).

To ensure that all *Tomentella* spp. sequences that we generated belonged to clades with at least some members that could support *C. odontorhiza*, we first aligned forward and reverse sequences using Sequencher version 3.1 (Gene Codes, Ann Arbor, Michigan, USA), aligned multiple sequences manually using Se-Al version 1.0 (Rambaut 1996), and conducted phylogenetic analysis using Paup 4.11, beta version (Swofford 2002). Using parsimony analysis, we first examined phylogenies generated using only the ITS1 and only the ITS2 fragment separately to detect possible chimeras (e.g., Parrent and Vilgalys 2007). Two taxa were removed based on dramatic differences in phylogenetic placement between ITS1 and ITS2 analyses, and phylogenetic analysis was conducted on the full sequences with the addition of outgroup taxa to provide a root for the tree.

In order to identify related taxa and place our Thelephoraceae sequences in a phylogenetic framework, we obtained the top five matches from GenBank to each of our sequences using our public web tools (*available online*).[7] Because our sequences from roots, soil, and orchids were of variable lengths and included many identical or near-identical sequences, we allocated our sequences together with the GenBank matches into operational taxonomic units (OTUs) by assembling them into contiguous sequence at 98% sequence similarity in Cap3 (Huang and Madan 1999). One representative from each taxonomic unit was then chosen to represent each OTU for tree building. The phylogenetic alignment of tomentelloid fungi included 649 characters of which 323 were constant and 228 were parsimony informative. Likelihood analysis was conducted in Garli version 0.951 (Zwickl 2006) under a GTR + I + G model (general time reversible model with proportion invariant sites and gamma distribution of rates across sites) and search termination after 10 000 generations without improvement. The same analysis was repeated with termination threshold dropped to 5000 for 100 bootstrap replicates. We also carried out an equally weighted heuristic parsimony search in PAUP*b10 (Swofford 2002) with tree bisection reconnection (TBR) branch swapping, 10 random addition replicates, and maxtrees set to 10 000. One thousand parsimony bootstrap replicates were performed in PAUP under the same conditions.

Statistical comparisons of fungal communities

We tested for differences in abundances of each tomentelloid OTU recovered from orchid mycorrhizae, ectomycorrhizae, and soils (Table 1) using the two-sided Fisher's exact test of homogeneity (Fisher 1935) in the R package, Version 2.6.1 (The R Foundation for Statistical Computing 2007; Becker et al. 1988). In addition, we compared tomentelloid assemblages across these samples, taking sequence divergence into account in two ways. We created an input file in haplotypic DNA format for Arlequin (Excoffier et al. 2005) containing three "populations": orchids, ectomycorrhizae, and soil. The most complete sequence representative for each OTU

[7] ⟨http://www.borealfungi.uaf.edu⟩

TABLE 1. Abiotic measurements in dense and sparse subplots.

Subplot type	Bulk density, mean ± SE (g)	Moisture, mean ± SE (%)	pH, mean ± SE	PAR, mean ± SE (%)
Dense	8.34 ± 0.46	36.8 ± 1.4	5.56 ± 0.11	58.5 ± 17.5
Sparse	9.05 ± 0.40	35.3 ± 1.6	5.61 ± 0.16	23.6 ± 9.4

Notes: Bulk density is given as g dry soil per 2.5 cm diameter × 5 cm deep soil core. Soil moisture is given as percentage by mass. Photosynthetically active radiation (PAR) is given as percentage of photosynthetic photon flux density. PAR differs significantly between dense and sparse subplots ($P = 0.03$); all other measurements are not significantly different (all $P > 0.15$).

was then replicated across the three populations along with its count (abundance) in each sample type. First, standard calculations for "molecular diversity" were carried out, which gave values for the mean pairwise sequence divergence (π) and its standard deviation within each population. This approach provides an estimate of the phylogenetic diversity found in each assemblage of fungi, as described in Taylor et al. (2004). Second, we carried out an AMOVA in which F_{st} based upon sequence divergences was calculated in order to test whether the three populations of tomentelloid fungi differed genetically. Permutation tests (1000 replicates) were carried out to test whether the resulting F_{st} values were significantly different from zero. F_{st} indicates the similarity in alleles (inbreeding coefficient F) between a subpopulation (s) relative to the total population (t). Where $F_{st} = 0$, the populations are genetically identical; where $F_{st} = 1$, they are completely different.

Seed germination

To relate soil DNA results to seed packet studies, which are more commonly used to assess environmental suitability for orchid growth, we placed 10 packets containing *C. odontorhiza* seeds (as per Rasmussen and Whigham 1998) in each of three pairs of adjacent subplots in the study plot in January 2001. In each pair of subplots one supported sparse and the other dense *C. odontorhiza* in 2000. We also placed 40 additional seed packets outside the main study plot. One location 50 m and one location 100 m from the center of each outer edge of the study plot received five seed packets each. After three years (February 2004) we collected five of the seed packets in each subplot and three of the five seed packets in each 50-m and 100-m location to assay for seed germination and protocorm development. Nine months later (November 2004) we collected and scored the remaining seed packets.

RESULTS

Inflorescences, reproductive effort, and survival

The number of flowering plants in the study plot fluctuated from year to year (Fig. 1), and using a stepwise regression, the annual number of flowering plants was significantly negatively related only to winter mean temperature ($r^2 = 0.30$, $P = 0.044$; M. K. McCormick, D. F. Whigham, J. P. O'Neill et al., *unpublished data*). In contrast to the total number of inflorescences, inflorescences as a proportion of the previous year's inflorescences was significantly positively related to growing-season rainfall (Fig. 2a; $P = 0.027$) and negatively related to winter mean temperature (Fig. 2b; $P = 0.03$, combined $r^2 = 0.75$).

The number of flowers per plant (range 6.46–9.29) was not significantly related to any climate variable (all

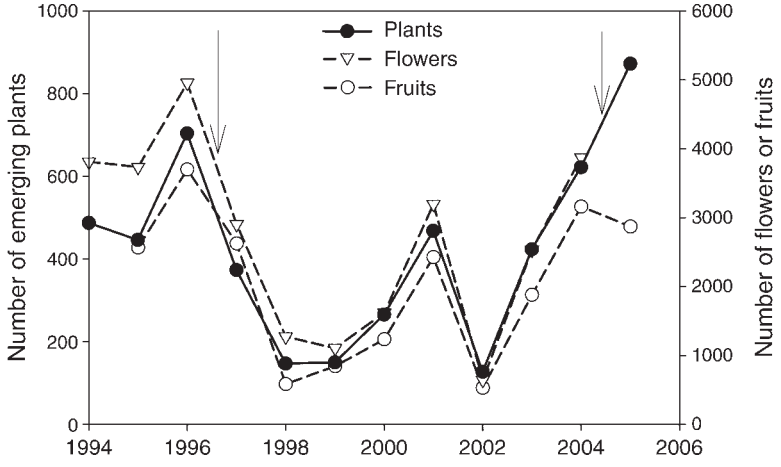

FIG. 1. The number of emerging plants of the mycoheterotrophic orchid *Corallorhiza odontorhiza* (solid symbols and solid line) and the number of flowers (open triangle and dashed line) and fruits (open circles and dashed line) that they produced in the study plot over 12 years. Arrows indicate the two years when underground survival was monitored (1996–1997, 2004–2005).

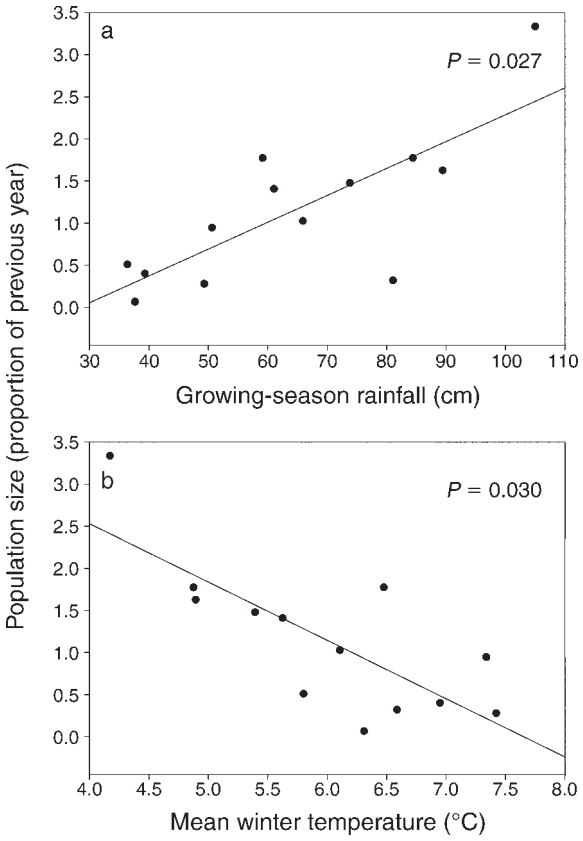

FIG. 2. The significant relationships between the change in the number of emerging *Corallorhiza odontorhiza* plants from year t to year $t+1$ and (a) growing-season rainfall and (b) mean winter temperature.

$P > 0.15$). The proportions of flowers that became fruits ranged from 0.443 to 0.888 and was negatively related to both winter precipitation in the previous year and to growing-season temperature ($P = 0.012$ and $P = 0.015$, respectively, $r^2 = 0.58$; M. K. McCormick, D. F. Whigham, J. P. O'Neill et al., *unpublished data*).

To determine the survivorship of flowering plants and the relationship between survival and flowering the following year, we tracked the survival of 100 randomly chosen plants over two different years (see *Methods: Inflorescences, reproductive effort, and survival*). Fifteen percent of the plants marked in November 1996 survived to November 1997 but none reflowered. In contrast, 84% of the plants marked in November 2004 survived to November 2005, and 19% reflowered. We noted in our 1996–1997 study that many of the rhizomes that did not survive appeared to be eaten by small mammals, based on the abundance of tunnels in the same locations as the marked plants.

Flowering plant distribution

Flowering plants were consistently absent from some parts of the study plot during the years that individuals were mapped (Fig. 3). In other instances, flowering individuals appeared year after year in very similar locations, suggesting that the presence of an individual in one year was related to the presence of individuals in subsequent years. Using O-ring statistics, we found that plants were statistically more clumped than random from one year to the next at distances < 50 cm (in some years up to 2.5 m). At greater distances, plant distribution was similar among years (Fig. 4), indicating that at larger scales the distribution of flowering plants

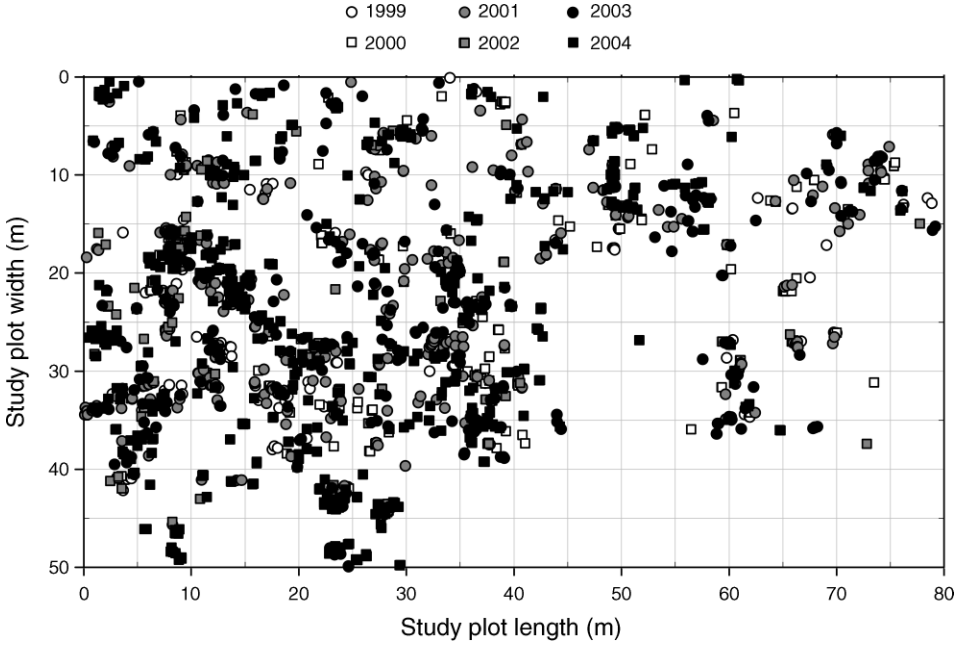

FIG. 3. A map of all *Corallorhiza odontorhiza* plants emerging in the 50×80 m study plot for each year between 1999 and 2004.

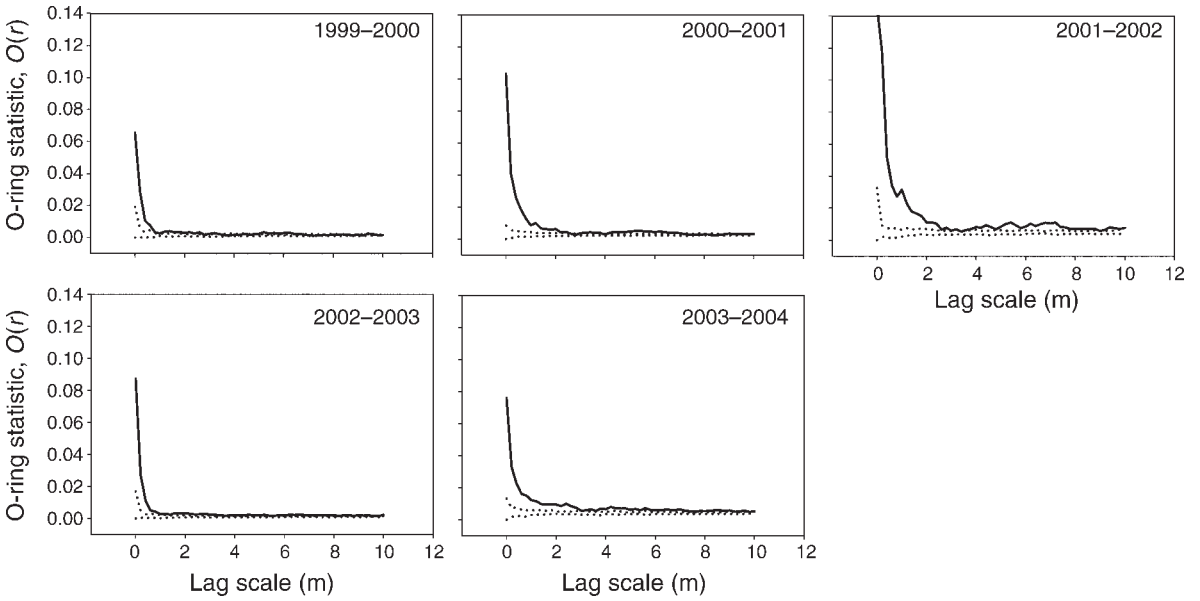

FIG. 4. O-ring statistics (Programita; Wiegand and Maloney 2004), where r represents the radius of the ring, for each pair of years calculated using year 1 fixed and year 2 random. The lag scale refers to the mean distance between pairs of points being compared within a distance class. Solid lines show the calculated test statistic. Dotted lines indicate 95% CI for random distribution. Test statistic values >95% CI indicate significantly clumped plant distribution; those <95% CI indicate significantly more regular plant distribution than random.

across the study plot was explained by an underlying pattern that did not change from year to year.

Abiotic factors, ECM trees, root tips, and mycorrhizal fungi

Soil bulk density, pH, and moisture did not differ significantly between the dense and sparse sections of the study plot (Table 1), but photosynthetically active radiation (PAR) was significantly higher ($P = 0.03$, $r^2 = 0.12$) in areas with dense *Corallorhiza odontorhiza*. Subplots with lower PAR and dense *C. odontorhiza* had more large ectomycorrhizal (ECM) trees than subplots with sparse or no *C. odontorhiza* (basal area = 0.88 ± 0.54 m^2 vs. 0.29 ± 0.15 m^2; all results reported as mean ± SE unless otherwise noted) ANOVA on log-transformed data, $P < 0.001$). However, dense and sparse subplots had similar basal areas of ECM trees (3.67 ± 2.51 m^2 vs. 2.39 ± 1.13 m^2; ANOVA on log-transformed data, $P = 0.68$). Subplots with higher densities of *C. odontorhiza* had more ECM root tips per sample in both summer (347.4 ± 93.2 root tips vs. 109.0 ± 23.5; ANOVA on log-transformed data, $P < 0.001$) and fall 2005 (210.8 ± 29.2 vs. 109.6 ± 33.2; ANOVA on log-transformed data, $P = 0.003$).

Using direct PCR, we obtained PCR product from 374 of 414 root tips using the plant-specific primer pair ITS1-P/ITS4. Although only some (only root tips hosting *Tomentella*) of these PCR products were sequenced, this provided a positive control for our direct PCR technique and for our PCR reactions. PCR reaction failure, when it occurred, generally resulted from roots that had been refrigerated more than one week after collection. The tomentelloid-selective PCR primer pair (SSU1318-Tom/LSU-Tom2) amplified *Tomentella* spp. was not as specific as SSU1318-Tom/LSU-Tom4 (Taylor and McCormick 2008), so sequencing was needed to determine which roots were colonized by *Tomentella* spp.

Tomentella *spp. on ECM root tips*

Morphotyping in fall 2005 identified eight types that were composed exclusively of *Tomentella* spp. Five of the nine most abundant morphotypes were composed of *Tomentella* spp., while the remaining three *Tomentella* morphotypes were infrequently encountered. *Tomentella* spp. were quite common across the entire study plot, colonizing 40.7% ± 5.1% of all ECM root tips sampled in fall 2005. Contrary to our expectations, the proportion of root tips colonized by *Tomentella* spp. did not differ between dense and sparse parts of the study plot ($P = 0.881$), with *Tomentella* spp. colonizing 40.0% ± 6.6% of root tips in dense subplots and 41.6% ± 8.2% of ECM root tips in sparse and no-plant subplots. Despite colonizing equal proportions of root tips in dense and sparse subplots during fall 2005, subplots with dense *C. odontorhiza*, which had more total ECM root tips, had more root tips colonized by *Tomentella* spp. per core than subplots with sparse *C. odontorhiza* (103.7 ± 24.6 root tips vs. 55.9 ± 18.3; ANOVA on log-transformed data, $P = 0.047$). Sequences from root morphotypes in the fall sampling demonstrated that morphotypes did a good job of separating fungal genera, as single

morphotypes generally yielded fungal sequences belonging to a single genus. However, within each genus, morphotypes generally contained several taxa (operational taxonomic units [OTUs]), and single fungal taxa on different host tree species produced multiple morphotypes. Because lumping of morphotypes during the summer sampling resulted in many morphotypes containing both *Tomentella* and other genera, we were unable to analyze whether the abundance of root tips colonized by *Tomentella* spp. also differed among dense and sparse parts of the study plot in summer 2005. All of the available ECM trees (*Quercus* spp., *Carya* spp., *Fagus grandifolia*; GenBank accession numbers EU646134-EU646185), hosted *Tomentella* spp., approximately in proportion to their occurrence in sampled areas of the study plot (GenBank accession numbers EU625846-EU625886 for *Tomentella* spp.). OTU E, the most common *C. odontorhiza* associate, was similarly widely distributed, occurring on *Quercus* spp., *Carya* spp., and *F. grandifolia*.

The phylogenetic alignment of tomentelloid fungi included 649 characters of which 323 were constant and 228 were parsimony informative. The likelihood search required 39 700 generations to complete and yielded a tree with $-\ln = 7435.50$. The equally weighted heuristic parsimony search yielded 488 most parsimonious trees of 1386 steps (consistency index = 0.342, rescaled consistency index = 0.200). Most *Tomentella* spp. amplified from ECM tree roots, including those from both dense and sparse or no-plant parts of the study plot, fell within the clades of fungi that associated with *C. odontorhiza* sampled from across the study plot. However, very few belonged to OTU E, the clade that was associated with 50% of the sampled *C. odontorhiza* plants (Fig. 5, Appendix E). OTU E was found associated with *C. odontorhiza* plants across the study plot, but the small number of samples precluded spatial analysis. On ECM root tips, OTU E was only found adjacent to orchids. Other *Tomentella* OTUs were occasionally found associated with *C. odontorhiza* plants and were found on a wide variety of root tips (Fig. 5).

Tomentella *spp. in the soil*

The *Tomentella* specific primer pair SSU1318-Tom/ITS-Tom4 amplified *Tomentella* spp. from all 32 soil samples from across the study plot. In each case a single amplicon was obtained, and the majority of amplified fungi fell within the clades associated with *C. odontorhiza* plants (Fig. 5), indicating that potential associates were present throughout the study plot. However, only a single soil amplicon belonged to OTU E, the most common *C. odontorhiza* associate.

Other primer pairs also amplified *Tomentella* spp. but included a range of other fungi as well. In every case when *Tomentella* spp. were amplified by multiple primer pairs, they had identical sequences.

Cloning amplicons produced diverse *Tomentella* spp. in some samples and none in others (Fig. 5). Three samples from subplots with dense and one from a subplot with sparse *C. odontorhiza* had multiple *Tomentella* taxa. The only dense subplot without *Tomentella* spp. was Soil D-D8202, and this may have been due to a partial cloning failure, as only one clone was successfully identified from this subplot. Two of the sparse subplots had no *Tomentella* spp. identified in cloning. Most *Tomentella* taxa amplified fell within the range of *Tomentella* spp. found associated with *C. odontorhiza* within the study plot (Fig. 5), but only one fell within OTU E. All direct *Tomentella* sequences and examples of each *Tomentella* clone sequence were deposited in GenBank (EU625887–EU625924).

Fisher's exact test for homogeneity of counts in a contingency table of tomentelloid OTUs associated with orchid samples, ectomycorrhizal roots, and soil samples very strongly rejected the null hypothesis ($P < 1 \times 10^{-16}$), showing that the tomentelloid assemblages recovered from these samples are quite different. Similarly, the AMOVA analysis revealed significant F_{st} values (population differentiation) between the tomentelloid sequences recovered from the orchid compared with those from roots and soils (Table 2). In contrast, F_{st} between root and soil samples was not different from zero. Hence, both count-based and sequence-diversity-based analyses suggest that *C. odontorhiza* associates with a different array of tomentelloid fungi than occur at the site overall. Mean pairwise sequence divergence (π, the number of base substitutions) was highest in the root population (75.25 ± 33.06 base substitutions), intermediate in the orchid population (57.67 ± 25.34 base substitutions), and lowest in the soil population (35.91 ± 15.98 base substitutions), although the standard deviations of all estimates overlapped.

Seed germination

Seed packets placed in adjacent sparse and dense 5×5 m subplots in the study plot in January 2002 were collected in February and November 2004. However, in the two years between seed packet placement and collection, two of the three sparse subplots supported moderate to dense aggregations of plants, and two of the three dense subplots had few plants. Within the study plot only one subplot with seed packets consistently supported few or no plants. Similarly, only one consistently supported a high plant density. Germination was most frequent in the consistently dense location (all 10 seed packets had protocorms) and least frequent in the consistently sparse location (no seed packets had protocorms; Appendix F). However, germination also occurred at three locations that were 50 m from the main study plot (20–60% of seed packets; seed packets at the fourth 50-m location were not recovered), and in one seed packet at one of the four locations that was 100 m from the main study plot in areas with few or no *C. odontorhiza*. Unfortunately, fungal symbionts were not identified in protocorms from these seed packets, but we have previously found germinating seeds to associate

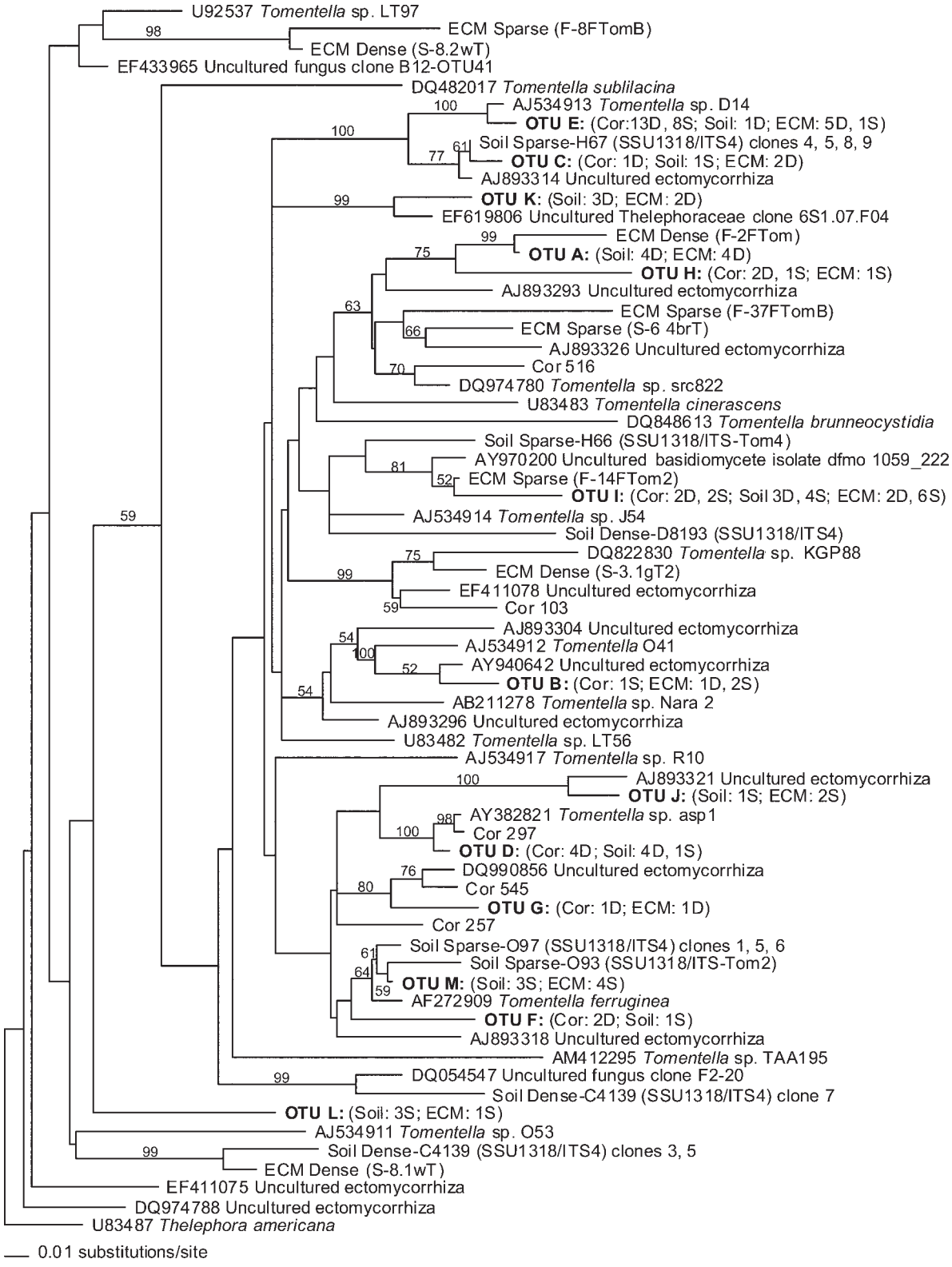

FIG. 5. Phylogenetic tree of the nrITS region amplified using a range of tomentelloid-selective primers. The tree was rooted to *Thelephora americana*. Taxon names from ectomycorrhizal root samples begin with "ECM," followed by the region of the study plot sampled (sparse or dense). Following that, in parentheses, root tip labels include one letter indicating sample time (F, fall; S, summer) followed by a hyphen and a unique sample name. Taxon names beginning with "Cor" followed by a number were

with the full range of *Tomentella* spp. associated with adult plants (M. K. McCormick, *unpublished data*).

Discussion

The importance of climate and how it may modify interactions between species has been identified as a major area of ecology for which knowledge is largely missing (Agrawal et al. 2007). For mycoheterotrophic plants, factors affecting flowering and those affecting plant distribution often appear to be inseparable because the only time that plants are visible is when they are flowering. In addition to providing information on the importance of climatological variables on the abundance of flowering individuals for a mycoheterotrophic orchid, this case study is the first to examine explicitly both the distribution of an orchid and also members of the fungal community that are necessary for its survival and growth. By evaluating the dynamics of the *Corallorhiza odontorhiza* flowering in response to factors at several scales, the results of these studies provide insight into the range of factors that interact to determine annual variation in the flowering abundance and distribution of individuals.

Inflorescences, reproductive effort, and survival

There have been relatively few studies in which the relationships between climatological factors and the dynamics of orchid populations have been examined, and the studies that have been published suggest that there is no clear pattern. Long-term population studies of *Ophrys apifera* (Wells and Cox 1991), *Gymnadenia conopsea* (Øien and Moen 2002), *Tipularia discolor* (Whigham and O'Neill 1991), and *Cypripedium calceolus* (Kull and Kull 1991) found no discernable relationship between plant density or number of plants flowering and variation in climatic factors. In contrast, Pfeifer et al. (2006) found that weather conditions explained ~50% of the yearly variation in flowering of *Himantoglossum hircinum*. Temperature and precipitation, especially drought, are the two factors that have most often been shown to be responsible for annual changes in populations of terrestrial orchids (e.g., Inghe and Tamm 1988, Wells et al. 1998, Willems 2002, Reddoch and Reddoch 2007).

The most likely interpretation of the pattern we observed in the number of flowering individuals of *C. odontorhiza* is that greater precipitation in the growing season prior to flowering resulted in greater survival, growth, and reflowering of belowground coralloid rhizomes. The driest season in Smithsonian Environmental Research Center (SERC) forests is the summer, especially late summer and early autumn. Research on *Goodyera pubescens* in these same forests has shown that drought causes both increased plant mortality and decreased access to mycorrhizal fungi, forcing plants to switch fungi (McCormick et al. 2004). The negative relationship between drought and mycorrhizal interactions might be especially important in a completely mycoheterotrophic species such as *C. odontorhiza*, which depends completely on fungal connections between individual orchids and associated tree or shrub species. If fungal connections are lost during dry periods as fungal hyphae contract and die back (Querejeta et al. 2007), it seems likely that orchid mortality would increase because the fungal ability to convey carbon and water would be lost. Accordingly, during the two years when we monitored *C. odontorhiza* survival and reflowering, we found higher mortality and lower reflowering following the dry 1997 growing season (precipitation 36 cm) than following the relatively moist growing season in 2005 (precipitation 61 cm).

Plant survival (15%) was lower for rhizomes in the cohort that flowered in 1996 compared to the 2004 cohort (84%). Based on results of our ongoing studies of the *C. odontorhiza* study plot, we believe that much of the increase in number of inflorescences may have been the result of clonal reproduction and reflowering of dormant plants (i.e., mature plants persisting below ground; e.g., Lesica and Steele 1994, Shefferson et al. 2003). Analyses of this study plot suggest that dormancy is high among previously flowering plants, and reflowering is dependent on temperature and precipitation (R. L. Shefferson and M. K. McCormick, *unpublished data*). We have also found that the coralloid rhizomes of flowering individuals may propagate clonally by producing buds (as per Klimešová and Klimeš 2007) that are similar to propagules produced by other mycoheterotrophic plants (Domínguez et al. 2006). Preliminary studies indicate that buds are abundant (see seed packet data in Appendix F), and many persist relatively unchanged in size or appearance for more than one growing season without developing into a coralloid rhizome, thus forming a propagule bank. Contrary to the proposed function of buds in other mycoheterotrophic plants (Klimešová 2007), these propagules do not appear to have mycorrhizal associations, evidenced by

←

amplified from *Corallorhiza odontorhiza* plants in fall 2005. Taxa amplified from soils are identified by "Soil" followed by study plot region (sparse or dense), sample name, (specific primer[s]), and a hyphen followed by the clone numbers represented by that sequence. When no clone numbers are included, the sequence was generated from a sample with a single fragment size. Every unique operational taxonomic unit (OTU) from our study is shown on a distinct branch. OTUs represented by multiple sequences are named "**OTU**" followed by a letter designation and codes for all constituent samples, while OTUs represented by a single sequence are simply designated by the sample code. The composition of each OTU is indicated in parentheses following each name. Composition is as follows: Cor indicates sequences from *C. odontorhiza*, Soil indicates sequences from soil, and ECM indicates sequences from ectomycorrhizal root tips. For each of these groups, the number of sequences from dense (D) and sparse (S) areas of the study plot are given. All sequences represented by each OTU are given in Appendix E. Taxon names preceded by a GenBank accession number are included for reference.

TABLE 2. Comparison of genetic diversity in the sets of tomentelloid fungi recovered from orchid (*Corallorhiza odontorhiza*) mycorrhizae, ectomycorrhizae, and soil in this study.

Source	Orchid		Ectomycorrhizal roots	
	Analogue of F_{st}	P	Analogue of F_{st}	P
Ectomycorrhizal roots	0.092	<0.00001		
Soil	0.16	<0.00001	0.0063	0.22

Notes: A DNA sequence-based analogue of F_{st} was calculated by AMOVA in Arlequin (Excoffier et al. 2005). Notice that the set of fungi recovered from orchid mycorrhizae differs genetically from the sets of fungi forming ectomycorrhizae and found in soil. F_{st} indicates the similarity in alleles (inbreeding coefficient F) between a subpopulation (s) relative to the total population (t). Where $F_{st} = 0$, the populations are genetically identical; where $F_{st} = 1$, they are completely different.

the lack of pelotons (M. K. McCormick and J. P. O'Neill, *personal observation*), and may be a response to loss of mycorrhizal fungi or a pathogenic attack. We are currently studying the role of these buds in *C. odontorhiza* and do not yet know how or when they may transition to flowering plants.

The proximate factors that link climatological factors with survival and growth of orchids remain unknown, but the results of this research and previous research on *Goodyera pubescens* (McCormick et al. 2006, Diez 2007) suggest that a key factor is the maintenance of a functional link between orchids and mycorrhizal fungi. McKendrick et al. (2000*a*) also clearly demonstrated the importance of maintaining a functional linkage between the orchid and its plant host through the fungal intermediary. They also found that germination was dramatically lower in a drought year than in more moist years. Other researchers have found that the abundance of ectomycorrhizal (ECM) root tips (e.g., Swaty et al. 1998, Izzo et al. 2005) and the amount of extramatrical hyphae (e.g., Osono 2003, Staddon et al. 2003) decreased in response to drought. This supports the suggestion that growing-season precipitation affects orchid growth by altering fungal hyphal abundance directly or by affecting fungal resources via the nutrient status of ECM host trees.

Distribution of flowering plants

The spatial pattern of *C. odontorhiza* inflorescences within the study plot was relatively consistent from year to year (Fig. 3) and did not appear to be driven by abiotic factors at this scale. Vanhecke (1991) found that the locations of vegetative and flowering individuals of *Dactylorhiza praetermissa* varied somewhat from year to year, driven largely by abiotic factors, but similar to our study, some parts of his study grid never supported any plants. Our results suggest the parts of the study plot occupied by the highest densities of flowering plants were areas that consistently supported a high density of *Tomentella* spp. associated with a large number of ECM tree roots and areas that supported "preferred" *Tomentella* spp. If *C. odontorhiza* generally lives for many years, then patterns of ECM tree root abundance could create a consistent distribution of plants, such as that demonstrated by the consistent underlying year-to-year pattern revealed by the O-ring analyses. While it is likely that the majority of seeds fall beneath the maternal parent, some seeds are undoubtedly spread more widely and may remain viable for several years (Whigham et al. 2006), making it unlikely that a complete lack of seeds in sparse areas produced the current pattern. Another possible explanation for the consistent plant distribution pattern is the production of asexual propagules and rhizome fragmentation. The dynamics of asexual propagules and fragmentation of rhizomes could also explain some of the clumped nature of plant distribution from one year to the next as well the contraction to more clumped distribution in dry years. However, due to the nonlinear growth pattern of coralloid rhizomes, asexual reproduction likely only accounts for clumping at the smallest scales.

Tomentella *spp. on root tips and in soil*

Contrary to our predictions, *Tomentella* spp. within the overall clade of *C. odontorhiza* fungi were present throughout the study plot and on all available ECM trees. This observation suggests that *C. odontorhiza* distribution within the study plot was not likely limited by distribution of potential host fungi. However, plant distribution could have been limited by the distribution of preferred fungi, such as operational taxonomic unit E (OTU E), within the broad tomentelloid clade or by the abundance of fungal hosts, as reflected by the abundance of ECM roots colonized by *Tomentella* spp. OTU E colonized fully half of the *C. odontorhiza* plants examined across the study plot (Fig. 6), but was only found on root tips or in soil immediately adjacent to *C. odontorhiza* plants in dense subplots. The ECM root tips colonized by OTU E included *Carya* spp., *Quercus* spp., and *Fagus grandifolia*, suggesting that host specificity does not explain the distribution of this taxon. These observations suggest that this preferred fungus was more abundant and widespread in dense than in sparse subplots, although our sampling of tomentelloid communities in soil was limited. Furthermore, the array of tomentelloid OTUs recovered from orchid mycorrhizae was distinctly different from those on tree roots or in soil, further supporting the concept of orchid preference. We speculate that orchid growth is accelerated by a combination of the preferred species of tomentelloid

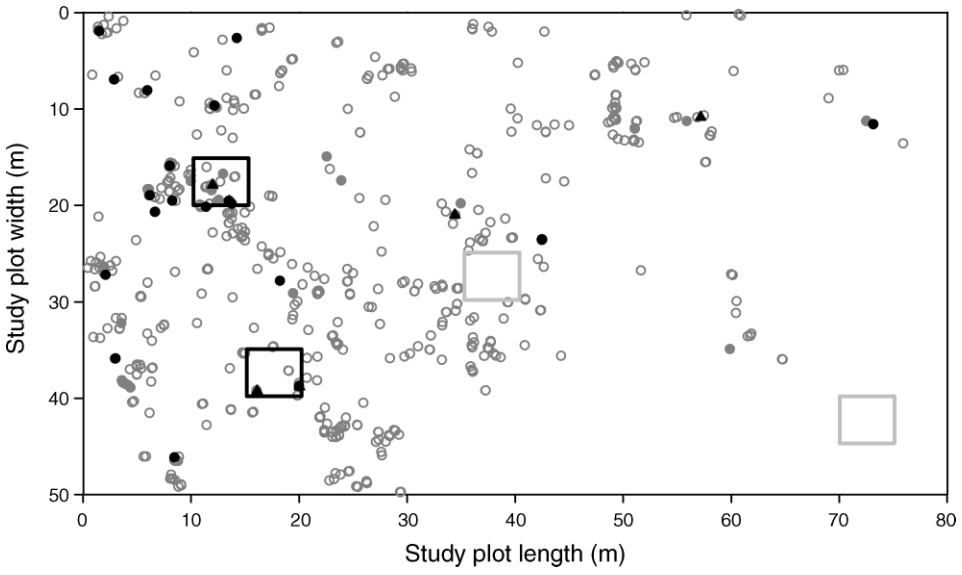

FIG. 6. Distribution in the 50 × 80 m study plot of fungi belonging to operational taxonomic unit E (OTU E), the most common fungus associated with *Corallorhiza odontorhiza*, and other OTUs. Solid black symbols indicate root tips (triangles) or *C. odontorhiza* rhizomes (circles) in which OTU E was amplified. Other OTUs amplified from *C. odontorhiza* rhizomes are shown as solid gray circles. Locations of unsampled *C. odontorhiza* plants (open circles) are shown for reference to plant distribution. Large open squares indicate subplots in which ECM root tips and/or soil contained fungi from OTU E (black outline) or only other OTUs (gray outline). ECM root tip samples that were not associated with *C. odontorhiza* plants (fall samples in sparse subplots) were not mapped beyond the subplot level; ECM root tips with OTUs other than E are not shown.

fungi, abundant ECM roots colonized by this fungus, and connection to large host trees. We cannot rule out that there is a positive effect of the orchid on OTU E as in monotropes (Bidartondo et al. 2000), but we feel that it is unlikely.

The examination of ECM root tips in this study took place during a "good" year for *C. odontorhiza*, with average levels of rainfall during the growing season and a resulting large number of emerging flowering plants. During 2002, a drought year, the increased strength of spatial correlation at short distances suggested that emerging plants were restricted to locations that were consistently especially good for plant growth and flowering, perhaps associated with a host tree that was resistant to drought. However, the same pattern could be produced if the only plants that flowered during dry years were those that had been clonally produced. During a second drought year, 2007, the number of flowering plants in the study plot declined to only 16. All 16 plants flowered in areas with historically dense *C. odontorhiza* and all occurred <10 cm from the location of a plant in one of the two previous years. That climatic effects on plants in the study plot largely affected a change in the number of inflorescences from one year to the next rather than affecting the total number of plants flowering suggests that dynamics in this study plot are driven by factors influencing flowering from a long-lived propagule bank or from persistant rhizomes to a greater extent than by plant survival. Because plant persistence requires longevity of fungi and ECM root hosts beyond the two or more years needed for a germinating seed to reach maturity (based on many years of seed packet studies in this study plot; e.g., Whigham et al. 2006), this implies that flowering dynamics may be strongly influenced by fungal dynamics, which in turn may be influenced by host plant dynamics.

Other biotic interactions may also contribute to orchid population dynamics. It is possible that pathogens not examined in the present study played a role in determining plant distribution. In other years we examined plants that flowered outside the main study plot and found that many of the plants were infected by an apparently pathogenic fungus belonging to the genus *Ceratobasidium* and were overcome by the pathogen before successfully setting seed. While some *Ceratobasidium* spp. form mycorrhizae with other orchids, this fungus has only been found in blackened, decayed sections of *C. odontorhiza* rhizomes, never in healthy rhizomes, so it is unlikely that it is functioning as a mycorrhiza. An exhaustive survey has not been conducted, and we have not tested for the presence of this pathogen in the soil, but this infection has not been found within the main study plot, so it is unlikely that it governs plant distribution within the study plot.

In this study we were able to examine the distribution of mycorrhizal fungi independent of the conditions needed for seed germination and to do this in a study plot where the long-term distribution of flowering plants was known. Furthermore, we demonstrated that the distribution of flowering *C. odontorhiza* was likely explained by the proximity of abundant ECM tree roots for the *Tomentella* spp. to colonize, which may reflect

the ability of a fungus to access nutrients (e.g., Agerer 2001, Kjøller 2006, Parrent and Vilgalys 2007) and by the distribution of a preferred clade in the genus *Tomentella*.

Seed germination

All combinations of PCR primers demonstrated that *Tomentella* spp. within *C. odontorhiza*-associated clades were present in areas of the study plot with few or no *C. odontorhiza*. However, germination occurred most strongly in seed packets placed in subplots supporting consistently dense *C. odontorhiza* flowering, less so in subplots supporting variable numbers of flowering plants, and the least in areas consistently supporting no flowering plants. Some germination did occur in areas that rarely supported flowering plants, so *Tomentella* spp. present in the soil may, at least sporadically, support germination even in areas that do not support flowering plants. These results suggest that many areas without flowering plants may nevertheless support a substantial number of unseen plants that may flower in the future.

Similarly, in a seed packet study of *Corallorhiza trifida*, McKendrick et al. (2000*b*) found that seed germination was more consistent in plots with adult plants than in plots with no adult plants. Diez's (2007) studies of *Goodyera pubescens* and Jacquemyn et al.'s (2007) study of *Orchis purpurea* also found strong relationships between adults and seed germination. In our study plot, PCR-based techniques also demonstrated that potential host fungi were more widespread than the long-term distribution of adult plants, independent of conditions appropriate for seed germination. Furthermore, we found that the abundance of potential host fungi, or at least their colonization of ECM roots, was a better indicator of adult distribution than simply the presence of appropriate fungi, although the presence of fungi belonging to OTU E might be an even better indicator.

The *Tomentella* spp. needed by *C. odontorhiza* are considered obligately ectomycorrhizal. While this means they are largely absent from forests without ECM trees, they appear to be able to colonize all of the ECM trees present in this area. Many forest areas at SERC have appropriate ECM trees but have no *C. odontorhiza*. Germination of *C. odontorhiza* seeds does occur in these forests but may occur only infrequently, as Whigham et al. (2006) found that seeds took four years to germinate in several SERC sites that did not have *C. odontorhiza* plants, only germinating after a year of relatively high rainfall (2000–2001) that also produced increases in the number of plants flowering in this study plot (Fig. 1).

While other plants are increasingly being found to associate with limited mycorrhizal communities (e.g., Vandenkoornhuyse et al. 2006, Wubet et al. 2006) and mycorrhizal diversity has been related to plant community diversity (e.g., van der Heijden et al. 1998), the extent to which plant distributions are driven by microbial distribution is unknown. In a related ongoing study, we found that germination of three green, photosynthetic terrestrial orchid species that associated with specific saprotrophic fungi was also driven by a combination of fungal abundance and fungal distribution (M. K. McCormick, D. F. Whigham, D. L. Taylor et al., *unpublished data*), suggesting the limitation of other orchids by conditions that support abundant fungal growth and the distribution of preferred taxa rather than strictly by presence or absence of potential host fungi. This suggests that orchids and perhaps many other plants with specific fungal requirements may be limited by the consistent abundance of preferred mycorrhizal fungi rather than strictly by fungal distribution.

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APPENDIX A

Photographs of the study plant and study site (*Ecological Archives* M079-022-A1).

APPENDIX B

A description of other plant species and soil characteristics of the study site (*Ecological Archives* M079-022-A2).

APPENDIX C

Flowering and climatic data for the *Corallorhiza odontorhiza* study plot during the years 1994–2007 (*Ecological Archives* M079-022-A3).

APPENDIX D

Sequencing efforts for different pairs of PCR primers (*Ecological Archives* M079-022-A4).

APPENDIX E

Taxa identified in clones of samples from dense and sparse areas of the study plot (*Ecological Archives* M079-022-A5).

APPENDIX F

Germination in seed packets placed in and around the main study plot (*Ecological Archives* M079-022-A6).