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Does mycorrhizal specificity affect orchid decline and rarity?[1]

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- *Premise of the study:* Orchids rely on mycorrhizal fungi for seed germination, and many species maintain associations during later stages in their life cycle. Because of the critical dependence of orchids on fungi it has been suggested that the degree of mycorrhizal specificity may be associated with rarity and long-term survival of orchid species, especially in highly degraded or fragmented landscapes. To test this hypothesis, we compared mycorrhizal communities in two species that differed significantly in decline in Belgium and other parts of Europe.
- *Methods:* Mycorrhizal associations were investigated in five populations of *Anacamptis morio* and *Dactylorhiza fuchsii* in Belgium. ITS-based DNA arrays were used for simultaneous detection and identification of a wide range of basidiomycetous mycorrhizal fungi. Mycorrhizal specificity, measured as phylogenetic diversity, was assessed for each population and compared between species.
- *Key results:* For both species, the degree of phylogenetic relatedness of the mycorrhizal partners was low, and both species were associated with a large number of fungal lineages related to clades of the Tulasnellaceae family. Contrary to expectations, the species that was apparently resilient to decline was associated with fewer fungal operational taxonomical units than the declining species was, and the phylogenetic relatedness of mycorrhizal communities among populations was higher in the stable than in the declining orchid.
- *Conclusions:* Although our results do not present detailed insights into the causes of orchid persistence, they do suggest that orchid rarity and persistence are not necessarily related to fungal diversity and that other factors may be more important in determining orchid persistence.

Key words: mycorrhizal associations; Orchidaceae; phylogenetic relatedness; specificity; Tulasnellaceae.

As the Earth is facing rapid global environmental changes (e.g., Walther, 2010), many rare or threatened species are expected to suffer increased risks of extinction in the near future. To conserve biodiversity, we need to define relevant approaches for conservation of endangered species. With more than 27 000 accepted species (The Plant List, 2010), the family of Orchidaceae represents around 10% of the angiosperms, making it, together with the Asteraceae, one of the largest families within the plant kingdom (Chase, 2005). Although this huge diversity is mainly concentrated in the tropics and in the biodiversity hotspots described by Myers et al. (2000), orchids are widespread and occur all around the world, from the Arctic Circle to the islands off the Antarctic (Luer, 1975; Jones, 1988). Despite their ubiquity, orchids have an important intrinsic rarity (Swarts and Dixon, 2009) and comprise one of the angiosperm families with the largest proportion of endangered species.

Because management tactics aiming at conserving individual orchid species largely depend on the species under consideration, thorough understanding of the processes leading to the decline of orchid species is essential. In general, two types of factors have been discerned that can lead to the decline and extinction of orchid species (Swarts and Dixon, 2009). Whereas extrinsic factors most often have an anthropogenic origin and reduce abundance either directly through habitat loss and collecting or indirectly by accelerating environmental and habitat changes, intrinsic factors refer to natural biotic processes limiting the abundance and distribution of species. While the first category is often well understood, biotic interactions between species can be quite complex and form intricate networks (Swarts et al., 2010, Jacquemyn et al., 2011; Waterman et al., 2011) that are more difficult to comprehend.

Orchids rely on symbiotic associations for two critical steps in their life cycle (Smith and Read, 2008; Waterman and Bidartondo, 2008; Waterman et al., 2011). One of them is the association with pollinators for successful sexual reproduction. The floral diversity in orchids is well known, and many species are specialists, attracting one or a few pollinators (Paulus and Gack, 1990; Waterman et al., 2011). The second association involves mycorrhizal fungi. Because most orchid species have seeds that lack endosperm (Arditti and Ghani, 2000), germination of seeds and further establishment of the seedlings require mycorrhizal fungi that provide the carbohydrates necessary for the early growth of the plant (Smith and Read, 2008; Rasmussen and Rasmussen, 2009), a process that is referred to as symbiotic germination. The symbiotic association with mycorrhizal fungi often continues during the adult stage, and mature orchids can

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range from autotrophy to full mycoheterotrophy (McKendrick et al., 2002; Cameron et al., 2008; Zimmer et al., 2008). There is a continuum from specialist plants associating with a narrow range of closely related mycorrhizal partners (Otero et al., 2004; Ogura-Tsujita and Yukawa, 2008; Roche et al., 2010; Swarts et al., 2010) to generalist species associating with a broad range of mycorrhizal fungi, sometimes belonging to distantly related clades (Otero et al., 2002; Stark et al., 2009; Jacquemyn et al., 2010, 2011).

Although specialized associations in both pollination and mycorrhizal associations could have played a central role in the unparalleled diversification of the Orchidaceae (Waterman and Bidartondo, 2008), strong specificity toward either pollinators or mycorrhizal fungi can come at a serious cost and affect the long-term viability of orchid species, particularly in our present-day, human-dominated landscapes. Since mycorrhizal associations have been shown to be a crucial factor determining the distribution and abundance of orchids (McCormick et al., 2012), a high degree of dependence to a fungal partner can be expected to increase the decline of rare species or species unable to switch fungal partners when a change in ecological conditions occurs (Swarts and Dixon, 2009). Recent analyses of mycorrhizal specificity in the genus *Caladenia* have, for example, shown that narrow specificity was related to rarity in this genus (Swarts et al., 2010). On the other hand, no link between high intrinsic rarity and mycorrhizal specificity was observed in the genus *Drakaea* (Phillips et al., 2011). In this case, the fungal partner was widely distributed and abundant, which can be expected to facilitate the encounter of orchid seeds with its obligate mycorrhizal fungi. Additionally, a species having specialized interactions with one or few fungi could benefit from more efficient exchanges with the latter, leading to enhanced growth and better development of the plant (Otero et al., 2005). However, evidence of mycorrhizal specialization affecting orchid decline and rarity is still rare, mostly because data investigating the relationship between decline and mycorrhizal specificity are largely lacking.

In this study, we investigated whether orchid persistence was related to mycorrhizal specificity in the orchid species *Anacamptis morio* (L.) R. M. Bateman, Pridgeon & M. W. Chase and *Dactylorhiza fuchsii* (Druce) Soó. Both species are terrestrial, photosynthetic orchids that grow in similar ecological conditions, sometimes co-occurring in the same sites. However, the two species have shown pronounced differences in decline during the last few decades, with populations of *A. morio* dramatically decreasing in several regions in Europe, whereas the distribution of *D. fuchsii* has remained more or less stable (Jacquemyn et al., 2005; Kull and Hutchings, 2006). In England for example, *A. morio* declined by about 49% between 1930 and 2000, while *D. fuchsii* decreased only about 14% (Kull and Hutchings, 2006). For a similar period in Estonia, where human pressure is weaker, the presence of *A. morio* decreased by 30%, while *D. fuchsii* incurred no loss at all (Kull and Hutchings, 2006). We hypothesize that, if mycorrhizal specificity is the driving factor determining orchid decline and rarity, *A. morio* should have a much higher degree of specificity toward its mycorrhizal associates (phylogenetic diversity of fungal associates; see Thompson, 1994; Taylor and Bruns, 1999; Taylor et al., 2003) than *D. fuchsii* does.

To test this hypothesis, we investigated in detail the fungal communities associated with *A. morio* and *D. fuchsii* in different Belgian populations and determined the degree of mycorrhizal specificity at the population scale using phylogenetic diversity (PD) as a measure of the phylogenetic relatedness. We also calculated the standardized effect size (SES) for PD to compare the observed phylogenetic relatedness with the expected patterns for null fungal communities and to determine whether the observed communities were phylogenetically clumped or overdispersed (evenness).

MATERIALS AND METHODS

***Study species*—**Species of the genus *Anacamptis* are widely distributed in the Euro-Mediterranean area, limited northward to Scandinavia, and spread eastward as far as Iran (Kretzschmar et al., 2007). Since the reorganization of the genus *Orchis*, the genus *Anacamptis* includes 16 species (Bateman et al., 2003), two of them (*A. morio* and *A. pyramidalis*) being found in Belgium. *Anacamptis morio* (the green-winged orchid, previously known as *Orchis morio*) is divided into six subspecies (Kretzschmar et al., 2007), which are found across most of Europe (from Portugal to Ukraine) and in some parts of the Mediterranean coastal zone. The most widespread of these subspecies, *A. morio* subsp. *morio*, is found in most parts of Europe. The southern limits of its distribution area are less clear due to confusion with *A. morio* subsp. *picta* (Bournérias et al., 2005). It can be found on both wet and dry soils ranging from slightly acid to basic (with a preference for nutrient-poor alkaline soils). It occurs in various habitats including grasslands, meadows, or very bright woods (Bournérias et al., 2005; Kretzschmar et al., 2007). In Belgium, it used to be quite common, but it has declined substantially between 1930 and 2000 (Jacquemyn et al., 2005). The species disappeared in more than 80% of the locations where it was previously known to occur, and in Flanders the species has probably gone extinct (T. Ceulemans, Division of Plant Ecology and Systematics, KU Leuven, personal communication).

The *Dactylorhiza* genus includes over 25 species (Bateman et al., 2003). Study of its taxonomy is quite complex due to high morphological variation of many taxa and the numerous intra- and intergenus hybrids. The species are widely distributed across Europe and can also be found in temperate Asia. *Dactylorhiza fuchsii* occurs in most of the Belgian territory and can be found in open to semiopen habitats, mostly on alkaline substrates. It grows in a wide variety of environments (dry grasslands, open woods on calcareous soil, meadows or swamps) (Bournérias et al., 2005). Unlike *A. morio*, the species has suffered no dramatic decline in Belgium and may even have enlarged its distribution area during the last decade (Van de Vijver, 2006).

***Sampling and molecular assessment of mycorrhizal fungi*—**Sampling was conducted in spring (April–May) 2009. For *A. morio*, root samples of 21 individuals were collected in a total of five populations spread over Belgium, whereas 18 individuals of *D. fuchsii* were investigated among another six populations (Table 1). The collected roots were surface-sterilized (30 s submergence in 1% sodium hypochlorite, followed by three 30-s rinses in sterile distilled water) and checked for mycorrhizal colonization using light microscopy. For each individual, 0.5 g of colonized root pieces was used to extract DNA using the UltraClean Plant DNA Isolation kit (Mo Bio Laboratories, Solana Beach, California, USA) following the manufacturer's recommendations. Obtained DNA was diluted 10 times before further processing.

The mycorrhizal community was assessed using a previously developed internal transcribed spacer (ITS)-based DNA array, enabling the simultaneous detection and identification of 23 or 21 operational taxonomic units (OTUs) based on a threshold of 97% and 95% of sequence similarity, respectively. Those OTUs were previously found associating with individuals of over 16 species of the genus *Orchis*, *Anacamptis*, and *Gymnadenia*, including *A. morio* (Jacquemyn et al., 2011). To simultaneously assess the mycorrhizal diversity on *D. fuchsii*, we extended the array with 10 additional OTUs that were detected in five species of the genus *Dactylorhiza* (including *Dactylorhiza fuchsii*, *D. incarnata*, *D. majalis*, *D. maculata*, and *D. praetermissa*) (Jacquemyn et al., 2012) using methods outlined in Lievens et al. (2010). To this end, first clone libraries were constructed for five individuals per species following PCR amplification with the Tulasnellaceae-specific primers ITS1-OF and ITS4-Tul (Taylor and McCormick, 2008). In a preliminary phase of this study, the performance of multiple primer pairs targeting different taxonomical levels, including the primer sets ITS1/ITS4, ITS1-OF/ITS4-OF, and ITS1-OF/ITS4-Tul, was evaluated. The results showed that the primer combination ITS1-OF and ITS4-Tul was the most efficient primer pair for these samples because it gave the most consistent amplification with high yields. In addition, many

samples were negative for the other primer pairs (data not shown). Ninety-six clones were randomly picked from each library and sequenced using the M13 forward primer. DNA sequences were aligned using the program MEGA4 (Tamura et al., 2007; http://www.megasoftware.net) under the Clustal W algorithm and manually edited. Sequences were shortened to conserved motifs identified in the regions flanking each sequence and grouped into OTUs using the program Mothur (Schloss et al., 2009), based on a 95% sequence similarity threshold. To identify the different OTUs, we queried representative sequences of each OTU (accessions JX649075–JX649095) against GenBank using the BLAST program (Table 1). Next, for each OTU, the existing DNA array was enlarged with a set of four specific detector oligonucleotides (Lievens et al., 2003, 2006), resulting in a DNA array capable of detecting 31 fungal OTUs based on a 95% cut-off value (Appendix S1, see Supplemental Data with the online version of this article). DNA arrays were produced as reported previously (Lievens et al., 2003, 2006, 2010), and all oligonucleotides were spotted in duplicate.

For DNA array analysis, the fungal ITS regions obtained from the samples of the different populations were amplified using both primer pairs ITS1-OF/ITS4-OF and ITS1-OF/ITS4-Tul (Taylor and McCormick, 2008) and simultaneously labeled with alkaline-labile digoxigenin (0.15 mmol/L digoxigenin-11-dUTP mix; Roche Diagnostics GmbH, Mannheim, Germany). These primers were shown to be specifically effective against a wide range of Basidiomycota and Tulasnellaceae, respectively (Taylor and McCormick, 2008). For the first primer set, DNA samples were amplified according to the following thermal cycling profile: initial denaturation at 94°C for 2 min; followed by 35 cycles of 45 s at 94°C, 45 s at 58°C and 45 s at 72°C; with a final elongation step at 72°C for 10 min. For the second primer set, the DNA samples were amplified according to the same protocol except for the annealing conditions, which were 65°C for 15 s. The resulting labeled amplicons from both reactions were subsequently combined and used for DNA array hybridization as previously described (Lievens et al., 2003, 2006). All hybridizations were performed twice to check for consistency of the results.

***Data analysis*—**To investigate phylogenic relationships between the different OTUs detected in the roots of both orchid species, two representative ITS sequences for each OTU were chosen to construct three phylogenetic trees representing three fungal groups. Additional orchid mycorrhizal fungi sequences (mainly based on Girlanda et al., 2011) were included as well. Sequences were aligned using the program Clustal_X 2.1 (Thompson et al., 1997) and the trees were computed using the program MrBayes 3.2 (Ronquist et al., 2012). Difficulties of alignment due to important phylogenetic distances between the OTUs detected (Roberts, 1999) forced us to separate the Tulasnellaceae and Ceratobasidiaceae members to construct phylogenies. Based on Girlanda et al. (2011), we separated the Tulasnellaceae in two subgroups (A and B). Based on the corrected Akaike information criterion (AICc) (Sugiura, 1978) calculated in the program Kakusan 4 for Windows operating system (Tanabe, 2011), the GTR+G nucleotide substitution model was selected as the best model for tree computation. Two simultaneous, independent runs per fungal group were performed for 5 000 000 generations starting from random trees. Trees were sampled every 500 generations, resulting in a total of 10 001 trees per run from which the first 2500 (25%) were discarded as the burn-in phase. Based on the remaining sampled trees, 50% majority rule consensus trees were calculated, enabling the use of Bayesian posterior probabilities (BPP) as node support.

Phylogenetic measures of specificity have proven to be an efficient way of quantifying specificity (Taylor et al., 2003; Shefferson et al., 2007, 2010). It differs from the simple count of fungal symbiotic species by including information about the phylogenetic distances between the fungal associates. The relatedness of the associated mycorrhizal fungi allows assessing whether host plants rely on closely related fungi (specialized interactions) or a group of genetically distant fungi (generalist interactions). The Picante package (Kembel et al., 2010) in the program R 2.13 (R Development Core Team, 2011) was used to quantify the degree of specificity of the mycorrhizal associations within and among sampling sites for both species. To do so, we coupled phylogeny with community data to determine the local fungal community phylogenetic structures. The phylogenetic distance (PD) (Faith, 1992) was first calculated per population, and standardized effect sizes (SES_{PD}) were then inferred to correct the effect of sample size. A *t* test was performed to assess whether the number of OTUs per population and PD values differed significantly between species. The statistical tests were performed with the program SPSS Base 17.0 for Windows (SPSS, Chicago, Illinois, USA).

RESULTS

***Mycorrhizal associates and fungal phylogeny*—**Using a cut-off value of 95%, 15 OTUs were detected associating with the two orchid species. BLAST analysis revealed that most OTUs belonged to the Tulasnellaceae (Table 1), and two nontulasnelloids belonged to the Ceratobasidiaceae. Seven of the Tulasnellaceae OTUs clustered in subgroup A as defined by Girlanda et al. (2011) (Fig. 1), whereas the other six belonged to subgroup B (Fig. 2). The ceratobasidioid OTUs appeared to be in different groups of the Ceratobasidiaceae tree found in the same study (Fig. 3).

In the five populations of *A. morio* investigated, 13 OTUs were detected (Table 1). OTU C1 was the only Ceratobasidiaceae OTU

TABLE 1. Phylogenetic affiliation of the different operational taxonomic units (OTUs) based on ITS sequences (cut-off value of 95%) detected in *A. morio* and *D. fuchsii* roots.

			Phylogenetic relationship			
OTU	Detected in	Sequence length (bp)	Family	Closest match in GenBank (accession no.)	Sequence identity (%)	Related to
OTU A1	Both sp.	728	Tulasnellaceae	Uncultured *Tulasnella* clone 1124a (FJ788890)	94	Tul A4 [a]
OTU A2	Both sp.	705	Tulasnellaceae	*Tulasnella irregularis* isolate C3-DT-TC-2 (GU166423)	97	—
OTU A3	*A. morio*	782	Tulasnellaceae	Uncultured *Tulasnella* clone 18tu-10 (HM230650)	97	Tul A1 [a]
OTU A4	*A. morio*	753	Tulasnellaceae	Uncultured *Tulasnella* mycobiont of *Riccardia multifida* clone 9592B (EU909305)	98	Tul A2 [a]
OTU A5	Both sp.	745	Tulasnellaceae	*Tulasnella calospora* strain MAFF P305801 (DQ388041)	96	Tul A1 [a]
OTU A6	*D. fuchsii*	759	Tulasnellaceae	*Tulasnella* sp. 145 (AY373276)	97	Tul A2 [a]
OTU A7	Both sp.	737	Tulasnellaceae	*Tulasnella calospora* strain MAFF P305805 (DQ388045)	98	Tul A1 [a]
OTU B1	*A. morio*	660	Tulasnellaceae	Uncultured Tulasnellaceae isolate CT96 (GQ241740)	96	OTU 1 [b], OTU 2 [b], OTU 3 [b]
OTU B2	*A. morio*	699	Tulasnellaceae	Uncultured Tulasnellaceae isolate 451 (EU195344)	97	OTU 6 [b]
OTU B3	Both sp.	677	Tulasnellaceae	Uncultured Tulasnellaceae isolate A1.14 (EU583697)	98	OTU 7 [b]
OTU B4	*A. morio*	701	Tulasnellaceae	Uncultured Tulasnellaceae isolate CT100 (GQ241745)	96	OTU 10 [b]
OTU B5	*A. morio*	683	Tulasnellaceae	Uncultured Tulasnellaceae isolate S4.4 (EU583714)	99	OTU 12 [b], Tul B6 [a]
OTU B6	*A. morio*	663	Tulasnellaceae	Uncultured Tulasnellaceae isolate 4065 (AY634130)	91	OTU 18 [b]
OTU C1	*A. morio*	658	Ceratobasidiaceae	Uncultured Ceratobasidiaceae isolate 7837.2.OR (EU668239)	99	OTU 11 [b], Cer 7 [a]
OTU C2	*D. fuchsii*	729	Ceratobasidiaceae	*Ceratobasidium* sp. L9Rh-col6 (HM117643)	97	OTU 23 [b], Cer 4 [a]

[a] Girlanda et al., 2011
[b] Jacquemyn et al., 2011

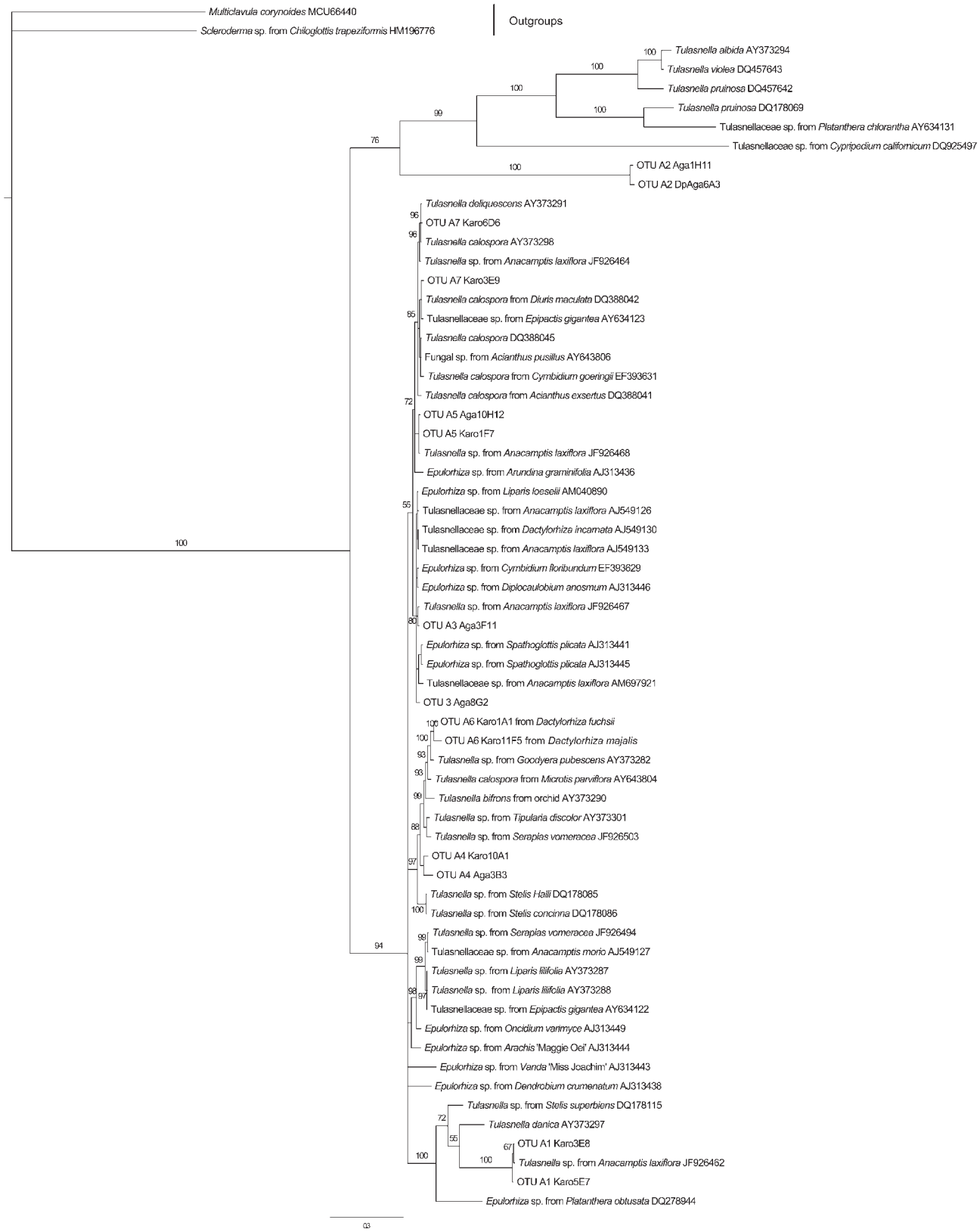

Fig. 1. Bayesian 50% majority consensus tree based on internal transcribed spacer (ITS) sequences of Tulasnellaceae fungi (subgroup A from Girlanda et al., 2011). The tree was computed under the GTR+G substitution model (5 000 000 generations run) and includes representatives of European, American, and Australian meadow and forest photosynthetic orchids, tropical terrestrial and epiphytic orchids, nonorchid species, and fungal strains and fruitbodies. *Multiclavula corinoides* and *Scleroderma* sp. were used as outgroup taxa. Branch support: Bayesian posterior probabilities (BPP).

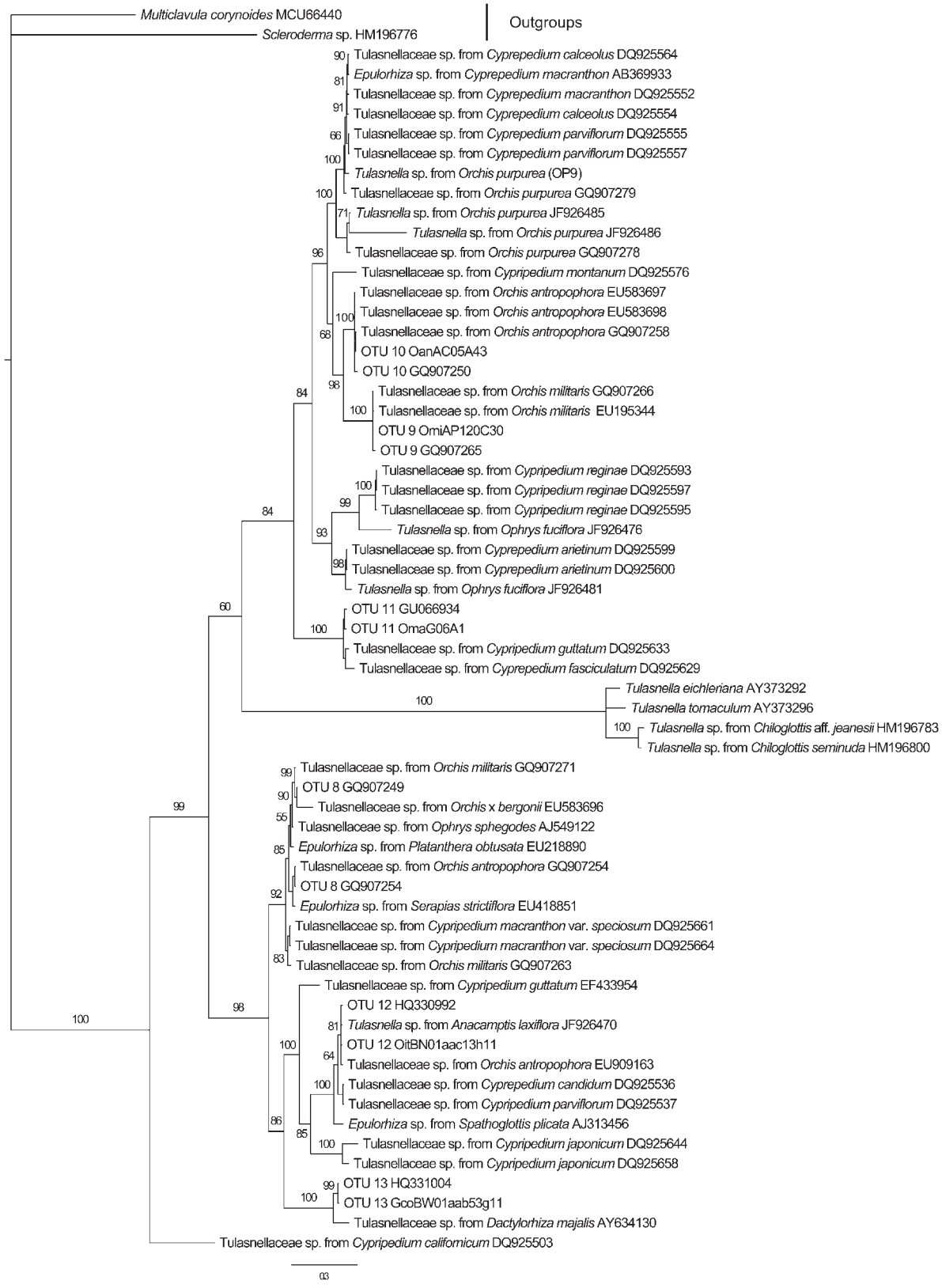

Fig. 2. Bayesian 50% majority consensus tree based on internal transcribed spacer (ITS) sequences of Tulasnellaceae fungi (subgroup B from Girlanda et al., 2011). The tree was computed under the GTR+G substitution model (5 000 000 generations run) and includes representatives of European, American, and Australian meadow and forest photosynthetic orchids, tropical terrestrial and epiphytic orchids, nonorchid species, and fungal strains and fruitbodies. *Multiclavula corinoides* and *Scleroderma* sp. were used as outgroup taxa. Branch support: Bayesian posterior probabilities (BPP).

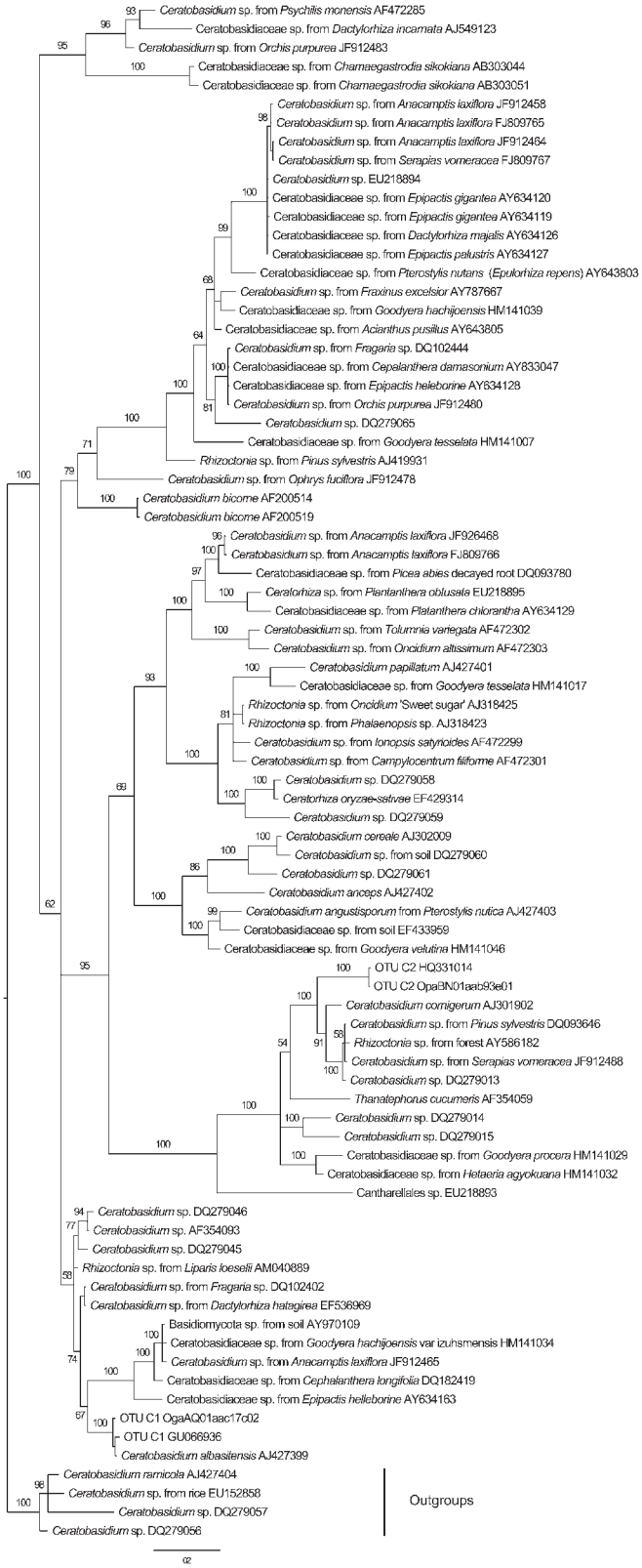

Fig. 3. Bayesian 50% majority consensus tree based on internal transcribed spacer (ITS) sequences of Ceratobasidiaceae fungi. The tree was computed under the GTR+G substitution model (5 000 000 generations run) and includes representatives of European, American, and Australian meadow and forest photosynthetic orchids, tropical terrestrial and epiphytic orchids, nonorchid species, and fungal strains and fruitbodies. *Ceratobasidium ramicola*, *Ceratobasidium* sp. (EU152858), *Ceratobasidium* sp. (DQ279057), and *Ceratobasidium* sp. (DQ279056) were used as outgroup taxa. Branch support: Bayesian posterior probabilities (BPP).

associating with *A. morio*. The species has three main fungal partners (representing more than 10% of the mycorrhizal associations each): OTU A2 (23%), OTU B3 (17%), and OTU A7 (13%) (Fig. 4). Another four OTUs were frequently found (OTU A5, OTU C1, OTU B2, and OTU B4, representing 34% of the associations together), and the remaining six accounted for 13% of the cases where symbiotic associations with mycorrhizal fungi were established.

Dactylorhiza fuchsii associated with seven different OTUs (Table 1). As for *A. morio*, most of the mycorrhizal associates detected for the species belonged to the Tulasnellaceae, with only OTU C2 belonging to the Ceratobasidiaceae. The four main OTUs encountered represented more than 90% of the observed associations (OTU A1, OTU A5, OTU A6, and OTU A7, Fig. 4). The three occasionally occurring OTUs represented merely 9% of the associations for this species.

A single *A. morio* individual was found to associate with two to six different fungi, while *D. fuchsii* was found to associate with one to five fungal OTUs per single plant (data not shown). OTU A2 and A7 were the only fungal partners detected in each population of *A. morio*, while three were found in all the *D. fuchsii* populations investigated (OTU A1, OTU A6, and OTU A7). On the other hand, up to six mycorrhizal partners (OTU A3, OTU A4, OTU B1, OTU B5, OTU B6, and OTU C1) for *A. morio* and three (OTU A2, OTU B3, and OTU C2) for *D. fuchsii* were found in a single population. *Anacamptis morio* did not associate with significantly more OTUs per population than *D. fuchsii* did (mean ± SD = 5.6 ± 1.34 and 4.0 ± 0.89 respectively; $t = 2.28$, $P = 0.06$).

***Community structure*—**The phylogenetic distance results based on the Tulasnellaceae fungal partners are given in Table 2. In *A. morio*, PD values of the tested populations were high (>2.3), whereas in most populations of *D. fuchsii*, PD values were close to 1.0 (Table 2). PD values differed significantly between the two species (mean ± SD = 2.70 ± 0.26 and 1.23 ± 0.56; $t = 5.734$, $P = 0.001$). Looking at the standardized effect sizes, there is also a clear difference between the two orchid species. In *A. morio*, most SES_{PD} values, which indicate the difference in phylogenetic distances between the observed communities and the null communities were positive, highlighting phylogenetic evenness as compared to their respective null communities. In contrast, SES_{PD} values for *D. fuchsii* were all negative, and significant for the community of Han-Sur-Lesse ($SES_{PD} = -3.85$; $P = 0.02$).

DISCUSSION

***Mycorrhizal associates*—**We showed that *A. morio* and *D. fuchsii* associated with members of Tulasnellaceae and Ceratobasidiaceae, which are the main mycorrhizal partners for terrestrial orchids (Rasmussen, 2002; Dearnaley, 2007; Yukawa et al., 2009). The Tulasnellaceae highlighted in our study belonged to two separate clades, subgroup A and subgroup B (Fig. 1). While *A. morio* associated with a total of 12 OTUs belonging to the two groups, *D. fuchsii* associated mainly with fungi from the first group (five of its six associating Tulasnellaceae). Additionally, both orchid species associated with a lineage related to the Ceratobasidiaceae (OTU C1 for *A. morio* and C2 for *D. fuchsii*). Only few OTUs could be accurately matched with existing ITS sequences of *Rhizoctonia* fungi. Three OTUs were identified to the species level. OTU A2 matched with a sequence of *Tulasnella irregularis* (Table 1), a symbiont of epiphytic orchids both in natural populations (Warcup and Talbot, 1980) and horticultural settings (Nontachaiyapoom et al., 2010). It is the main mycorrhizal partner of *A. morio* and has been detected for *D. fuchsii* as an occasional partner (Fig. 4). OTU A5 and OTU A7 matched with *Tulasnella calospora*, a well-known orchid mycorrhizal fungus (see Roberts, 1999) that is considered as a generalist in this type of symbiosis. A7 is the only OTU that was found in all populations for both orchid species (data not shown).

Our observations for *A. morio* are remarkably consistent with recent results reported for the closely related *Anacamptis laxiflora* (Girlanda et al., 2011; Table 1). In this species, a total of nine different OTUs was described, the majority of them being *Tulasnellaceae* spp. Members of the Ceratobasidiaceae were also detected in the same study. These results together with ours suggest that members of the Tulasnellaceae are the primary associates in both orchid species, whereas members related to the Ceratobasidiaceae serve as sporadic associates. These associations are corroborated by laboratory experiments of Dijk and Eck (1995), who found good compatibility in the laboratory between *A. morio* and two Tulasnellaceae strains (*Epulorhiza repens*), but not with two Ceratobasidiaceae (*Ceratorhiza* sp.) strains.

***Mycorrhizal specificity and orchid decline*—**In accordance with the results on the genus *Orchis* (Jacquemyn et al., 2011) or *Drakaea* (Phillips et al., 2011) and in contrast with Swarts et al. (2010) for the endangered orchid species *Caladenia huegelii*, we found no indications that orchid rarity and decline were related to mycorrhizal specificity. In our case, the species that was most abundant and had hardly declined during the last decades (*D. fuchsii*) associated with a smaller range of fungal partners, both in terms of phylogenetic distance and number of associates than the declining species. The mean number of fungal associates per population was higher for the latter. In contrast to our prediction that phylogenetic diversity of mycorrhizal communities should be low in the declining species, we found high PD values for *A. morio*. On the other hand, in the relatively resilient *D. fuchsii*, most populations were specialized toward a small number of fungal OTUs and had similar low levels of PD. Only in one population (Han-Sur-Lesse) was significant clustering of the OTUs on the phylogeny detected.

Phillips et al. (2011) cited another pattern of mycorrhizal specificity that is not based on associations with fungi from a narrow phylogenetic range. In this case, orchids associated locally with a small range of fungi, but with many different fungal partners along the species distribution range. This pattern has been observed for several orchid species (McKendrick et al., 2002; Martos et al., 2009) and allows a locally specialized species to remain an ecological generalist. In such a pattern, the association with rare fungi could also cause orchid species to be more susceptible to decline, even at a small scale. However, in our study, *A. morio* individuals associated with a maximum of six OTUs per population (not shown), some of which appeared to be generalist fungi. For example, mycorrhizal associations were observed with the very common, generalist orchid mycorrhizal partner *T. calospora*. Regardless of the taxonomic problems in this taxon (Suárez et al., 2006), the two OTUs corresponding to *T. calospora* were related to strains (GenBank ID: DQ388041 and DQ388045) that were observed in the northern Andes of southern Ecuador. The fact that those strains were also present in Belgium indicates a wide distribution in very differing ecological conditions and environments. It also suggests that *A. morio* associates with possibly ubiquitous mycorrhizal

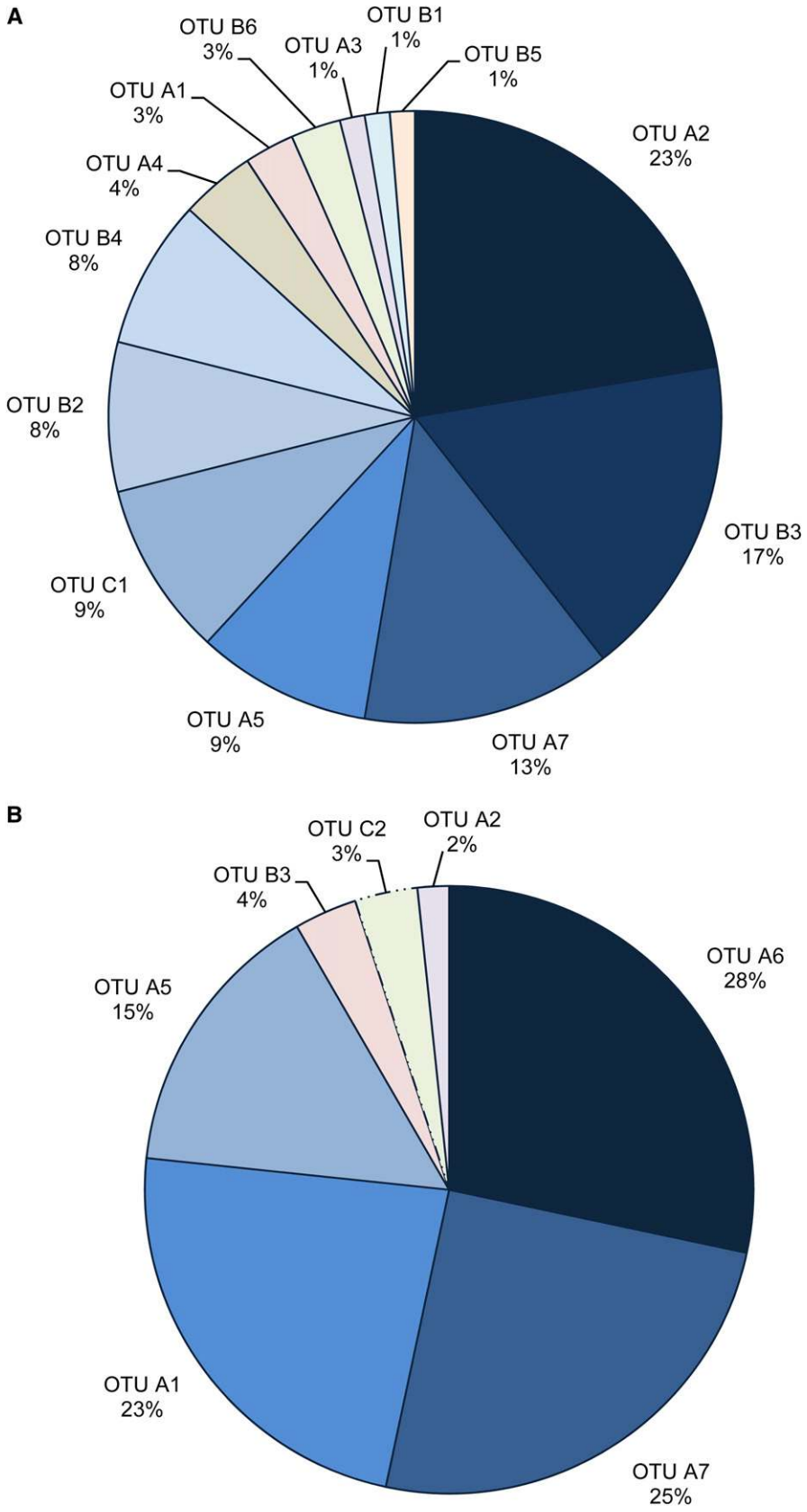

Fig. 4. Pie charts representing the relative abundance of the mycorrhizal operational taxonomic units (OTUs) detected in (A) *Anacamptis morio* and (B) *Dactylorhiza fuchsii*.

TABLE 2. Phylogenetic distance (PD) and standardized effect sizes (SES_{PD}) values in the sampled populations (only taking into account the Tulasnellaceae).

Location	Individuals sampled	Number OTUs	PD obs.	SES_{PD}	SES_{PD} *P*
Bonnerieux	8	5	2.68 [a]	0.62	0.78
Rochefort	4	7	2.85	0.33	0.57
Hour	4	5	2.68	0.66	0.75
Viroin A	2	4	2.30	0.02	0.28
Viroin B	3	7	2.99	1.11	0.86
Hobokense Polder	2	3	0.93 [a]	−1.47	0.17
Rechteroever	3	3	0.93	−1.32	0.21
Ter Yde	2	4	0.94	−2.41	0.09
Torfbroek	5	4	0.94	−2.62	0.07
Baronville	3	5	2.33	−0.44	0.16
Han Sur Lesse	3	5	1.32	−3.85*	0.02

Notes: Obs. = observed. Upper part: *A. morio*, lower part: *D. fuchsii*.
* Significant at $P < 0.05$.
[a] Association with a Ceratobasidiaceae, not taken into account in the calculations

fungi and therefore cannot be considered as a specialist as evoked by Phillips et al. (2011).

The reasons underlying *A. morio* decline are probably related to human-induced disturbances. Comparing temporal changes in distribution of a wide range of orchid species in Estonia and the United Kingdom, Kull and Hutchings (2006) showed that in a less densely populated region orchid decline was less pronounced for most species including *A. morio*. Human activities threaten orchids in a variety of ways, particularly through habitat fragmentation and altered environmental conditions (Swarts and Dixon, 2009). Investigating the effect of soil fertilization on *A. morio* and *D. fuchsii* growth ex situ, McKendrick (1996a) showed that *A. morio* was not significantly negatively affected by fertilizer application, but *D. fuchsii* was. In a related study, McKendrick (1996b) also showed that growth of *A. morio* was only slightly affected by increased shading, while *D. fuchsii* was more susceptible to this factor. These ex situ experiments seem to indicate that *A. morio* is less susceptible to environmental changes (in the case of fertilization and shading) than *D. fuchsii*. However, Silvertown et al. (1994) showed a significant adverse effect of low levels of fertilizer application in situ on *A. morio* flowering. The authors suggested that increased competition with the vegetation was the main problem in most treatments, while the application of phosphorus seemed to have toxic effects on the orchid. Similarly, Jersáková et al. (2002) showed that *A. morio* is susceptible to a lack of management of meadows and pastures where it is frequently found. Other human-mediated impacts such as habitat loss and fragmentation probably played a determinant role on the decline of *A. morio* in Belgium.

Early germination of the seeds and protocorm development are other factors that should be taken into account. They can be limited by a narrower range of associations with fungi that could differ greatly from the associations found in mature plants. McCormick et al. (2004) showed that the protocorms of *Tipularia discolor*, a photosynthetic terrestrial orchid, had greater fungal specificity than their adult relatives did. Similar observations were made for *Platanthera leucophaea*; *Ceratorhiza* isolates found in mature individuals could initiate seed germination for this orchid species, whereas *Epulorhiza* isolates were only found in mature plants and failed to initiate germination (Zettler and Piskin, 2011). Therefore, additional work should investigate the different stages of the orchid life cycle to assess potential effects of mycorrhizal specialization on species decline.

***Future perspectives*—**The results of this study do not support our initial predictions that orchid rarity is related to mycorrhizal specificity. First, in both the common and rare species, we found a rather low overall specificity toward mycorrhizal fungi. Second, the common *D. fuchsii* associated with fewer fungal OTUs than did the rare *A. morio*, and the phylogenetic relatedness of mycorrhizal communities among populations was higher in the common orchid than in the rare. However, our study focused solely on two species and cannot be considered as representative of the whole family. Future research focusing on interspecies variation (at different life stages) and species with different lifeforms (e.g., epiphytic species) are therefore necessary to understand thoroughly the impact of mycorrhizal partners on orchid decline. Comparing terrestrial orchids from temperate regions with tropical epiphytic orchids will be a first necessary step. Since most terrestrial orchids have aboveground photosynthetic organs that senesce during the winter and therefore strongly rely either on resources stored in the roots, tubers, and/or on their mycorrhizal partners for growth in the spring, most epiphytic orchid species retain their photosynthetic organs all year. One could therefore hypothesize that they are less dependent on a highly efficient fungal partnership, which, in turn, could favor a more generalist-type of association compared with terrestrial species. In any case, for the purpose of conservation of endangered or declining orchid species, mycorrhizal associations are a factor that cannot be neglected and thus need careful attention. A better understanding of the processes linking mycorrhizal fungi with orchid decline and rarity is of the highest importance to set up efficient conservation and reintroduction strategies and preserve the diversity of this huge but nonetheless sensitive family.

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