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Hidden diversity of endophytic fungi in an invasive plant[1]

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Fungal endophytes are important in plant ecology and common in plants. We attempted to test cointroduction and host-jumping hypotheses on a community basis by comparing endophytes isolated from invasive spotted knapweed (*Centaurea stoebe*, Asteraceae) in its native and invaded ranges. Of 92 combined, sequence-based haplotypes representing eight classes of Fungi, 78 occurred in only one of the two ranges. In the native range of *C. stoebe*, one haplotype of *Alternaria alternata* was clearly dominant, whereas in the invaded range, no haplotype was dominant. Many haplotypes were closely related to one another and novel. For example, six putative, new species of *Botrytis* were discovered as endophytes of *C. stoebe*, which has never been reported to have *Botrytis* spp.. Apparent differences between the two communities of endophytes were significant according to an analysis of similarity, but phylogenetic community structure did not differ significantly between the ranges. Both host-jumping and cointroduction of fungal endophytes likely took place during the spotted knapweed invasion.

Key words: Asteraceae; *Centaurea stoebe*; *Botrytis* phylogeny; diversity; endophytes; geographic origins of fungi; native and invaded ranges; spotted knapweed.

Endophytic fungi grow inside plant tissue without causing any obvious symptoms and are an important though widely neglected component of plant communities. Endophytes have recently been shown to be key elements in plant symbiosis, affecting host tolerance to stressful conditions (Redman et al., 2002; Rodriguez et al., 2004; Marquez et al., 2007), plant defense (Omacini et al., 2001; Bailey et al., 2006), plant growth (Ernst et al., 2003), and plant community biodiversity (Clay and Holah, 1999). However, the diversity, geographic distribution, and host specificity of endophytes remain largely unknown (Arnold et al., 2001; Otero et al., 2002, 2007; Ganley et al., 2004; van Bael et al., 2005; Arnold and Lutzoni, 2007; Higgins et al., 2007). Recent estimates of fungal diversity (Hawksworth, 1991, 2001; Cannon, 1997; Schmit and Mueller, 2007) suggest that more than 90% of fungal species are not described, and there are no specific estimates of the number of existing endophytes (Hyde et al., 2007).

The geographic distributions of specific fungi are under recent study (Taylor et al., 2006). For example, Gladieux et al. (2008) showed that *Venturia inaequalis*, causal agent of apple scab, is likely native to the same area in central Asia where apple itself was domesticated. Similarly, an Asian origin for the dry rot fungus *Serpula lacrymans* has been inferred from a study of its genetic variation (Kauserud et al., 2007). Intense interest in the origins of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, has resulted in a hypothesis that it originated in South Africa (Weldon et al., 2004). The human dermatophyte *Trichophyton rubrum* appears likely to have evolved in Africa, given that its genotypic diversity was highest there (Gräser et al., 2007). Fungal species endemic to Antarctica have been described recently (De Hoog et al., 2005). However, even phytopathogenic fungi, such as the *Ophiostoma* species that have caused global pandemics of Dutch elm disease in the past century, remain of uncertain geographic origin (Brasier and Buck, 2001). This uncertainty is surprising given the potentially devastating consequences of introducing a fungal pathogen to a new geographic range (e.g., the introduction into North America of the chestnut blight pathogen). In general, without baseline mycofloras from early in the Age of Exploration (15th century), it is possible that the current geographic distributions of fungi may reflect both recent homogenization events and original ranges. This possibility is especially true for endophytes that could have been dispersed during plant introductions, both deliberate and inadvertent (Cox, 2004).

In general, an exotic, invasive plant can either colonize its invaded range accompanied by fungi from its native range (i.e., the cointroduction hypothesis), or it can leave its native associates behind and acquire new associates (i.e., the host-jumping hypothesis). The ecological implications of these two scenarios for plant invasions are unknown. Invasive plants are less competitive and less abundant in their native ranges than in their invaded ranges (Broennimann et al., 2007), and endophytes could contribute to the greater competitiveness that is seen in the invaded ranges. For example, in the host's invaded range, cointroduced endophytes could act as novel weapons (Bais et al., 2003; Callaway and Ridenour, 2004; Cappuccino and Arnason, 2006) that would increase the competitiveness of their hosts by producing novel allelochemicals inhibitory to evolutionarily naive plants. Alternatively, endophytes acquired in the invaded range through host-jumping might enhance the competitiveness of invasive plants if those endophytes were

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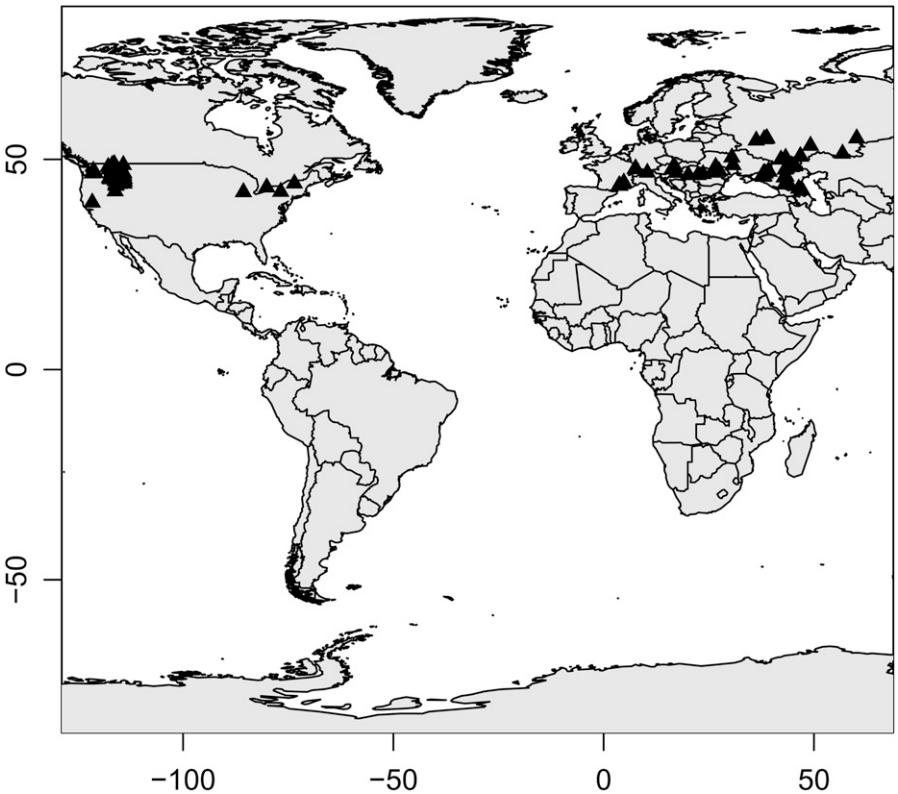

Fig. 1. Endophyte sampling sites of *Centaurea stoebe* in its native (Eurasia) and invaded (North America) ranges.

mutualists (i.e., the enhanced mutualism hypothesis: Richardson et al., 2000; Klironomos, 2002; Reinhart and Callaway, 2006). However, in spite of their importance as common symbionts of plants and potential contributors to invasiveness, the endophyte status of invasive plants is rarely determined, and the geographic origins of endophytes have never been conclusively investigated.

Centaurea stoebe L. s. l. (= *C. maculosa*, *C. pseudomaculosa*, *C. biebersteinii*, *C. micrantha*, Asteraceae), or spotted knapweed, is an established model system in invasion biology research (Ochsmann, 2000; Cox, 2004; Inderjit, 2005; DiTomaso and Healy, 2006; Nentwig, 2007). This Eurasian plant has a wide distribution in its native range: from western France to northwestern Kazakhstan, across the Ural Mountains, and extending into southwestern Siberia (Klokov, 1963; Czerepanov, 1994; Ochsmann, 2000, 2001). Its invasion in North America started in the late 1890s, and the plant now infests millions of hectares in North America causing more than 150 million U.S. dollars of economic damage annually (Pimentel et al., 2000; Story et al., 2006).

Our experiments to date (A. Shipunov, G. Newcombe, and A. Raghavendra, unpublished data) have indicated that endophytes can affect the competitiveness of *C. stoebe*. As both cointroduction and host-jumping of endophytes align with hypotheses of plant invasion that are based on enhanced competitiveness, we sought here to compare endophyte communities from the two ranges of *C. stoebe* and to test these hypotheses with traditional comparative and phylogenetic methods. In addition, we tried to shed some light on the extent of taxonomic novelty in the endophyte communities of one invasive plant species.

MATERIALS AND METHODS

Sampling—In 2003–2006, we collected *Centaurea stoebe* from sites in both its native and invaded ranges. We sampled a total of 53 populations of knapweed in the invaded range, primarily from the northwestern United States (i.e., Montana, Idaho, Washington, and Oregon), where knapweed infestation is common (Fig. 1), with additional sites in Canada (British Columbia and Ontario) and the eastern United States (Michigan and New York). In three cases, we repeated sampling from the same knapweed populations (all in the Palouse region of northern Idaho) in 2004 and 2006. We sampled a total of 49 populations in the native range, along a broad transect, from western Europe through the Ural Mountains and from Moscow (central Russia) to Dagestan in northeastern Caucasus.

All sampling was done according to a standard protocol. Sampling sites were at least 25 km apart. From each site, 5–16 plants were collected. Plants in any given location were usually sampled along a transect at 3-m intervals. Because the identification of spotted knapweed in its native range can be problematic (Ochsmann, 2001), we used a standard identification key (Ochsmann, 2000) for all determinations and photographed plants and flowering heads with bracts for most samples in situ. From each individual plant, 10–20 flowering heads with ripe achenes were collected. All collected seed heads were refrigerated to prevent loss or "storage succession" (Dugan and Lupien, 2002) of fungal symbionts.

Isolation and cultivation of fungi—Endophytes were isolated from achenes after ethanol–bleach surface sterilization of plant tissues (Schulz et al., 1993; method II: 30 s in 96% ethanol, 3 min in 15% NaOCl solution, and 30 s in ethanol), and cultured on potato dextrose agar (PDA). In all, 8763 achenes were used for isolation. Most of the endophyte isolates began to grow within the first 10 d of plating. The cultures were kept for one month or until it became clear that no more fungal isolates would appear. Each isolate received its own cultivation identification number (CID). After isolation, cultures were classified morphologically (microscopic and culture characters were used), and selected isolates (one per group) were subcultured. DNA from all pure isolates was sequenced (described next) to assist in species identification. All fungi were

TABLE 1. Haplotypes of endophytes of *Centaurea stoebe*, their abundance, taxonomic affinities, and GenBank accession numbers. The first GenBank number is for the ITS sequence; the second is for the *Alt a 1* sequence for Pleosporales, and for the heat shock protein 60 (*HSP60*) sequence for Helotiales (*Botrytis*). Asterisks indicate exact matches with NCBI GenBank BLAST search. Ordinal and class names are in accordance with the most recent classification of Fungi (Hibbett et al., 2007).

Haplotype	% Abundance in		Species	Order	Class	GenBank accessions
	Invaded range	Native range				
acr1617	0	0.08	*Acremonium strictum* *	Hypocreales	Sordariomycetes	EF682095
alt002a	16.6	6.46	*Alternaria tenuissima*	Pleosporales	Dothideomycetes	EF589849, EF589831
alt002b	10.39	43.54	*Alternaria alternata* *	Pleosporales	Dothideomycetes	EF589849, EF589830
alt002c	0.11	11.7	*Alternaria alternata*	Pleosporales	Dothideomycetes	EF589849, EF589832
alt002f	2.03	6.08	*Alternaria longipes*	Pleosporales	Dothideomycetes	EF589849, EF589833
alt002j	2.46	0	*Alternaria longipes*	Pleosporales	Dothideomycetes	EF589849, EF589828
alt002k	0.11	0	*Alternaria longipes*	Pleosporales	Dothideomycetes	EF589849, EF589829
alt002m	0	3.8	*Alternaria longipes*	Pleosporales	Dothideomycetes	EF589849, EF589847
alt002q	0	0.76	*Alternaria alternata*	Pleosporales	Dothideomycetes	EF589849, EF682121
alt002r	0	0.91	*Alternaria alternata*	Pleosporales	Dothideomycetes	EF589849, EF682122
alt076e	3.43	0	*Alternaria conjuncta*	Pleosporales	Dothideomycetes	EF589850, EF589836
alt076ea	0.11	0	*Alternaria oregonensis*	Pleosporales	Dothideomycetes	EF589850, EF589846
alt076u	0.64	0	*Alternaria oregonensis* *	Pleosporales	Dothideomycetes	EF589850, EF682120
alt1043e	0	0.46	*Alternaria conjuncta*	Pleosporales	Dothideomycetes	EF682096, EF589836
alt417c	0	0.99	*Alternaria alternata*	Pleosporales	Dothideomycetes	EF589851, EF589832
alt417t	0	0.08	*Alternaria metachromatica*	Pleosporales	Dothideomycetes	EF589851, EF682119
alt459b	1.07	1.9	*Alternaria alternata* *	Pleosporales	Dothideomycetes	EF589852, EF589830
alt459c	0	0.08	*Alternaria alternata*	Pleosporales	Dothideomycetes	EF589852, EF589840
alt674n	0.11	0	*Alternaria malorum*	Pleosporales	Dothideomycetes	EF589853, EF589848
alt704v	0.11	0	*Alternaria photistica*	Pleosporales	Dothideomycetes	EF682097, EF682124
alt840o	0.11	0	*Alternaria arborescens* *	Pleosporales	Dothideomycetes	EF682098, EF682125
alt842s	3	0	*Alternaria conjuncta*	Pleosporales	Dothideomycetes	EF682099, EF682123
amp1634	0	0.15	*Ampelomyces humuli*	Pleosporales	Dothideomycetes	EF682100
asp1375	0	0.3	*Aspergillus* sp.	Eurotiales	Eurotiomycetes	—
aub061	5.35	6.31	*Aureobasidium pullulans* *	Dothideales	Dothideomycetes	EF589854
bot017	0.86	4.26	*Botrytis cinerea* *	Helotiales	Leotiomycetes	EF589855, EU386602
bot079	2.89	1.67	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589856, EU386596
bot080	0.54	0	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589857, EU386597
bot093	3.32	0	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589858, EU386598
bot095	3.43	0	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589859, EU386599
bot109	0.32	0	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589860, EU386600
bot1093	0	0.15	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589855, EU386601
bot360	1.07	0	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589861, EU386603
bot361	0.43	0	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589862, EU386604
bot378	0.43	0.23	*Botrytis* sp.	Helotiales	Leotiomycetes	EF589863, EU386605
cla063	11.24	0.08	*Cladosporium herbarum* *	Capnodiales	Dothideomycetes	EF589865
cla280	0.43	0	*Cladosporium herbarum*	Capnodiales	Dothideomycetes	EF589866
cla307	3.85	1.06	*Cladosporium cladosporioides* *	Capnodiales	Dothideomycetes	EF589867
cop1342	0	0.08	*Coprinus* sp.	Agaricales	Agaricomycetes	EF682101
dia016	0	0.99	*Phomopsis* sp.	Diaporthales	Sordariomycetes	EF589868
dia1330	0	0.08	*Diaporthe helianthii*	Diaporthales	Sordariomycetes	EF682102
dia1465	0	0.23	*Diaporthe helianthii*	Diaporthales	Sordariomycetes	EF682103
dre1670	0	0.15	*Bipolaris* sp. *	Pleosporales	Dothideomycetes	EF682104
epi066	11.56	1.06	*Epicoccum* sp.	Pleosporales	Dothideomycetes	EF589869
epi1423	0	0.38	*Epicoccum* sp.	Pleosporales	Dothideomycetes	EF682105
epi278	0.32	0	*Epicoccum* sp. *	Pleosporales	Dothideomycetes	EF589870
epi328	0.86	0	*Epicoccum* sp. *	Pleosporales	Dothideomycetes	EF589871
epi497	0.64	0.46	*Epicoccum* sp.	Pleosporales	Dothideomycetes	EF589872
ere1339	0	0.23	*Eremothecium coryli*	Saccharomycetales	Saccharomycetes	EF682106
ere1574	0	0.08	*Eremothecium cymbalariae* *	Saccharomycetales	Saccharomycetes	EF682107
fus025	0	0.3	*Fusarium tricinctum* *	Hypocreales	Sordariomycetes	EF589873
fus026	0	0.61	*Gibberella avenacea* *	Hypocreales	Sordariomycetes	EF589874
fus027	0	0.23	*Fusarium tricinctum* *	Hypocreales	Sordariomycetes	EF589875
fus044	0	0.23	*Fusarium equiseti* *	Hypocreales	Sordariomycetes	EF589876
fus090	0.11	0	*Fusarium sporotrichioides*	Hypocreales	Sordariomycetes	EF589877
fus1003	0.11	0	*Fusarium* sp.	Hypocreales	Sordariomycetes	—
fus124	0.96	0	*Fusarium* sp. *	Hypocreales	Sordariomycetes	EF589878
fus1369	0	0.08	*Fusarium pulverosum* *	Hypocreales	Sordariomycetes	EF682108
fus396	0	0.23	*Gibberella avenacea*	Hypocreales	Sordariomycetes	EF589879
fus903	0	0.15	*Fusarium poae* *	Hypocreales	Sordariomycetes	EF682109
fus995	0	0.23	*Fusarium sporotrichioides* *	Hypocreales	Sordariomycetes	EF682110
geo682	0.11	0	*Geomyces vinaceus*	Onygenales	Eurotiomycetes	EF589880
hpx1341	0	0.08	*Hypoxylon serpens*	Xylariales	Sordariomycetes	EF682111
hyp678	0.11	0	*Trichoderma harzianum* *	Hypocreales	Sordariomycetes	EF589881
lep676	0.11	0	*Leptosphaerulina trifolii*	Pleosporales	Dothideomycetes	EF589882

TABLE 1. Continued.

Haplotype	% Abundance in Invaded range	% Abundance in Native range	Species	Order	Class	GenBank accessions
lew210la	0	0.08	*Alternaria metachromatica*	Pleosporales	Dothideomycetes	EF589883, EF589844
lew212lb	0	0.15	*Alternaria metachromatica*	Pleosporales	Dothideomycetes	EF589884, EF589842
lew245lc	0	0.23	*Alternaria metachromatica*	Pleosporales	Dothideomycetes	EF589885, EF589843
muc285	0.11	0	*Mucor fragilis*	Mucorales	Zygomycetes	EF589886
nem046	0	0.3	*Nemania aenea* *	Xylariales	Sordariomycetes	EF589887
nig200	0	0.15	*Nigrospora oryzae*	Trichosphaeriales	Sordariomycetes	EF589888
pen107	0	0.08	*Penicillium* sp.	Eurotiales	Eurotiomycetes	—
pen1245	0	0.08	*Penicillium* sp.	Eurotiales	Eurotiomycetes	EF682112
pen1615	0	0.23	*Penicillium brevicompactum*	Eurotiales	Eurotiomycetes	EF682113
pen333	0.43	0	*Penicillium biourgeianum* *	Eurotiales	Eurotiomycetes	EF589889
pez601	0.11	0	*Chromelosporium fulvum*	Pezizales	Pezizomycetes	EF589890
pho086	0.43	0	*Phoma herbarum*	Pleosporales	Dothideomycetes	EF589891
pho250m	0	0.08	*Phoma tracheiphila*	Pleosporales	Dothideomycetes	EF589892, EF589847
pho602z	2.03	0	*Phoma herbarum*	Pleosporales	Dothideomycetes	EF589893
pla366	0.21	0	*Plagiostoma euphorbiae*	Diaporthales	Sordariomycetes	EF589894
ple015p	0.43	0.76	*Pleospora herbarum*	Pleosporales	Dothideomycetes	EF589895, EF589837
pyr294	0.21	0	*Phaeangium lefebvrei*	Pezizales	Pezizomycetes	EF589896
ste1012w	0	0.08	*Stemphylium* sp.	Pleosporales	Dothideomycetes	EF682116, EF682126
ste1374w	0	0.61	*Stemphylium botryosum*	Pleosporales	Dothideomycetes	EF682117, EF682126
ste1428	0	0.3	*Stemphylium* sp.	Pleosporales	Dothideomycetes	EF682118
str337	0.54	0	*Stemphylium solani* *	Pleosporales	Dothideomycetes	EF589897
tri103	0.43	0	*Trichothecium roseum*	Hypocreales	Sordariomycetes	EF589898
ulo068d	2.14	0	*Ulocladium cucurbitae*	Pleosporales	Dothideomycetes	EF589899, EF589835
ulo068db	0.96	0	*Ulocladium cucurbitae* *	Pleosporales	Dothideomycetes	EF589899, EF589839
ulo068dc	0.21	0	*Ulocladium cucurbitae*	Pleosporales	Dothideomycetes	EF589899, EF589838
ulo068g	2.25	0	*Ulocladium atrum* *	Pleosporales	Dothideomycetes	EF589899, EF589841
ulo087i	0.21	0	*Ulocladium atrum*	Pleosporales	Dothideomycetes	EF589900, EF589845

grown at 19°C; "blue" (near-ultraviolet) light was used to induce sporulation (Dahlberg and Van Etten, 1982).

Sequence-based identification—More than 200 (288) pure cultures were selected for DNA extraction, amplification, and sequencing. Because most of our cultures did not adequately express diagnostic morphological characters, we used ITS sequence data to assist in species identification, specifically the region amplified with universal fungal primers ITS1 and ITS4 (White et al., 1990). PCR was carried out as follows: the reaction mixture in a total volume of 25 μL contained a final concentration of 1.5 mM $MgCl_2$, 0.5 mM dNTPs (Applied Biosystems, Foster City, California, USA), 10 pmol of each primer, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems), and 30–60 ng genomic DNA. Samples were incubated in a thermal cycler at 94°C for 4 min, followed by 35 cycles of 94° for 1 min, 54° for 1 min, 72° for 1 min; and finally 72° for 3 min. Single-band PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio, USA) according to the manufacturer's instructions. Purified PCR products were then sequenced in both directions with the ITS1 and ITS4 primers. Sequencing reaction products were then cleaned using differential precipitation with ethanol. DNA sequences were determined by an ABI 3130*xl* automated sequencer (Applied Biosystems). Sequences were then aligned to other sequences obtained from the GenBank database using the program Sequencher 4.5 (Gene Codes, Ann Arbor, Michigan, USA).

To improve the quality of identification for *Alternaria* and *Ulocladium* (Pleosporaceae), for which ITS sequences do not provide sufficient resolution, we chose the *Alt a 1* gene (*Alternaria* allergen gene). This gene aids resolution among species of these genera (Hong et al., 2005). The primers are described in Hong et al. (2005). PCR conditions for *Alt a1* amplification were 3.5 mM $MgCl_2$, 0.5 mM dNTPs (Applied Biosystems), 2 pmol of each primer, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems), and 30–60 ng genomic DNA, in a 25-μL reaction volume. Samples were incubated in a thermal cycler at 94°C for 4 min, followed by 35 cycles of 94° for 1 min, 55° for 1 min, 72° for 1 min; and finally 72° for 3 min. For better identification and subsequent phylogenetic analysis of fungal genus *Botrytis* (Helotiaceae) samples, we amplified the gene for heat-shock protein 60 (*HSP60*), which has given the best resolution in phylogenetic analyses of this genus (Staats et al., 2005). Primers and PCR conditions for *HSP60* are described elsewhere (Staats et al., 2005). For DNA-based identification, we used the standard NCBI GenBank (Bethesda, Maryland, USA) nucleotide BLAST search with unmodified options. Then we checked the results with 100% identity and used them as species designations for our endophytes. If sequence similarity was less than 100%, we used the most similar taxa for generic determination only. We did not use a 95% cutoff value as a species boundary (Arnold and Lutzoni, 2007) because in some instances fungi with just a single nucleotide difference could satisfy some species criteria (discussed later). Instead, we used the neutral term haplotype for all of our distinct sequences (Table 1). The name of each haplotype contains its generic abbreviation, CID number, and letter(s) for "Alt a 1" sequence, such as "alt002b" (*Alternaria* CID 002, "b" type of *Alt a 1*. Sequences for all unique haplotypes were deposited in GenBank (122 sequences, accessions EF589828-EF589908, EF682095–EF682126 and EU386596–EU386605, see also Table 1).

Data analysis—Traditional community analyses do not take phylogenetic distance between species into account. To take this into consideration, we used the program Phylocom 3.41 (Webb et al., 2007) to calculate the distance matrix between communities based on phylogenetic distances from a given phylogenetic tree. This tree (based on aligned ITS data) was calculated using the program PAUP* version 4.0b10 (Swofford, 2003) under maximum parsimony criterion with heuristic search. The search used the stepwise addition option and was repeated 1000 times from different starting points with tree-bisection-reconnection (TBR) branch swapping; all characters were equally weighted and unordered, and alignment gaps were treated as missing data. Finally, the 50% majority rule consensus tree rooted with the muc285 haplotype was constructed. The resulting tree (not shown) coincided well with a recently published phylogeny of fungi (Hibbett et al., 2007).

To understand the level of taxonomic novelty in our endophytes, we used data from our *Botrytis* haplotypes. *Botrytis* is an ideal taxon to use because all 22 recognized species of this genus have been sequenced. In addition to our sequences, we used GenBank data for 21 species (excluding *Botrytis ×allii*) (Staats et al., 2005). In total, we used 61 operational taxonomic units (OTUs). All sequences were aligned with the program CLUSTAL_X (Thompson et al., 1997) using gap opening cost = 15, gap extension cost = 6.66, and IUB weight matrix, then corrected by eye. The most parsimonious trees based upon HSP60 DNA sequences were obtained by heuristic search using TBR branch swapping on starting trees generated by random sequence addition (1000 replications)

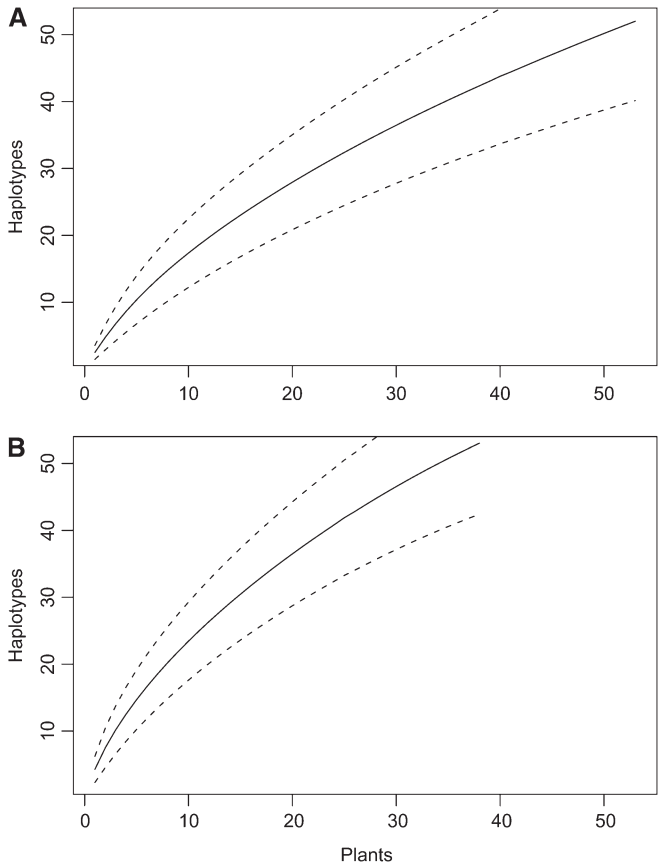

Fig. 2. Plant-based, haplotype accumulation curves for endophytes from the (A) invaded range and the (B) native range of *C. stoebe*. Dashed lines indicate 95% confidence intervals inferred using EstimateS software.

using PAUP* 4.0.10b (Swofford, 2003). Bootstrap values were evaluated by 500 replications using a heuristic search with simple sequence addition, TBR branch swapping, and MULTREES. The *HSP60* sequences of *Monilinia fructigena* and *Sclerotinia sclerotiniorum* were used as outgroups. The best-fit models of evolution for the data sets were chosen through a likelihood ratio test using the program MrModeltest version 2.2 (Nylander, 2004). Models obtained were used for Bayesian analyses using the Markov chain Monte Carlo method (MCMC). MCMC chains were run for 2 000 000 generations, sampling every 100th generation resulting in 20 000 trees. The first 5000 trees were discarded as burn-in, and the remaining 12 000 trees were summed to calculate the posterior probabilities. Functional characters were mapped onto the molecular phylogeny using the program MacClade version 4.06b (Maddison and Maddison, 2000).

For most statistical data analysis, the R statistical environment (R Development Core Team, 2007) and specifically its "vegan" package (Oksanen et al., 2007) were used. Haplotype-accumulation curves were generated for endophytes from each range of knapweed using the program EstimateS 8.0 (Colwell, 2006). Web GIS Google Earth (Google) was used to determine precise geographical coordinates for the collection sites of samples.

RESULTS

Endophyte diversity in native and invaded ranges—Some knapweed achenes did not yield any endophyte isolates. A few gave rise to multiple isolates, as can occur in endophyte studies (Sahashi et al., 1999). Overall, the numbers of isolates per plant and per achene were 8.1 and 0.26, respectively. Isolation frequencies per plant and per achene were higher in the native than in the invaded range of *C. stoebe*: 0.35 and 13.71 vs. 0.19 and 5.21, respectively. In all, 2291 fungal isolates and 92 combined (ITS + *Alt a 1* + *HSP60*) haplotypes were obtained (Table 1). On average, each site revealed five different haplotypes. Endophyte isolates belonged to five different classes of Pezizomycotina (Hibbett et al., 2007), as well as to Saccharomycetes (*Eremothecium* spp.), Agaricomycetes (*Coprinus* sp.) and Zygomycetes (*Mucor* sp.). Most of the endophytes (i.e., 88.7% of isolates from the native range and 83.8% from the invaded range) belonged to Dothideomycetes, in particular Pleosporales. Some haplotypes belonged to groups that are not commonly isolated as plant endophytes: *Coprinus* sp., *Mucor* sp., and Pezizales (Arnold and Lutzoni, 2007; Higgins et al., 2007). The most frequent genera of endophytes were *Alternaria*, *Botrytis*, and *Fusarium* (Table 1). Plant-based haplotype accumulation curves were not asymptotic, and the numbers of predicted haplotypes (Chao index: 82 ± 15 for the native range, and 135 ± 47 for the invaded range) were significantly greater than the number of observed haplotypes (52 and 50, respectively), especially for the invaded range (Fig. 2).

Despite similar numbers of haplotypes predicted by the Chao index of extrapolated species richness (Oksanen et al., 2007), endophyte floras of knapweed plants in the native and invaded ranges differed in the abundance of their most frequent haplotypes (Fig. 3). One haplotype, alt002b, dominated the community of the native range at 43.54% relative abundance (Table 1); no other haplotype in the native range exceeded 12% relative abundance. Alt002c and alt002a were the second and third most common haplotypes of the native range at 11.7% and 6.46%, respectively. In striking contrast, the invaded range lacked any dominant haplotype equivalent to alt002b in the native range. Instead, the invaded range was characterized by a number of more or less equal haplotypes, none of which exceeded 16% relative abundance: alt002a, epi066, cla063, alt002b, and aub061 were the top five, in descending order (Table 1). Most of the 92 haplotypes were relatively rare; 78 of the 92 haplotypes, or 85%, occurred in only one of the two ranges. The most abundant of the haplotypes that were restricted to one range only was alt002m at 3.8% abundance in the native range. Of the 78 one-range haplotypes, 69 were present in that one range at less than 1% abundance. Haplotypes such as bot079, ple015p, and epi497 that were present in both ranges at low frequency did not have a range preference, according to a one-sample proportion test (Fig. 3).

Haplotype accumulation curves (Fig. 2) revealed the high possibility of finding new endophytes with greater sampling. Given this finding and the relative rarity of most observed haplotypes, Chao dissimilarity indices were used (Chao et al., 2005). These indices take into account "unseen shared species" and thus reduce undersampling bias (Unterseher et al., 2008). They were used for calculating distances between endophyte communities. The matrix of distances were then used as the source of principal coordinates analysis, and the results were plotted on the two-dimensional subspace of the first two coordinates (Fig. 4A). In this plot, endophyte communities from the native and invaded ranges were more or less clearly separated (ANOSIM [ANalysis Of [dis]SIMilarity; Oksanen et al., 2007] values based on 1000 permutations: $R = 0.2303$, $P < 0.01$).

However, a different picture resulted from a similar principal coordinate analysis (PCO) ordination that was based on mean phylogenetic distances (Webb et al., 2007). In this case (Fig. 4B), ANOSIM differences were not significant ($R = -0.063$, $P = 0.56$). Three samples from eastern North America were

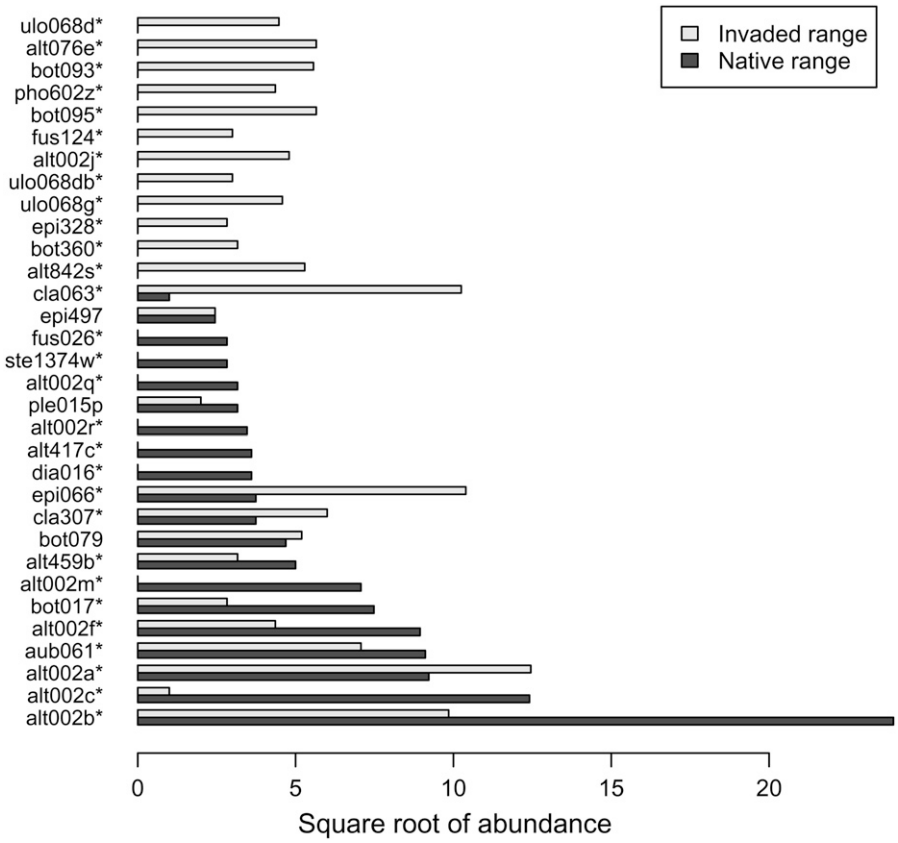

Fig. 3. Abundance of endophytes isolated at least seven times (this represents a threshold based on the distribution of isolation frequencies). Endophyte abundance is shown at square-root scale. Abbreviations on the left side are the names of haplotypes (Table 1) sorted by the abundance in the native range. Asterisks denote cases with a significant difference between ranges ($P < 0.05$) according to a one-sample proportions test with continuity correction.

located among Eurasian samples in both plots. This finding was supported with ANOSIM; differences between endophyte communities from the knapweed native range and the communities from eastern North America were not significant (R = 0.014, $P = 0.44$).

In three sites, our sampling was repeated after an interval of two years (2004 and 2006). Isolation rates in the repeated samples differed substantially (a more than twofold difference) between years. The positions of points belonging to these repeated samplings on the first ordination plot (Fig. 4A, points marked with italic numbers) suggest that the magnitude of difference between samples from various years is at least comparable with the magnitude of differences between samples from various localities. Nonparametric MANOVAs ("adonis" analysis: Oksanen et al., 2007) based on 1000 permutations and Chao dissimilarities indicated that community structure differed between years ($F = 2.557$, $P < 0.001$), but not among localities ($F = 1.004$, $P = 0.668$) nor for years nested in localities ($F = 3.6850e+15$, $P = 0.171$), suggesting that the difference between years is greater than the difference between samples.

Novelty among endophytes of Centaurea stoebe: the case of Botrytis—Of the ITS sequences, 32.8% were identical to sequences in GenBank, 29.9% had 99% identity, and 37.3% had 90–98% identity. For *Alt a 1* sequences, only 21.2% had identical sequences in GenBank, and 30.3%, 45.5%, and 0.03% sequences had 99%, 90–98%, or <90% identity, respectively. In some cases, even haplotypes with 99% similarity in ITS sequences demonstrated different distributions. As an example, *Cladosporium* haplotypes cla063 and cla307 were distributed differently in the two ranges of *C. stoebe* (Fig. 5).

Phylogenetic analysis of *HSP60* sequences for 61 *Botrytis* OTUs resulted in a well-resolved tree (Fig. 6). Of the 983 total characters, 185 were parsimony informative. The Bayesian and parsimony bootstrap analyses resulted in similar trees, consistent both in topology and in resolution with the *HSP60* tree from Staats et al. (2005 fig. 2). Most branches had strong bootstrap and Bayesian support, with the exception of the branch leading to bot360 and bot361 (Fig. 6). In many cases, branches leading to endophytes as terminal taxa (bot360 and bot361; bot079 and bot378) or branches supporting groups of related haplotypes (bot080, bot093 and bot109; bot095 and bot1093) were longer or at least comparable with branches leading to the recognized species of *Botrytis*. On the basis of relative branch lengths and node support, we identified six groups of novel *Botrytis* endophytes that should be regarded as undescribed species: (1) bot079, (2) bot378, (3) bot095 + bot1093, (4) bot080 + bot093 + bot109, (5) bot360 and (6) bot361. In contrast, the bot017 haplotype belonged to the *B. cinerea* group.

To determine if the new endophytes from *C. stoebe* provided insight into the evolution of *Botrytis*, we mapped both the distribution of host-plant group (monocots or eudicots) and fungal lifestyle (endophytic or parasitic) onto the cladogram (Fig. 7), as done previously by Staats et al. (2005, fig. 6B). This effort

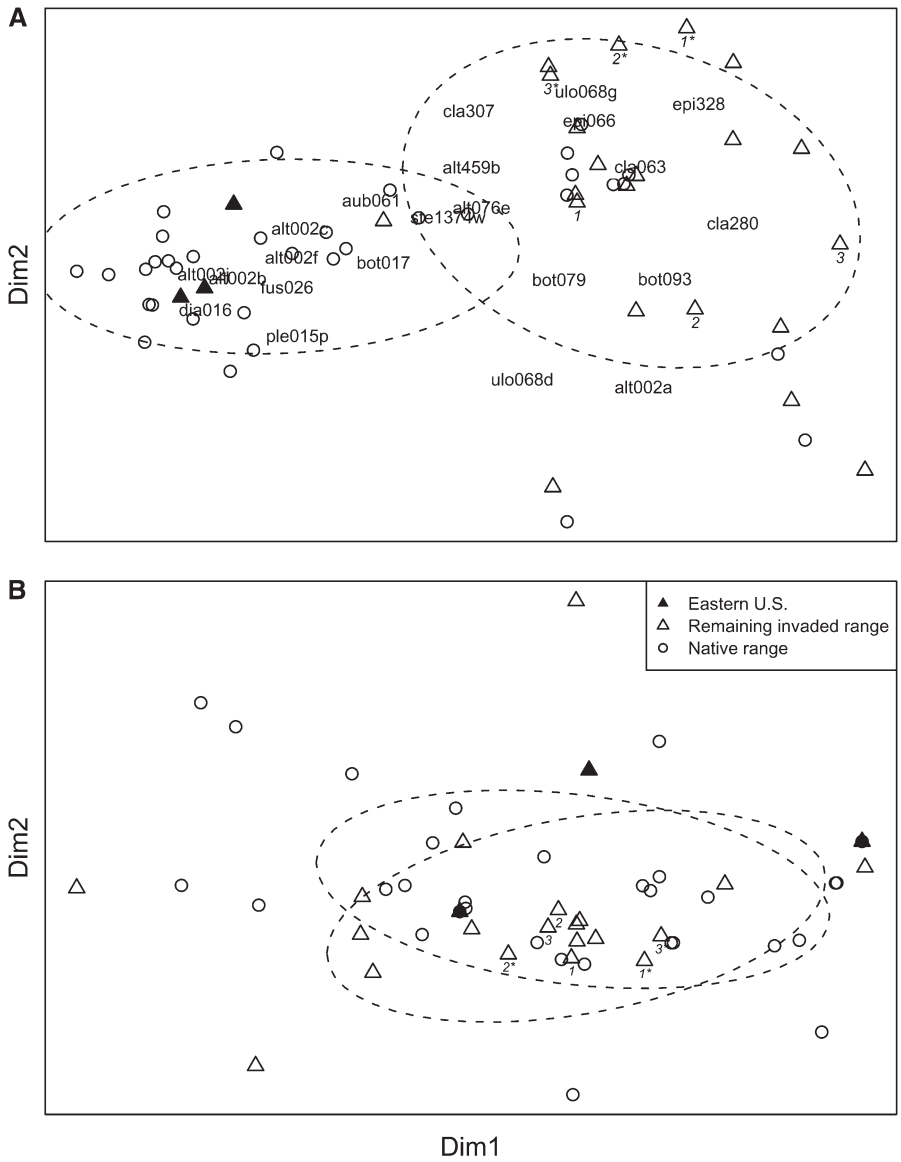

Fig. 4. Two principal coordinate analysis (PCO) ordinations of endophyte communities with (A) Chao coefficient of similarity based on occurrence data and with (B) phylogenetic distance inferred using the program Phylocom. Dashed lines indicate 55% confidence ellipses of the two expected groups: the endophyte communities from the native and invaded ranges of *C. stoebe*. Abbreviations in upright font indicate the position of species scores for the first ordination and represent the names of haplotypes (Table 1) that occurred in more than two localities. Abbreviations in italics indicate repeated samples from three locations (*1*, *2,* and *3*) and two years (asterisk for second year).

showed that the evolution of endophytism from parasitism has occurred at least twice in *Botrytis* (Fig. 7A). The transition from eudicots to monocots as hosts of *Botrytis* appeared to have evolved independently three times (i.e., at the nodes leading to *B. porrii*, the *B.* sp.-*B. squamosa* group, and the *B. aclada* group, see Fig. 7B).

DISCUSSION

Endophyte communities of plants are typically dominated by a few species or haplotypes. For example, *Venturia ditricha* was the dominant endophyte of *Betula pubescens* subsp. *czerepanovii* in Finland (Ahlholm et al., 2002), whereas a haplotype of *Lophodermium* s. l. dominated the endophyte community of *Pinus monticola* in its native range (Ganley and Newcombe, 2006). The endophyte community of *C. stoebe* in its native range followed this rule with a dominant member, the alt002b haplotype of *Alternaria alternata*, at 44% abundance, and 53 other haplotypes, all at less than 11.7% abundance including 14 singletons at 0.08% (Table 1). In contrast, the endophyte community of *C. stoebe* in its invaded range lacked a dominant member equivalent to alt002b, although the latter was present in the invaded range at 10.39% abundance and consisted of many rare haplotypes including 13 singletons at 0.11%. The lack of a dominant member of the community in the invaded range does not clearly support either cointroduction or host-jumping.

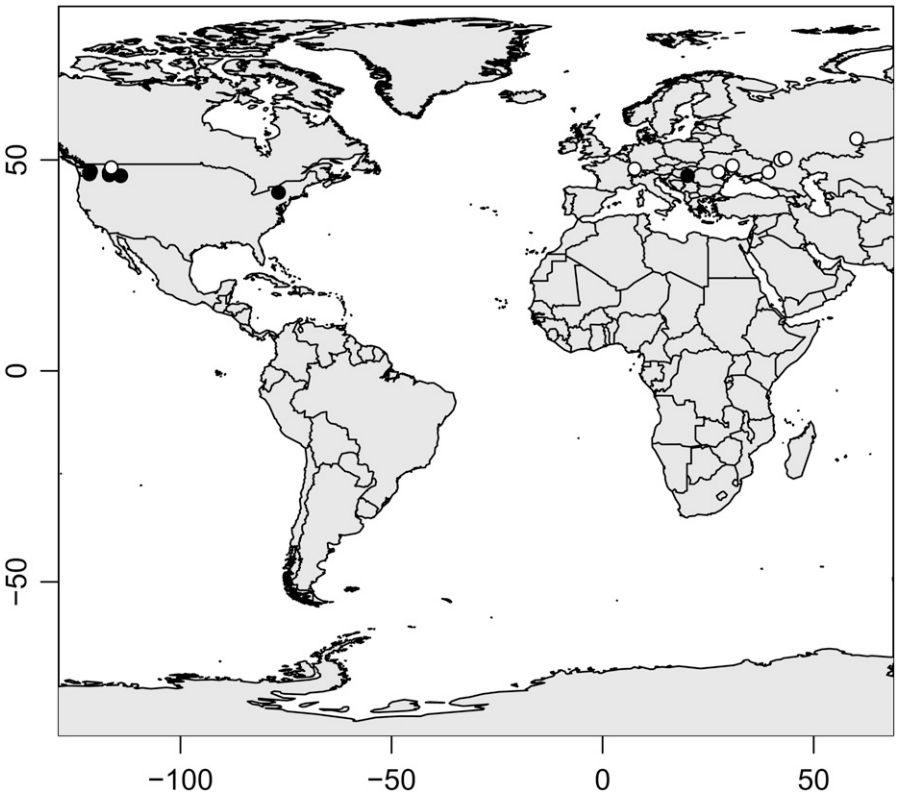

Fig. 5. Geographic distributions of two closely related haplotypes of *Cladosporium* that differed only by 1 bp. Haplotype cla063 = solid circles, cla370 = open circles.

Despite robust sampling, our accumulation curves were not asymptotic, suggesting that we have not exhaustively sampled endophyte species or haplotype diversity in *C. stoebe*. Further sampling would likely reveal more diversity, especially for the invaded range where twice the number of observed haplotypes was predicted by the Chao index of extrapolated species richness. Our preliminary environmental sequences (Arnold et al., 2007) based on the DNA extracted from a single plant (A. Shipunov, G. Newcombe, and A. Raghavendra, unpublished data) show that even plants with 0% isolation frequency from achenes contain DNA of some common endophyte haplotypes (like cla063) in vegetative parts. Thus, endophytes may be absent from the achenes of a plant that is otherwise colonized by endophytes, so further sampling of leaves, stems, or roots of *C. stoebe* might be more efficient than further sampling of achenes.

A low overall isolation frequency of 0.26 endophyte isolates per achene (i.e., 2291 isolates from 8863 achenes) suggested that competition among isolates within the small (i.e., 3–4 mm^3) achenes did not exacerbate undersampling. Repeated sampling from the same host populations is consistent with previous investigations of the seasonal fluctuations of endophyte communities (Sahashi et al., 1999; Gao et al., 2005).

Comparisons of communities like ours, with many rare members, may be misleading due to a bias in undersampling. Most recently, a new statistical approach to reduce undersampling bias, the Chao similarity index (Chao et al., 2005), has been developed. Using this approach (Fig. 4A), we found that the endophyte communities of the two ranges are distinct. The difference is greater than expected by chance, providing support for host-jumping. Host-jumping was also suggested by the high number of haplotypes that were found only in the invaded range (Table 1). At the same time, we cannot rule out cointroduction because 13 of 20 haplotypes frequent in the native range also occurred in the invaded range (Fig. 3).

In the phylogenetic ordination analysis (Fig. 4B), the difference between endophyte communities of the two ranges disappeared. In other words, phylogenetic patterns support the similarity between fungal communities from native and invaded ranges. This similarity is likely a result of both cointroduction of the most frequent endophytes (as discussed earlier) and host-jumping from a phylogenetically similar suite of species. The most outstanding example of the latter is five haplotypes of *Ulocladium* that were isolated from *C. stoebe* only in the invaded range (with individual abundances from 0.21 to 2.25). Phylogenetically, *Ulocladium* is a part of *Alternaria* s. l. (Hong et al., 2005), and these haplotypes were also isolated from plants native to the Pacific Northwest (A. Shipunov, G. Newcombe, and A. Raghavendra, unpublished data).

Interestingly, western North America and Europe can be distinguished by climatic niche spaces for *C. stoebe* (Broennimann et al., 2007). Although a minor focus in this study, the similarity of endophyte communities of *C. stoebe* in eastern North America and the native range in both ordinations is intriguing in this context. Climate matching has not been attempted for eastern North America and the native range of *C. stoebe*, but it may still be interesting to speculate on the role of climate in relation to abundance shifts in endophyte communities.

In sum, because both cointroduction and host-jumping were suggested by our data, endophytes of the invaded range in

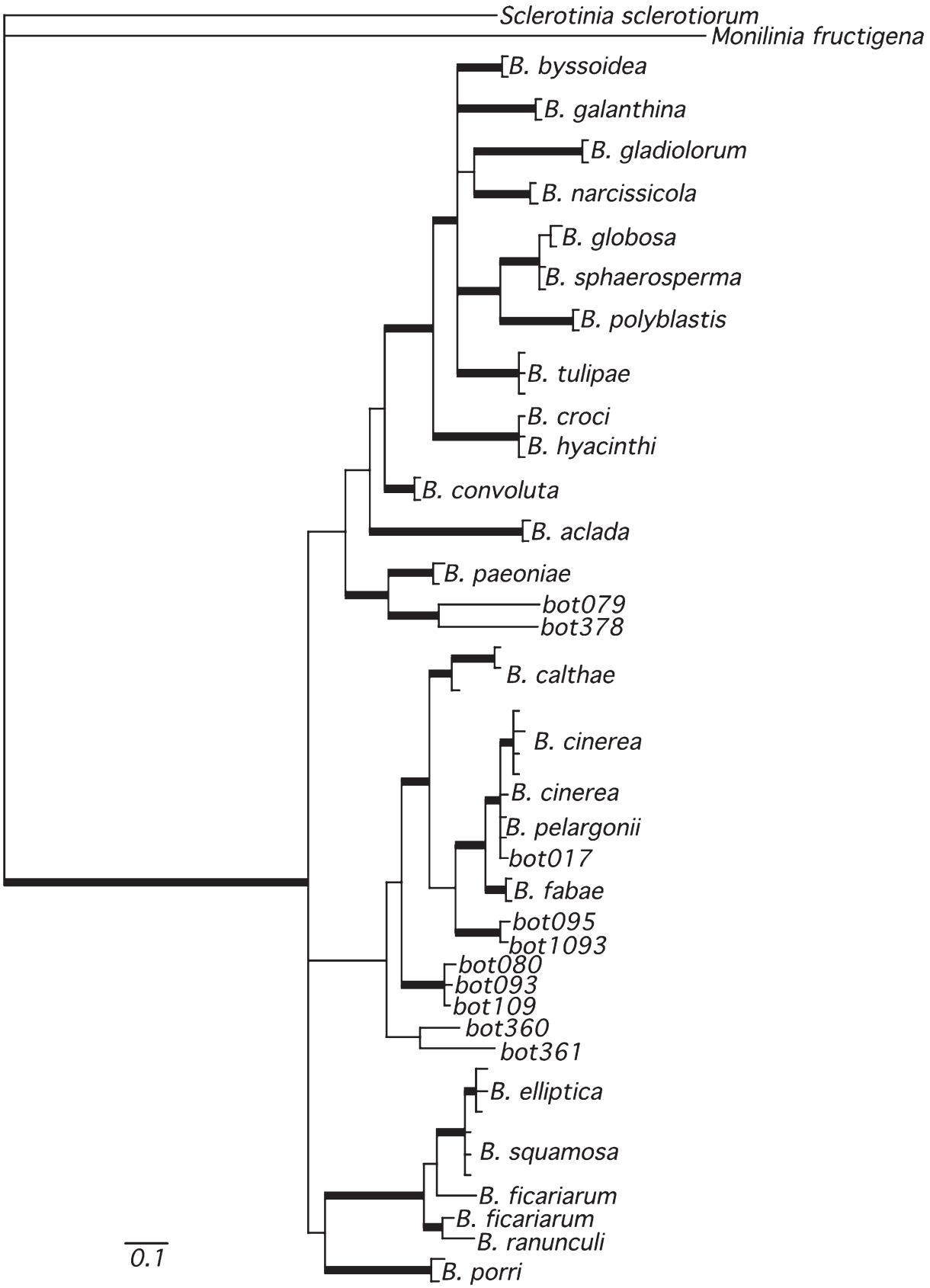

Fig. 6. Phylogenetic (Bayesian maximum likelihood 50% majority-rule consensus) tree for *Botrytis* (*HSP60* DNA sequences) from a two-million generation MCMC analysis. Nodes that received more than 65% bootstrap in parsimony analysis and 95% Bayesian support are in boldface.

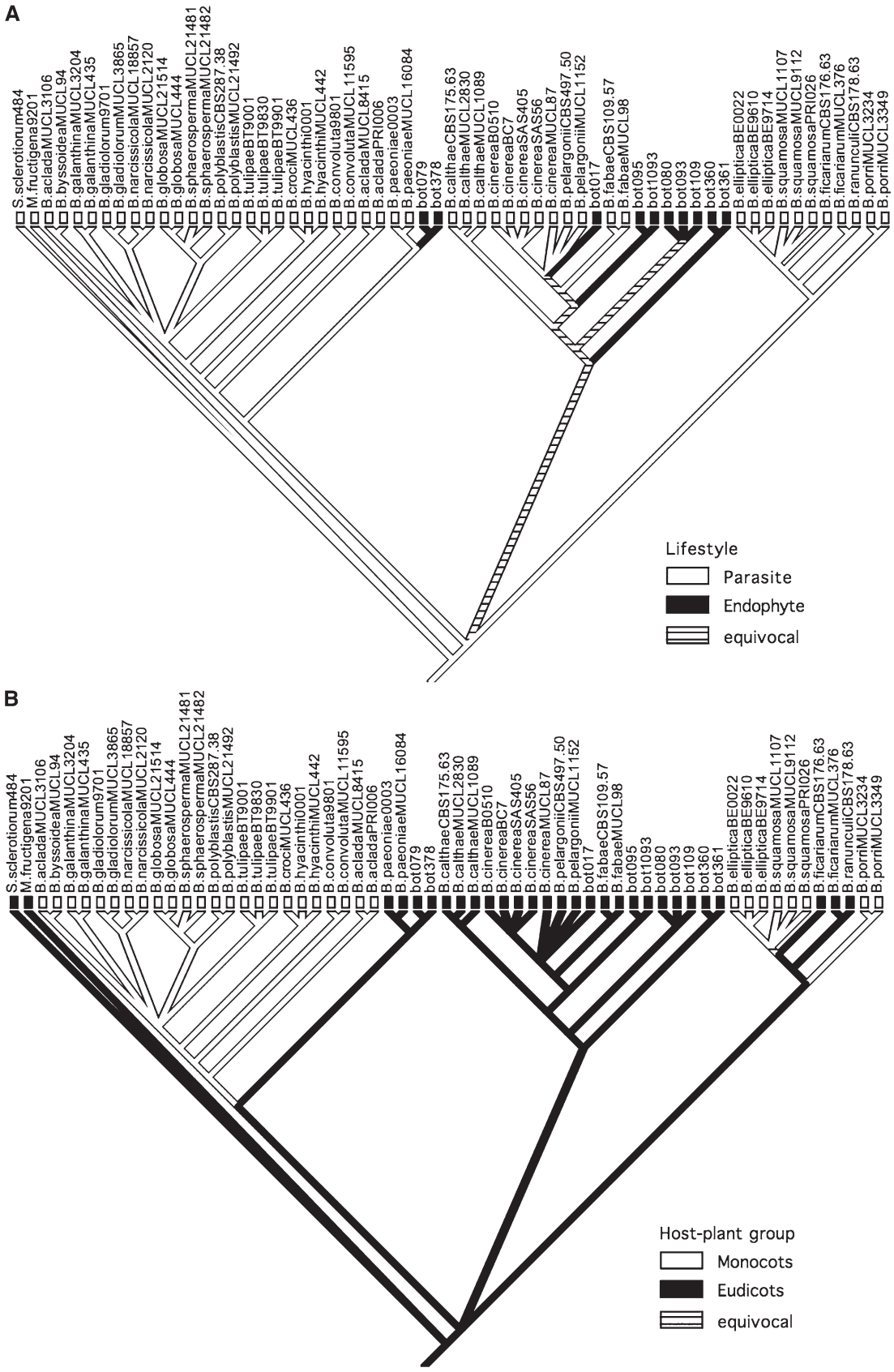

Fig. 7. Reconstructions of the character-state evolution of *Botrytis* mapped onto a Bayesian tree (the same topology as in Fig. 6) under an assumption of parsimony: (A) for lifestyle and (B) for host-plant group.

western North America may be a mixture of Eurasian endophytes that could function as novel weapons and North American endophytes that could enhance mutualism. Other studies of cointroduction vs. host-jumping of fungi have been similarly inconclusive (Tedersoo et al., 2007; White and Backhouse, 2007). Had our study revealed endophytes with known geographic ranges in either Eurasia or North America, we could then have drawn conclusions on cointroduction or host-jumping for individual haplotypes even if the community analyses were problematic due to undersampling bias. Although we did obtain endophytes (i.e., the asterisked haplotypes of Table 1) that matched named taxa in GenBank, none of these taxa could be linked to particular geographic ranges. The undescribed species that we isolated from achenes of *C. stoebe* (e.g., the six haplotypes of *Botrytis*) represent an outstanding example of species that, by definition, do not have known geographic ranges.

One third of the haplotypes from our study had less than 98% identity with their closest relatives from GenBank; this may be evidence that we have isolated more than 30 new species of fungi from *C. stoebe*. Species of fungi can be distinguished by 1-bp differences in their ITS sequences, particularly if there is also evidence for more rapid evolution of other genes (Schubert et al., 2007). Our phylogenetic analysis of the *Alternaria-Ulocladium* group suggests at least nine new lineages belonging to these genera (A. Shipunov, G. Newcombe, and A. Raghavendra, unpublished data). However, this conclusion is preliminary because sequence data exists for only a small fraction (< 25%) of the species described in this group (Pryor and Gilbertson, 2000; Hong et al., 2005). Therefore, we could have obtained endophytes that have been described but not yet sequenced.

In contrast, *Botrytis* represents a completely different situation. All 22 recognized species have been sequenced (Staats et al., 2005). Remarkably, we found six groups of novel *Botrytis* endophytes for which there appears to be phylogenetic evidence that they represent undescribed species (Fig. 6). These findings are especially noteworthy in light of the complete absence of any records of *Botrytis* as a pathogen of *C. stoebe* (Farr et al., 2008); *Botrytis cinerea* is the only *Botrytis* sp. that has ever been reported on *Centaurea*, and only on *C. cyanus* and *C. moschata*. This latter point is tempered somewhat by the fact that all the other 30 fungal genera reported in this study are "first reports" for *C. stoebe* globally (Farr et al., 2008). Interestingly, the addition of our endophytes to the *Botrytis* phylogenetic tree provided some new insights into the evolution of host specificity and lifestyle (Fig. 7). Contrary to Staats et al. (2005), it seems likely that there were no secondary transitions of *Botrytis* from monocots to eudicots, because eudicots appeared to be the primary and ancestral hosts for the genus. According to the character mapping, *Botrytis* endophytes appear to have evolved from parasites, which is not surprising (Carroll, 1988; Saikkonen et al., 1998).

Because the host ranges of knapweed endophytes are not known, it is impossible to estimate the ratio of endophyte to host species. However, our results proved that a thorough investigation of endophyte diversity from one plant species may substantially increase the number of known fungal species (e.g., six new species added to a genus, *Botrytis*, of 22 known species). Estimates of 1.5 million species (Hawksworth, 2001) or even 9.9 million species (Cannon, 1997) of fungi look feasible, given that there are approximately 250 000–420 000 species of vascular plants (Govaerts, 2001; Thorne, 2002).

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