Endophytic fungi as a biodiversity hotspot: the case of *Centaurea stoebe* (spotted knapweed)

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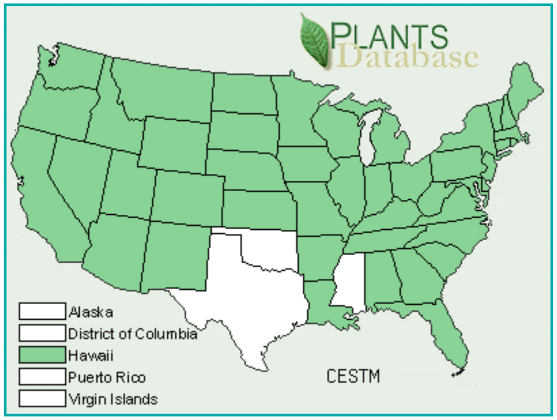
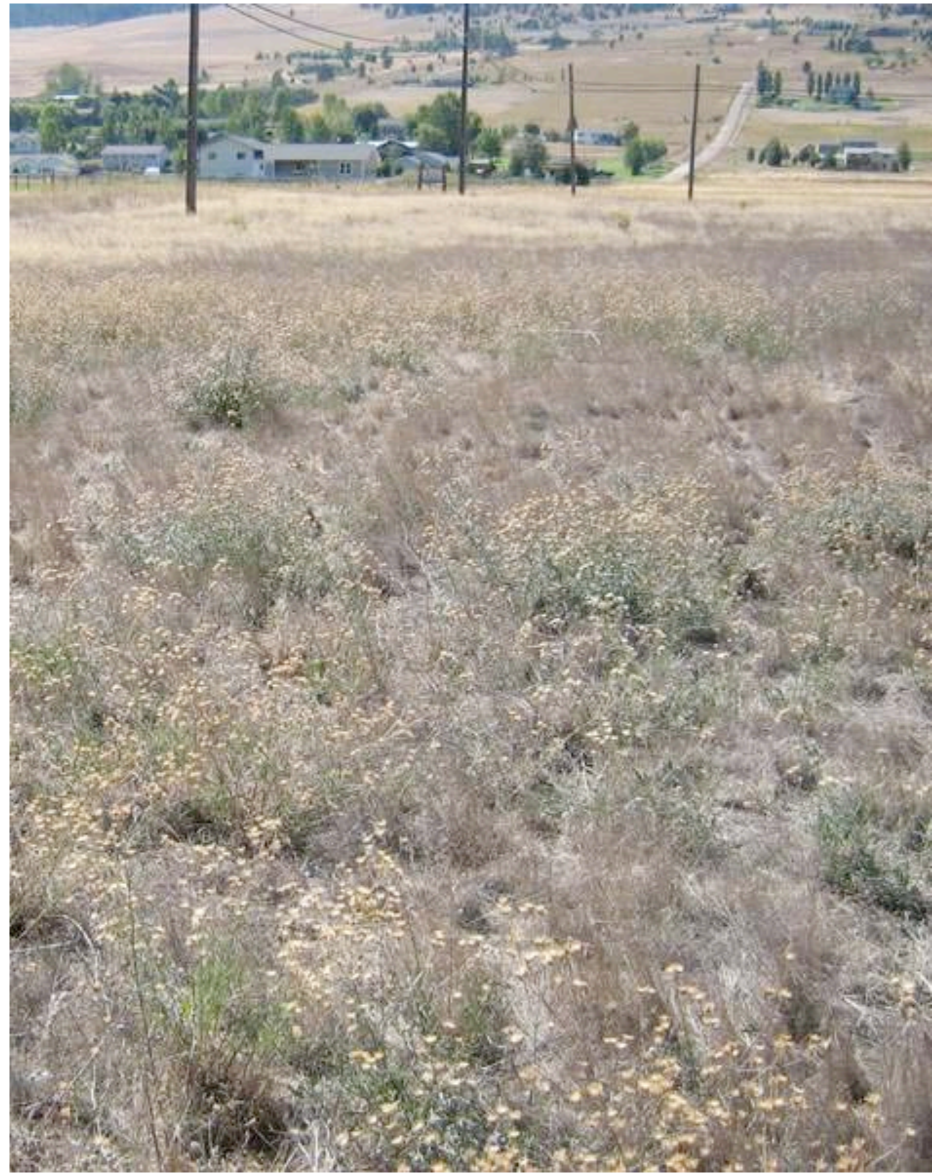
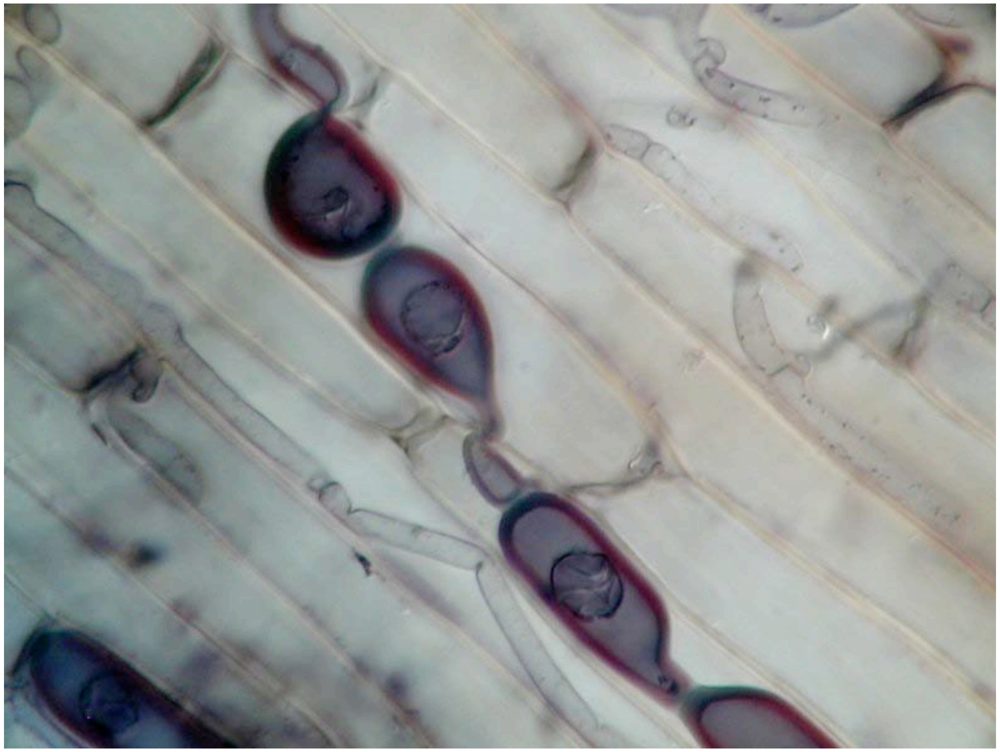
Spotted knapweed



Spotted knapweed (*Centaurea stoebe* L., also known as *C. maculosa*, *C. micrantha*, *C. biebersteinii*) is a noxious, invasive plant which was introduced into North America from Eurasia in 1890s.





Plant fungal endophytes

• Grow inside plant, but do not cause any symptoms

• Cryptic symbionts, inhabiting all plants

• Play lots of different roles, include host tolerance to stressful conditions, plant defense, plant growth, and plant community biodiversity

• One example of the economic importance of endophytes is **taxol**, well-known anticancer drug, which is not a product of *Taxus brevifolia* (yew) tree, but of its endophyte *Taxomyces andreana*

Anamorphs and teleomorphs

More than 1/3 of fungi do not normally express any sexual characters. They are **anamorphs**. Sometimes, some anamorphic fungi develop into sexual **teleomorphs** which have “more morphology” and can be properly classified. Before molecular era, all anamorphic fungi have been treated as “Deuteromycota”.
Most of knapweed endophytes are anamorphic ascomycetes.

Alternaria (anamorph, above), and *Lewia* (teleomorph, below) are the same organism.

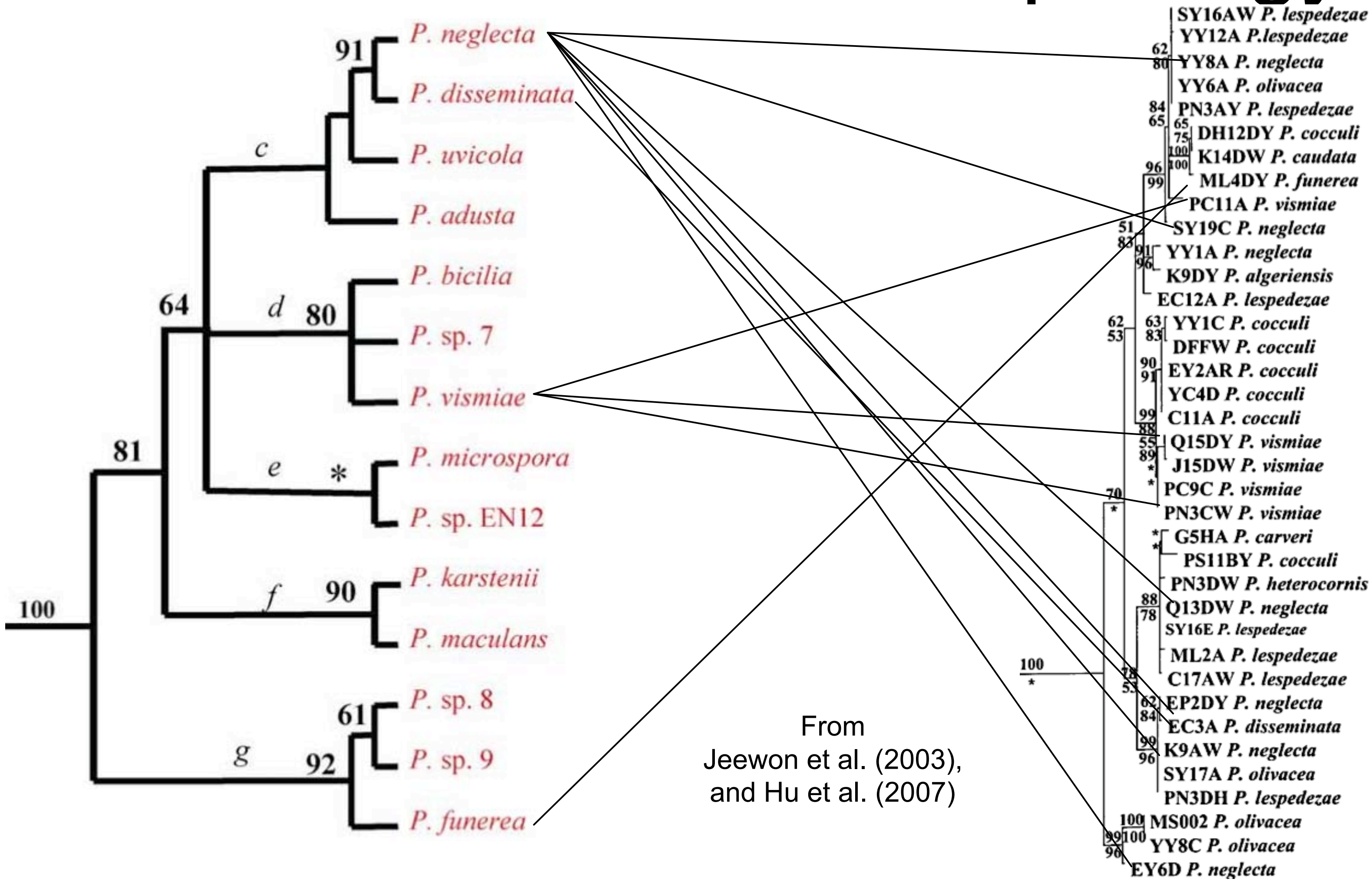
Fusarium tricinctum haploty	9
Gibberella avenacea haploty	8

Cladosporium macrocarpum st	6
Davidiella tassiana strain	6

BLAST search usually reveals mixed lists of ana- and teleomorph names

Pleomorphic fungi (with variable anamorph/teleomorph relationships) are one of the most painful problem for fungal taxonomy.

The weakness of morphology

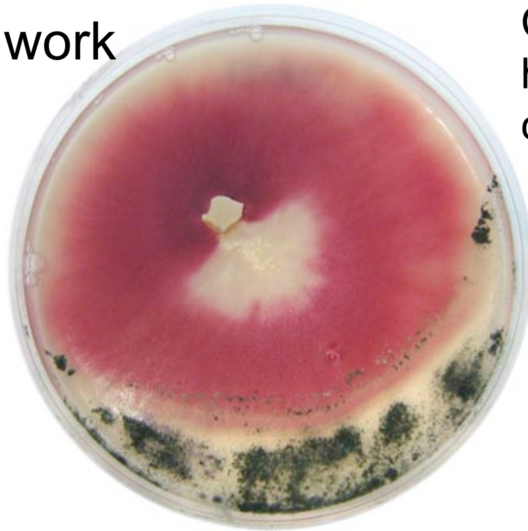


Pestalotiopsis example: morphology chosen as the only identification tool leads to highly tangled molecular tree. “Identify, then sequence” does not work for novel isolates. Thus, the identification of fungi depends on either high level of expertise, or on proper barcoding. This is probably because fungi stand between groups with “DNA-supported” and “DNA-unsupported” morphology.

The weakness of cultivation approach

"Isolate, cultivate, then sequence" is often not work properly because of:

- Contamination
- Mycoparasitism
- Non-cultivated fungi

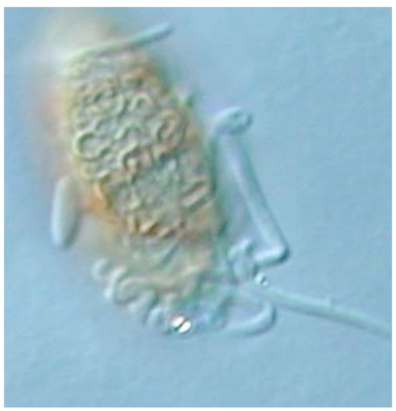


Contamination is the simplest case: here *Fusarium* culture have been contaminated with *Trichoderma*.

Fusarium sp. 6/97-54 ITS1,	8
Cordyceps sinensis genes fo	8

BLAST results: (above) consequence of contamination, (below) of mycoparasitism (*Bionectria* is well-known mycoparasite).

Bionectria ochroleuca isola	87
Fusarium oxysporum f. cuben	86
Bionectria ochroleuca strai	86



Different cases of mycoparasitism:

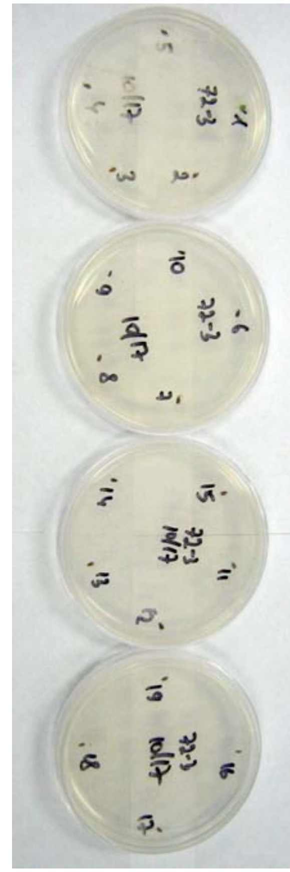
- *Ulocladium* + *Hydropisphaera fungicola* (two types of conidia);
- *Alternaria* + unknown fungus (coiling hyphae);
- *Phoma* + *Fusarium* (hyphae inside hyphae).

Our sampling

- 104 sites were sampled (49 from the invaded range and 55 from the native range), plus 10 populations of native North American plants (*Saussurea americana*, *Cirsium brevifolium*, *Festuca idahoensis* etc.)
- In each knapweed population, five plants were sampled, and from each plant, 20 seeds (i.e., achenes), for a total number of 100 seeds per site and 10,400 seeds in all
- Endophytes isolated from achenes – 2291 isolates
- Isolates grouped in 288 morphological groups (strains)
- Each group has been sequenced (ITS, “Alt a 1”, hsp60), 103 haplotypes obtained

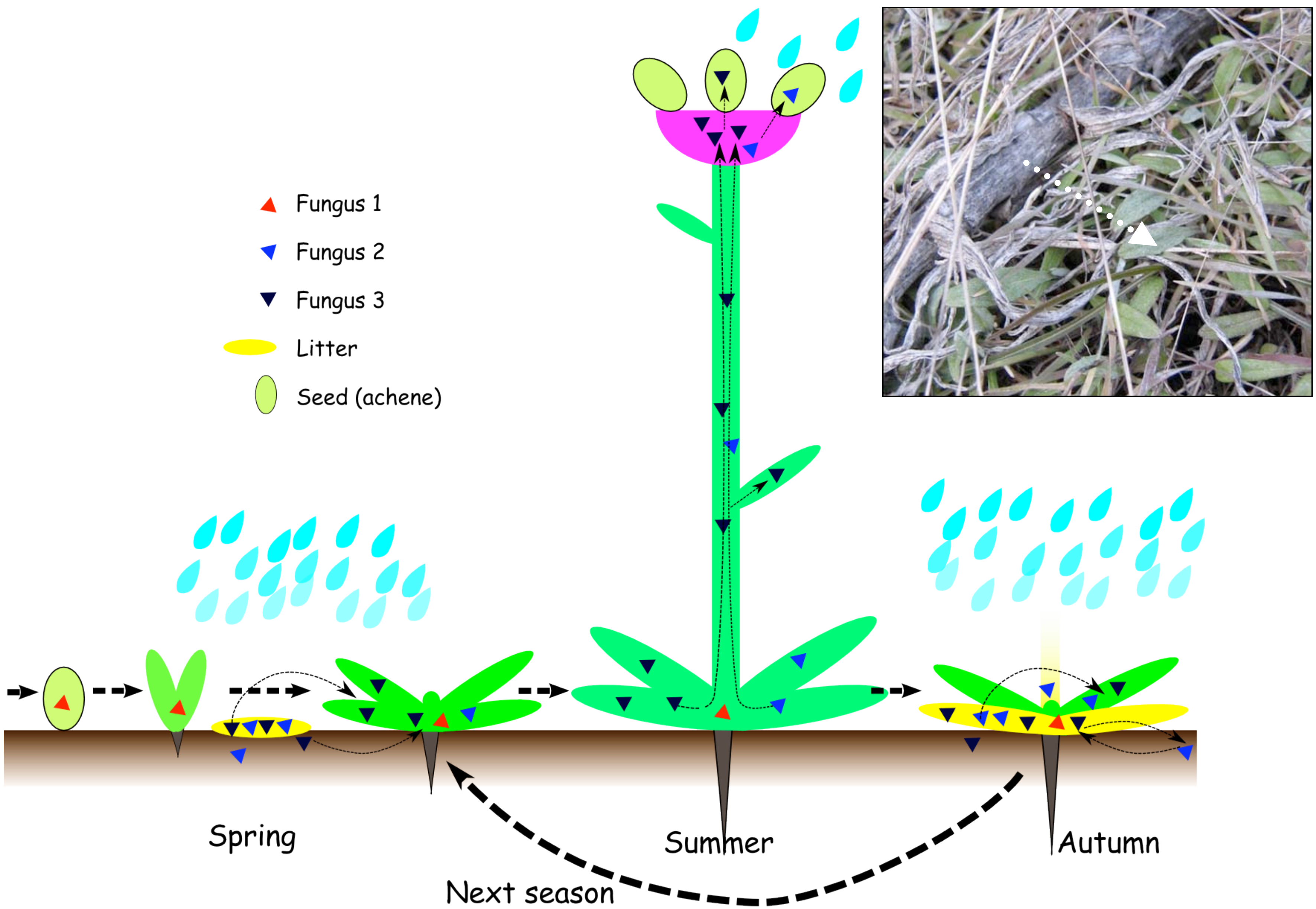
Isolation of endophytes

Surface sterilization

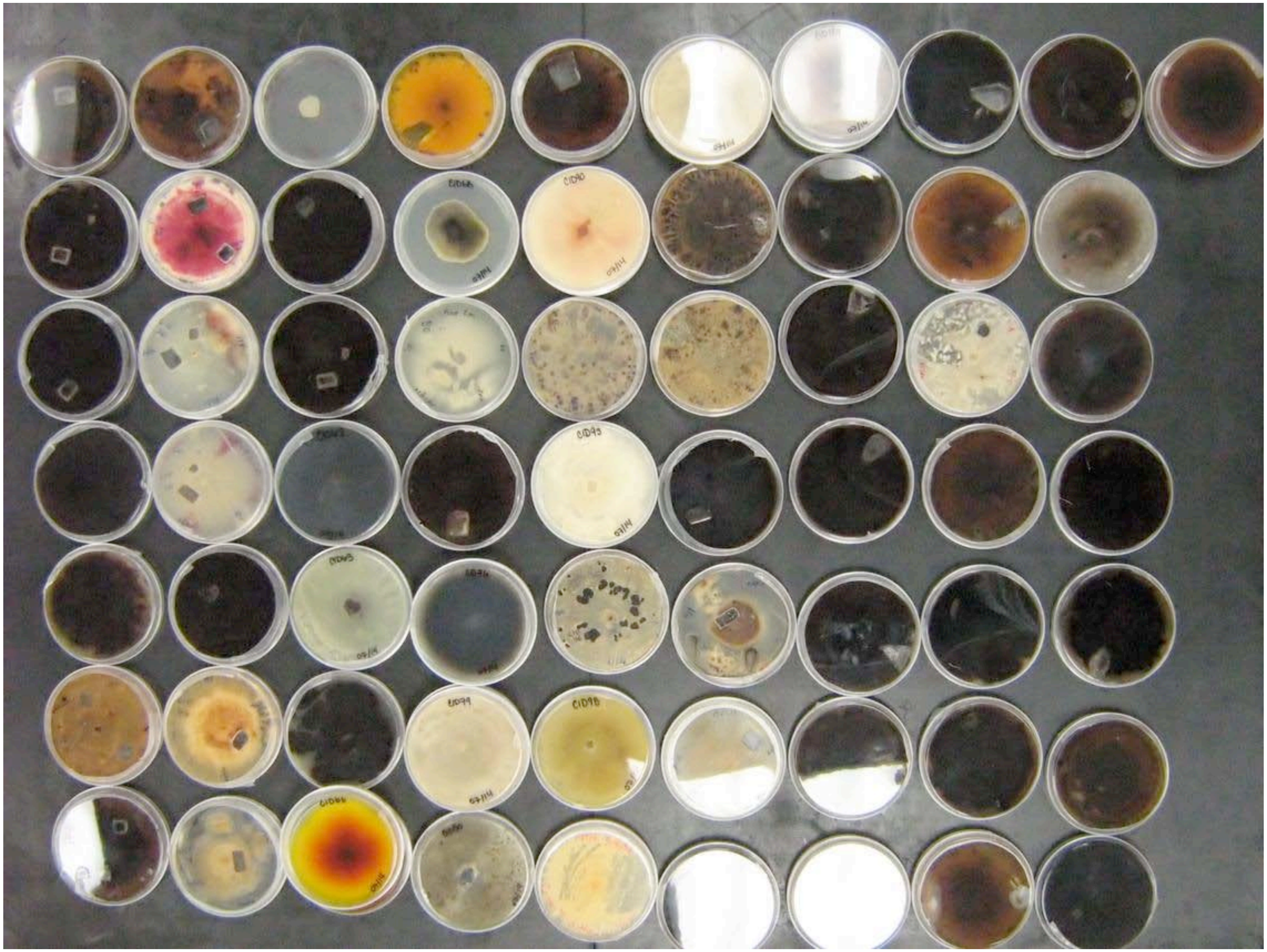


Plants → Flowering heads → Achenes → PDA (potato-dextrose agar) cultures

Life cycle of knapweed endophytes

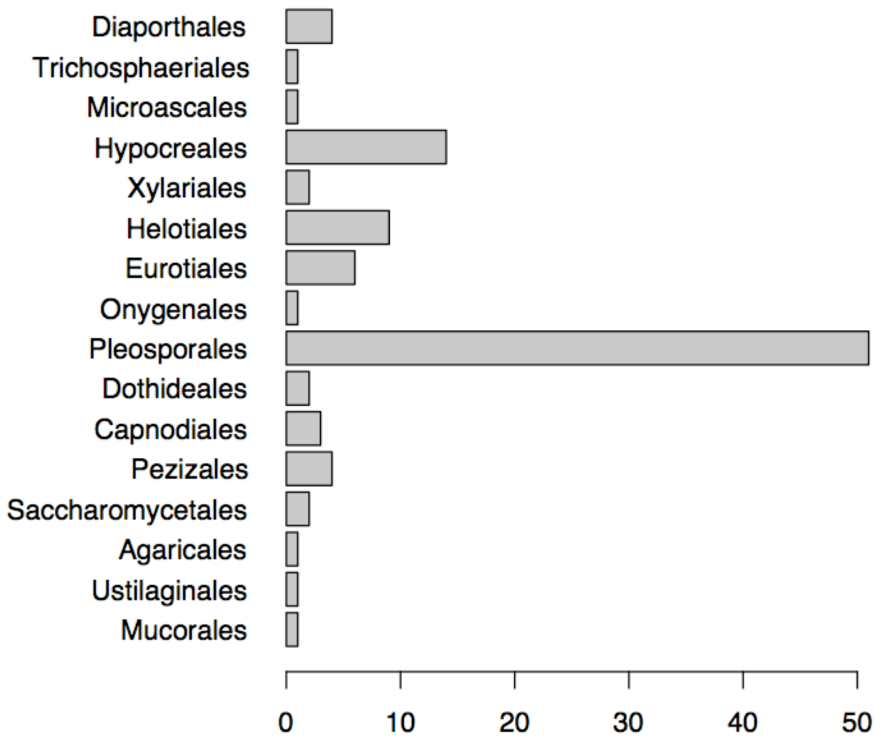


Diversity of cultures

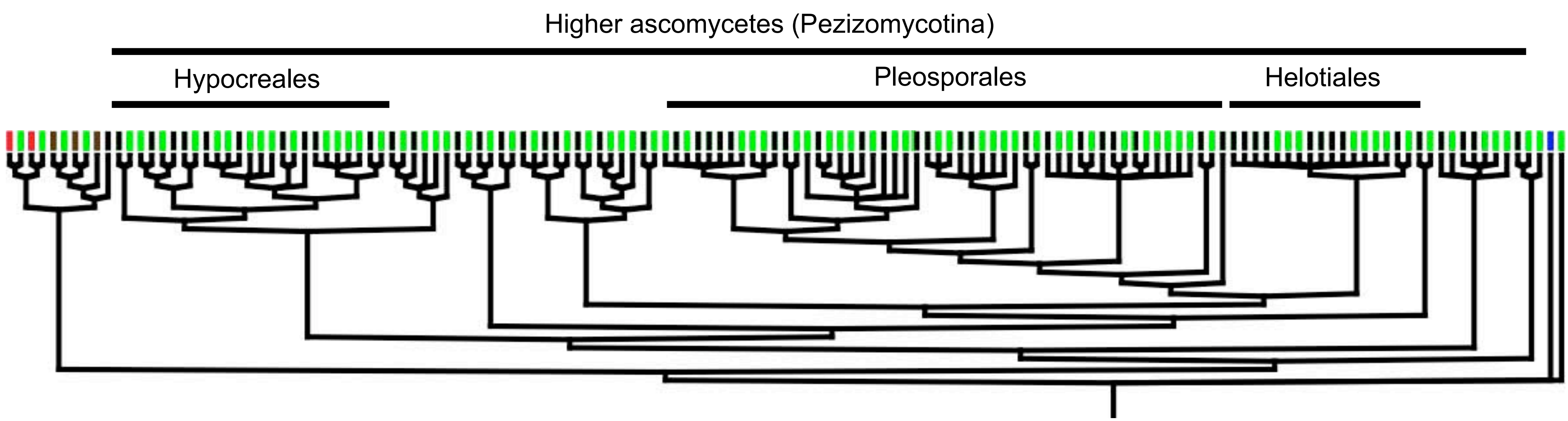


1/5 of our collection

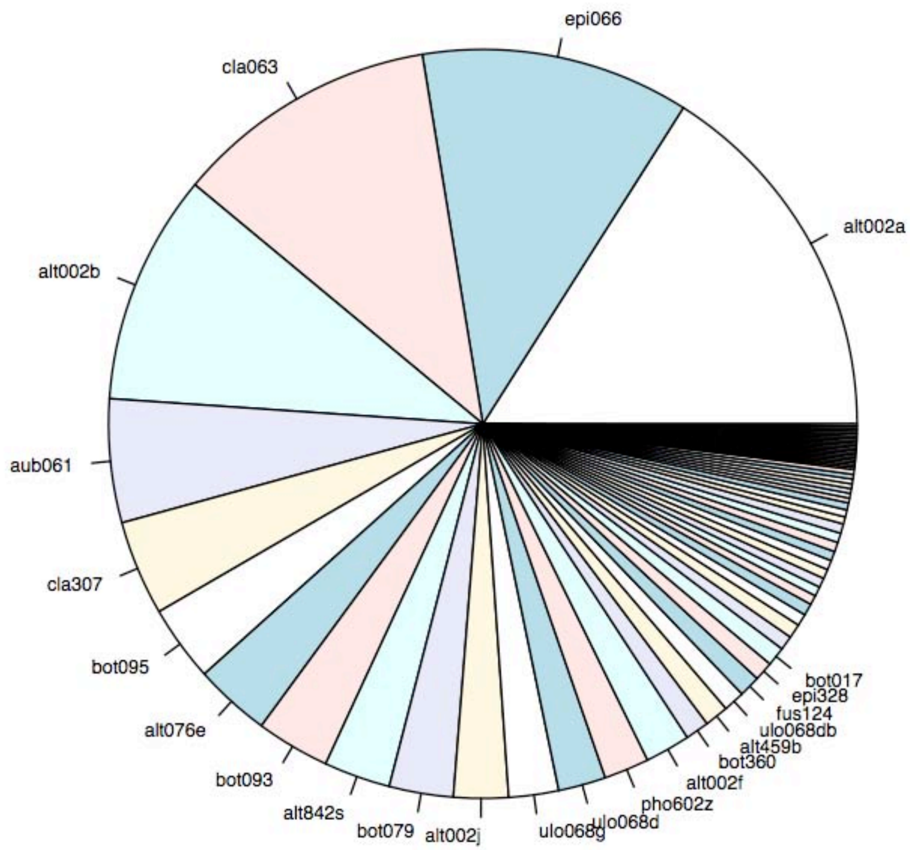
Our fungi



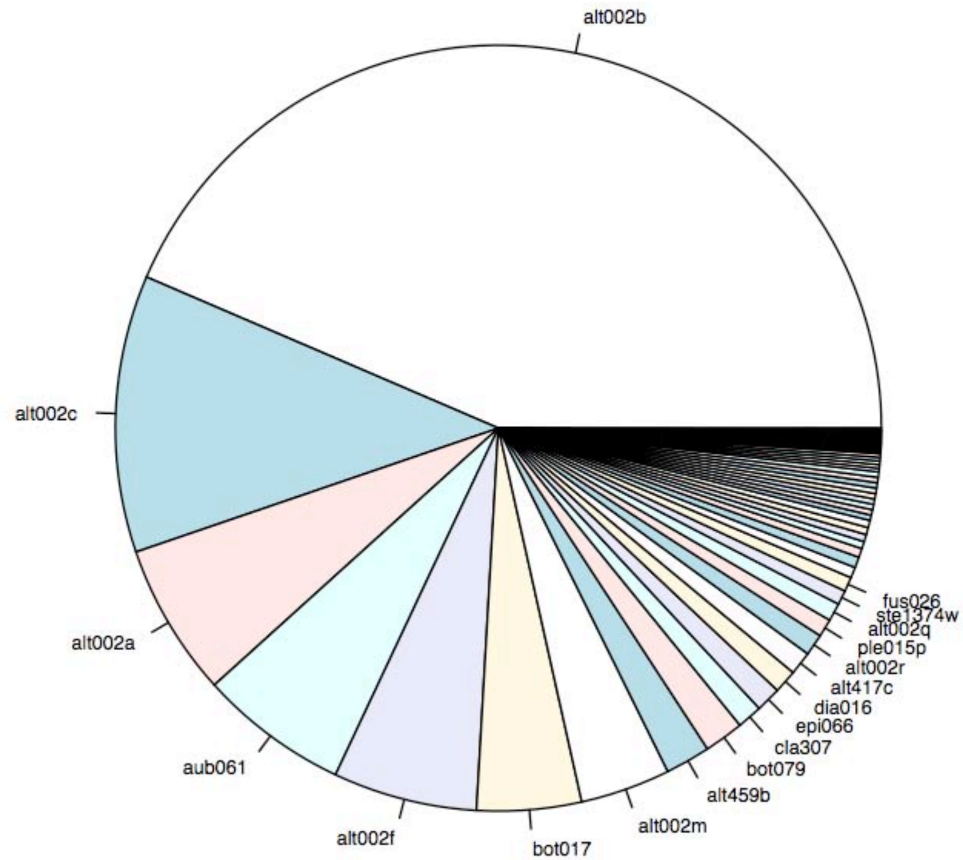
Most of them belong to anamorphic higher ascomycetes, and more than 50% – to Pleosporales



Two ranges

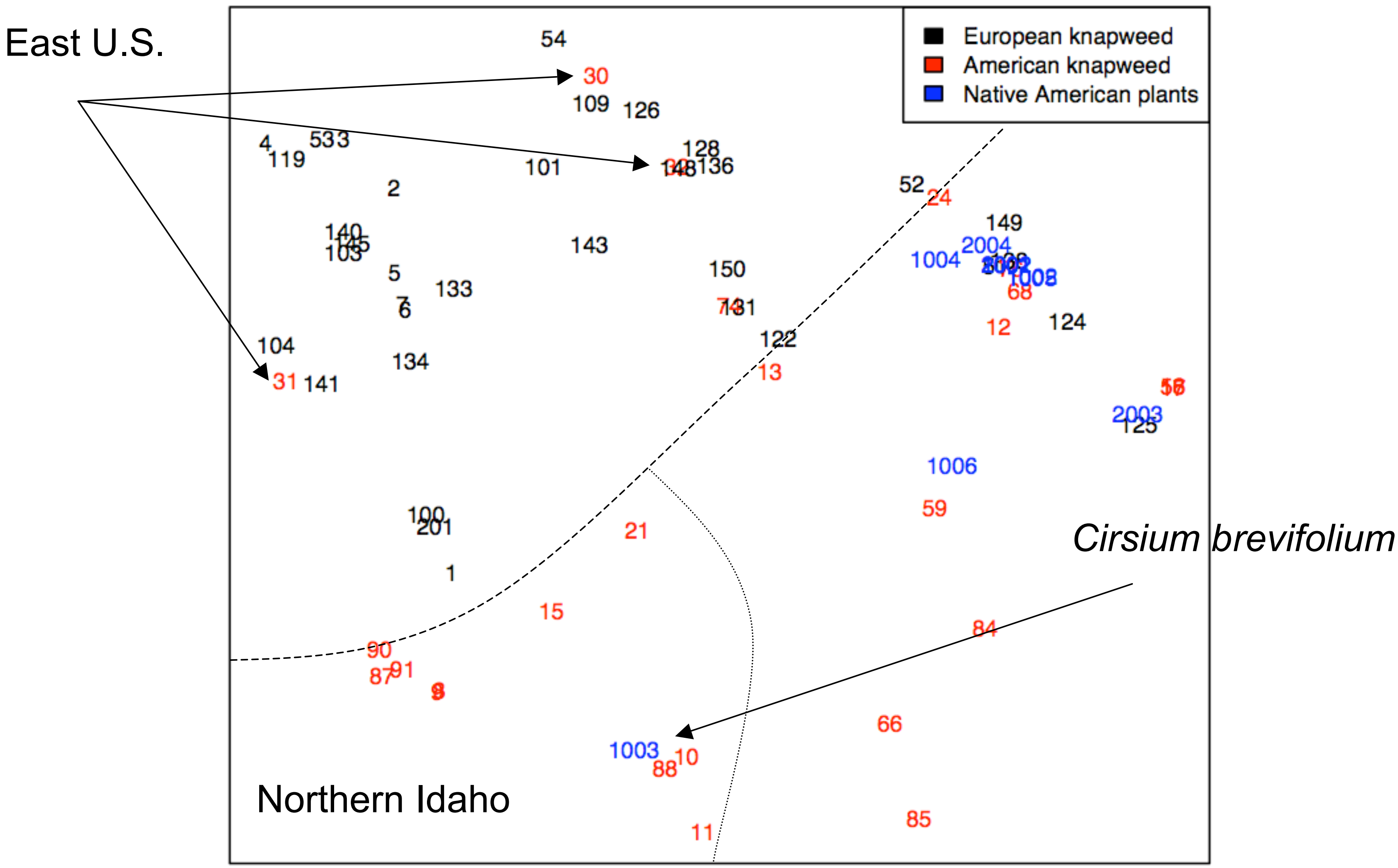


Invaded range
(N. America)



Native range
(Europe)

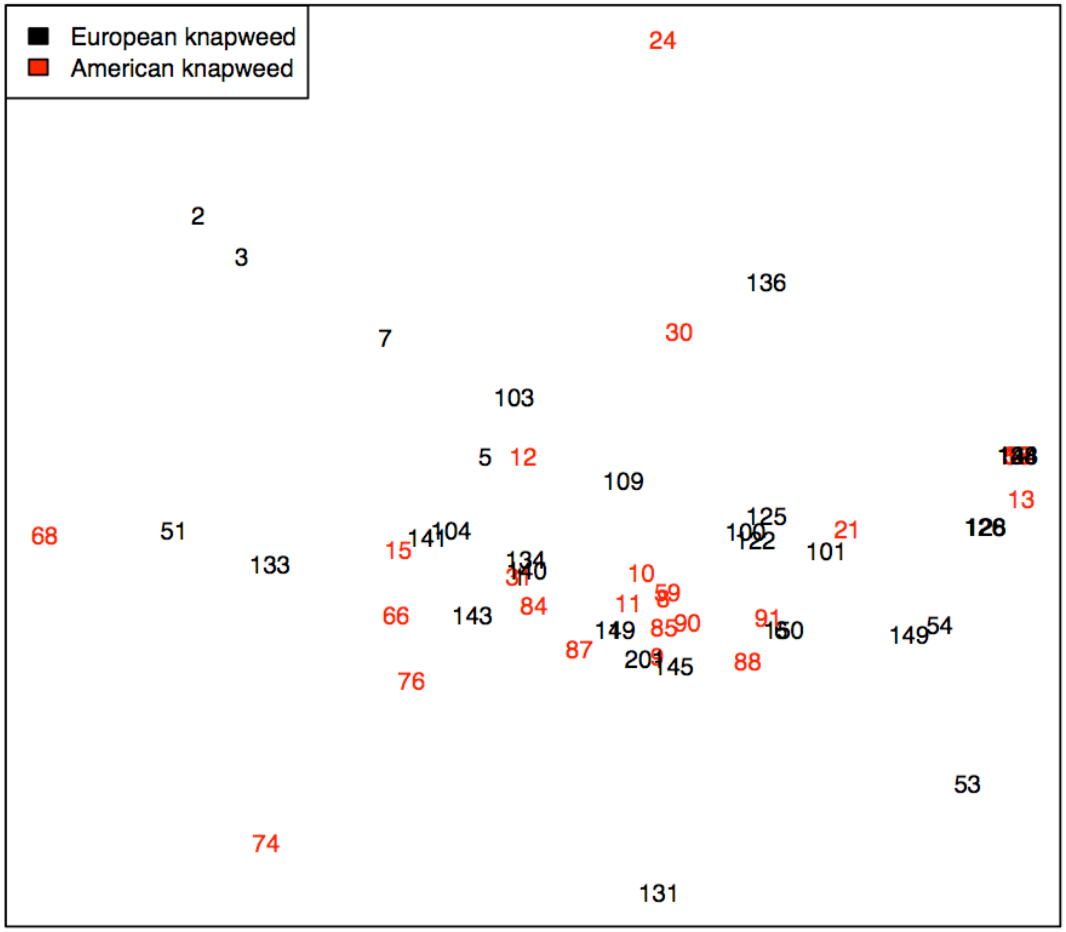
Ordination of communities



Principal coordinates analysis (PCO) of fungal communities based on **Chao similarity** indexes

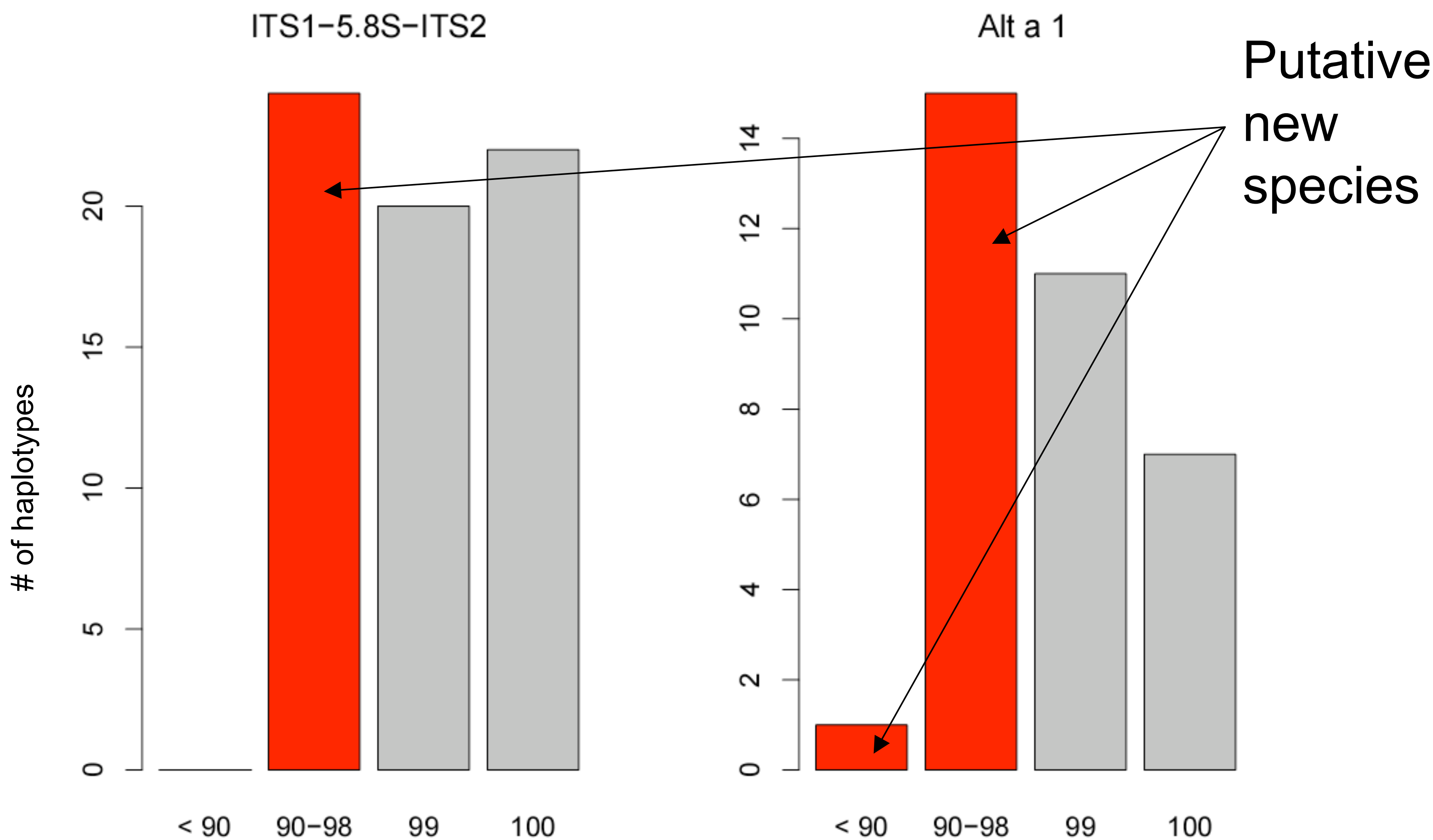
Phylogenetic ordination

Based on Phylocom (Web et al., 2007) output, ITS MP tree used for mean phylogenetic distances between samples



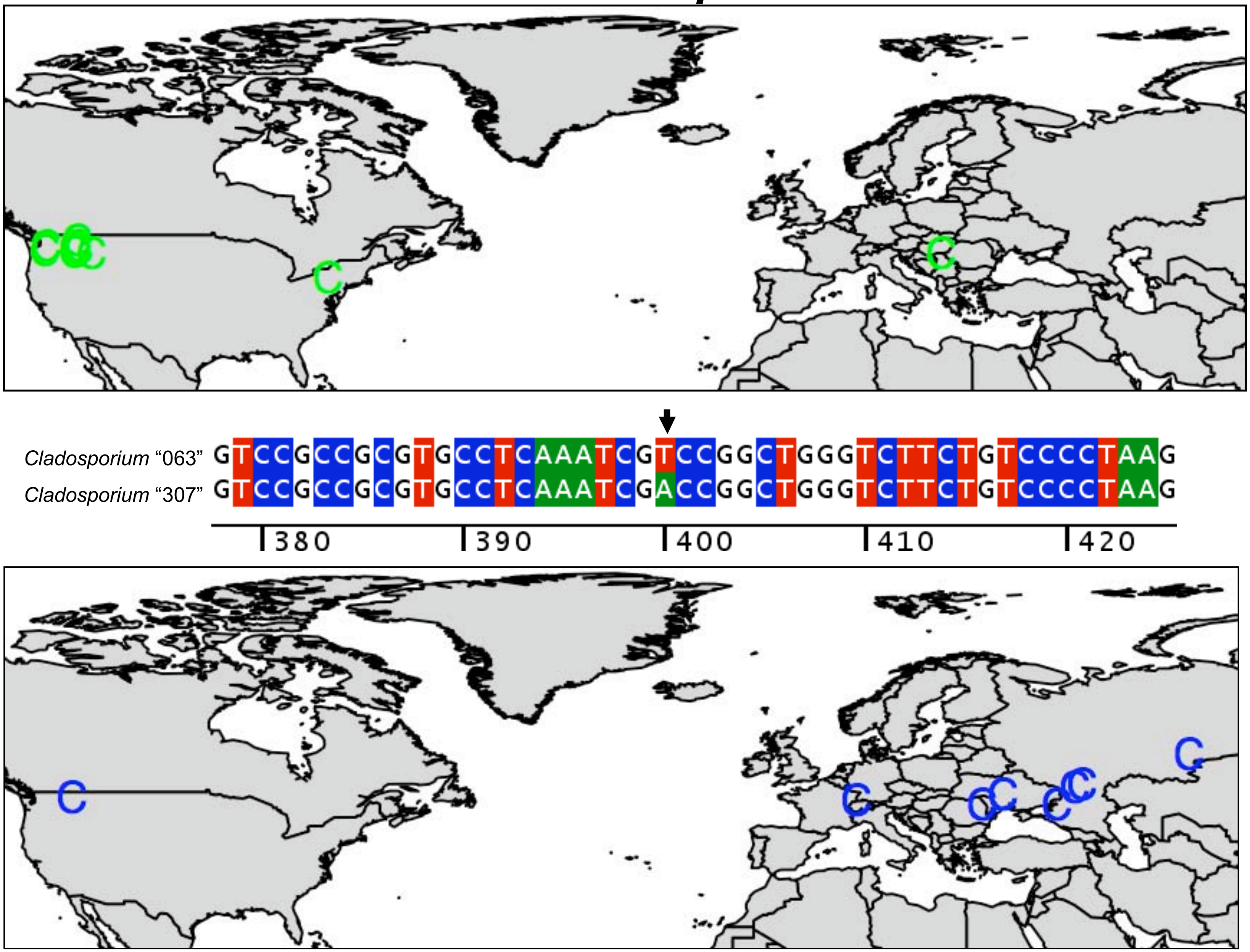
No clear difference between ranges: either (1) some American fungi were pre-adapted for knapweed because they were phylogeneticaly close to Eurasian endophytes; or (2) new fungal species aroused during invasion.

New species?



Current GenBank statistics: ~17,000 fungal species were sequenced – 22% of ~78,000 species described (Mueller, Schmit, 2006). Unfortunately, up to 30% of species descriptions are incorrect (Nilsson et al., 2006)

Two *Cladosporium*

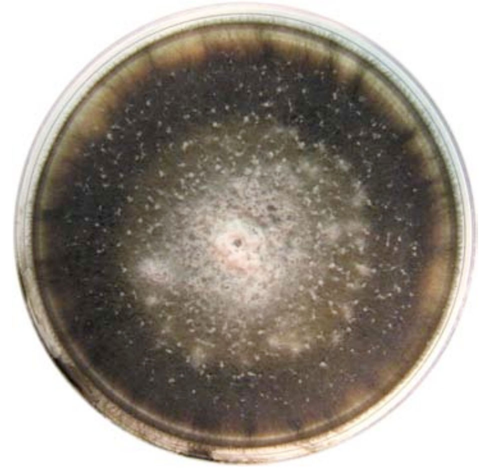


Alternaria/Ulocladium group

Important group of pant pathogens, sometimes also saprotrophs. Belong to Pleosporales. More than 100 species described, ~ 25% sequenced. *Lewia* is the teleomorph of *Alternaria*, teleomorph of *Ulocladium* is unknown.

Alternaria solani on tomato

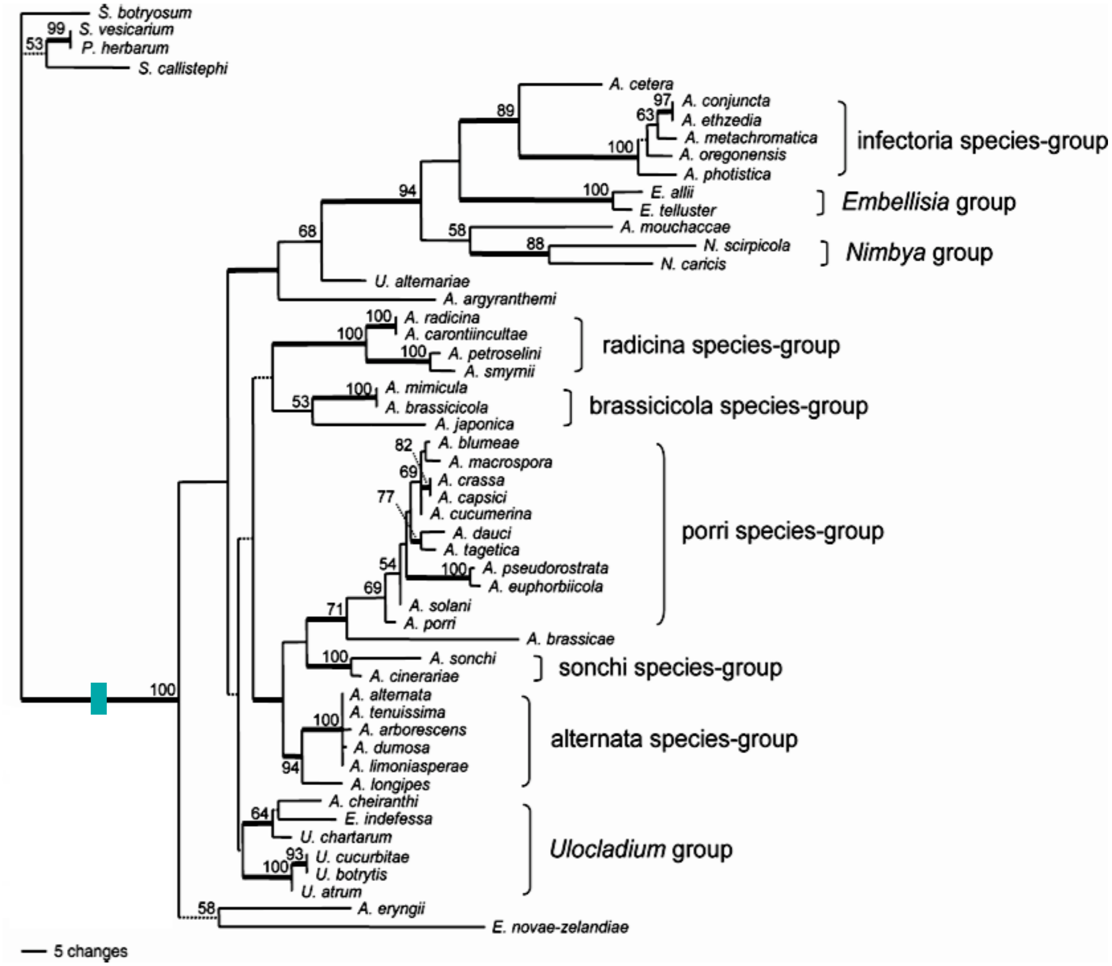
Alternaria conidia



Alternaria and *Ulocladium* PDA cultures

Ulocladium conidia

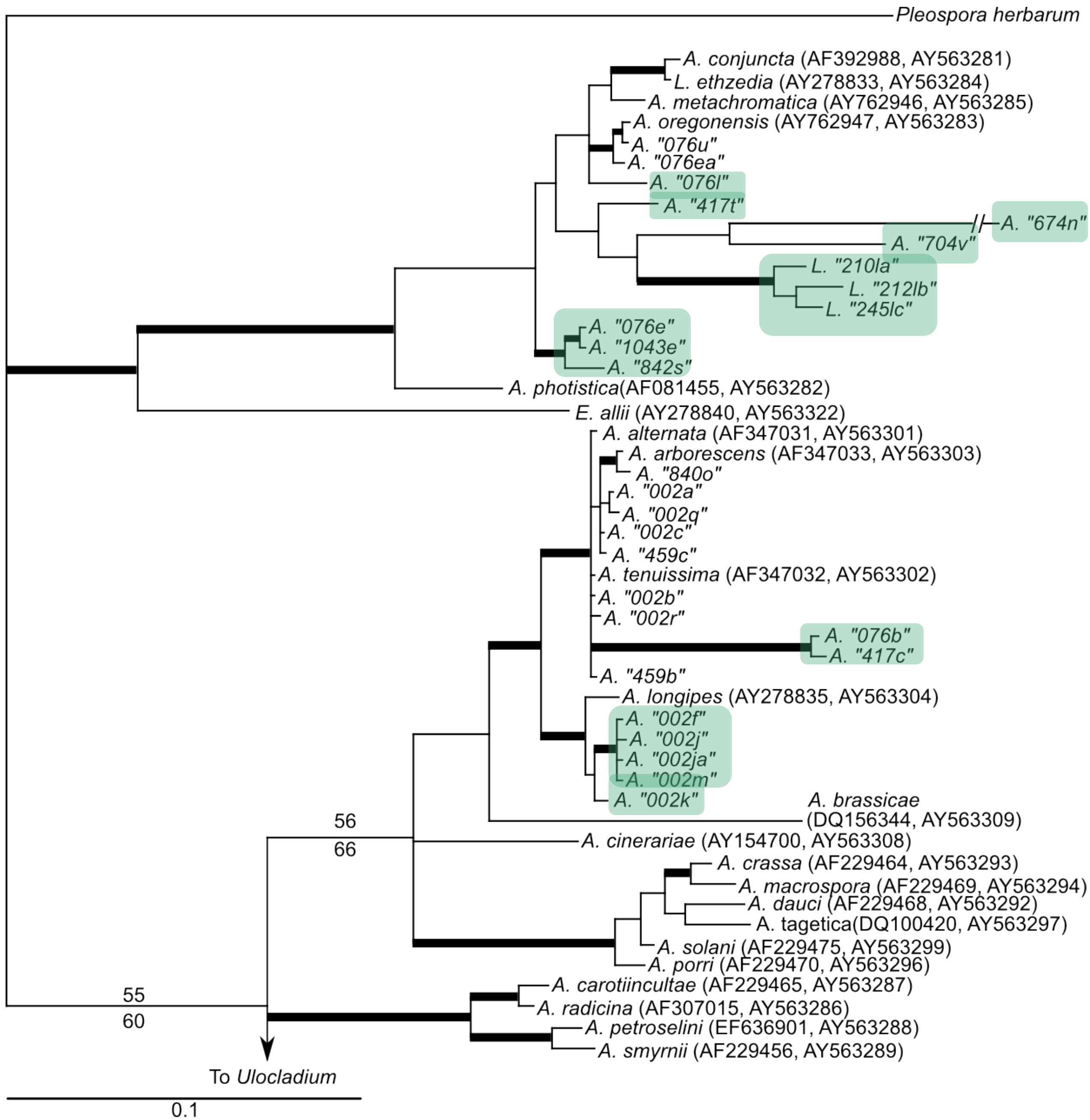
The case of *Alternaria*: without endopytes



"Alt a 1", the major *Alternaria* allergen, is a protein with no known function in fungal metabolism. It is also proved as a good marker for *Alternaria* phylogeny on the species level (Hong et al., 2005).

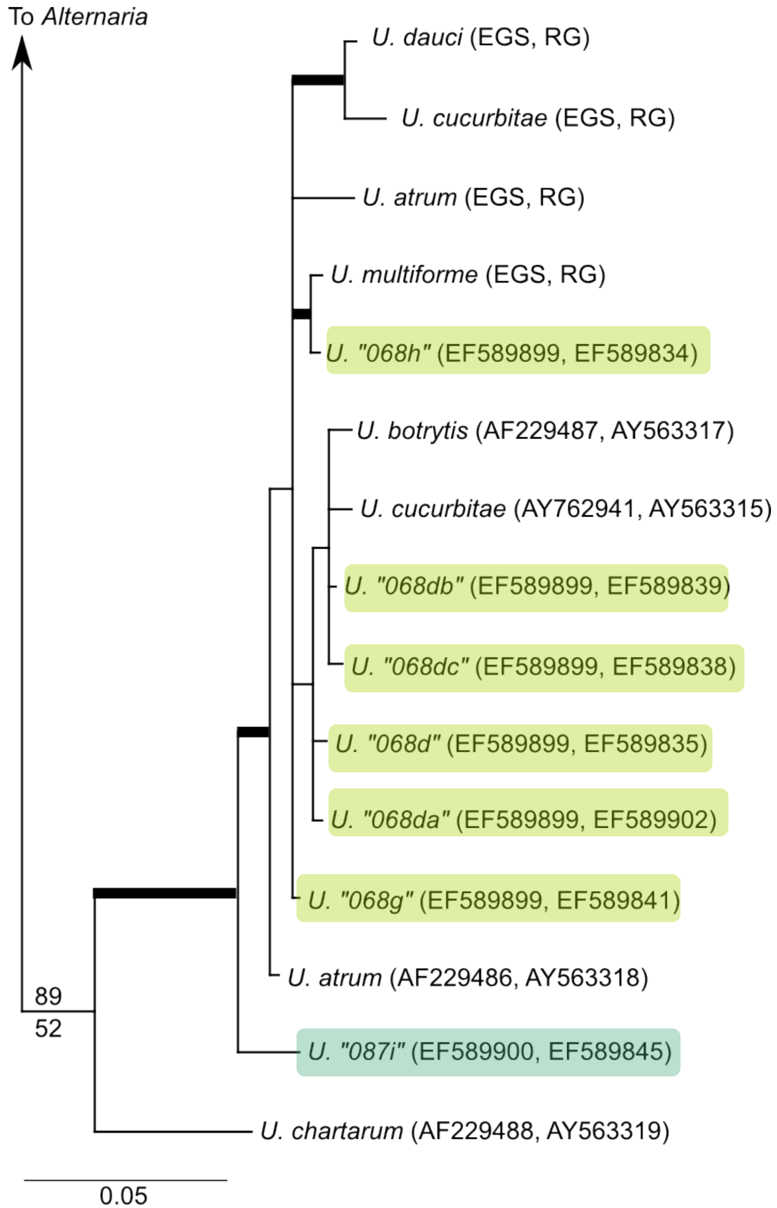
"Alt a 1" gene contains recently evolved spliceosomal intron.

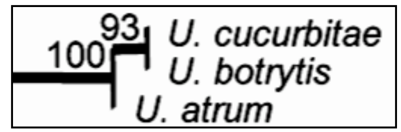
The case of *Alternaria*: with endophytes



At least 9 new branches, 40% of species with available ITS and “Alt a 1” sequences

The case of *Ulocladium*: with endophytes





In this case, the addition of endophytes mostly blurred the species relationship. This is probably because the diversity of *Ulocladium* have been overestimated by “splitters”.

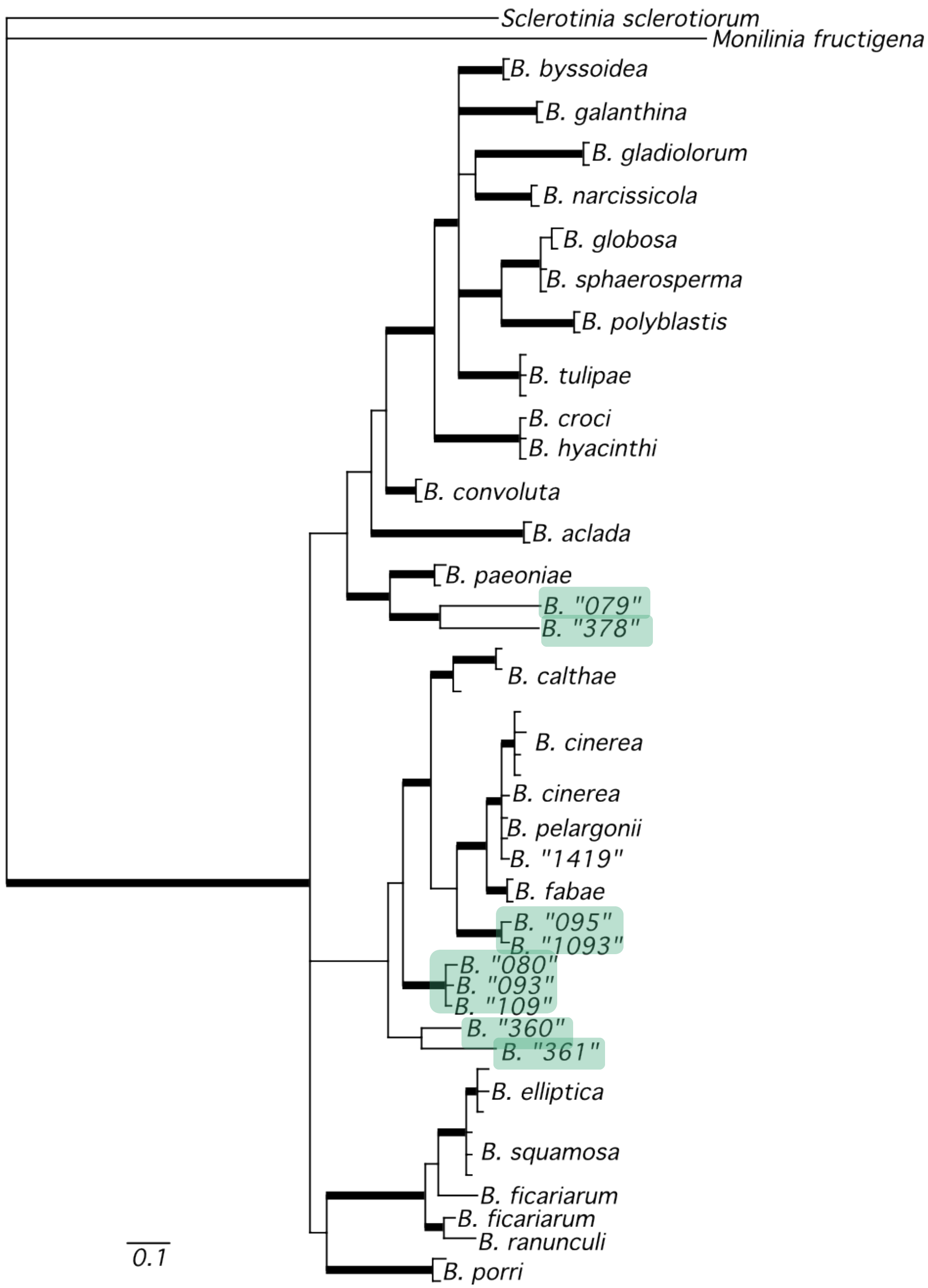
The case of *Botryrtis*

Botrytis blight of rose

Botrytis
(anamorph of *Botryitinia*, Sclerotiniaceae, Helotiales), is widespread and economically important genus of plant pathogens. Totally, 21 species have been described. As an exception, **all** species have been sequenced (Staats et al., 2005). hsp60 stands as a good marker of intrageneric evolution.

PDA culture and conidia

The case of *Botryris*: with endophytes

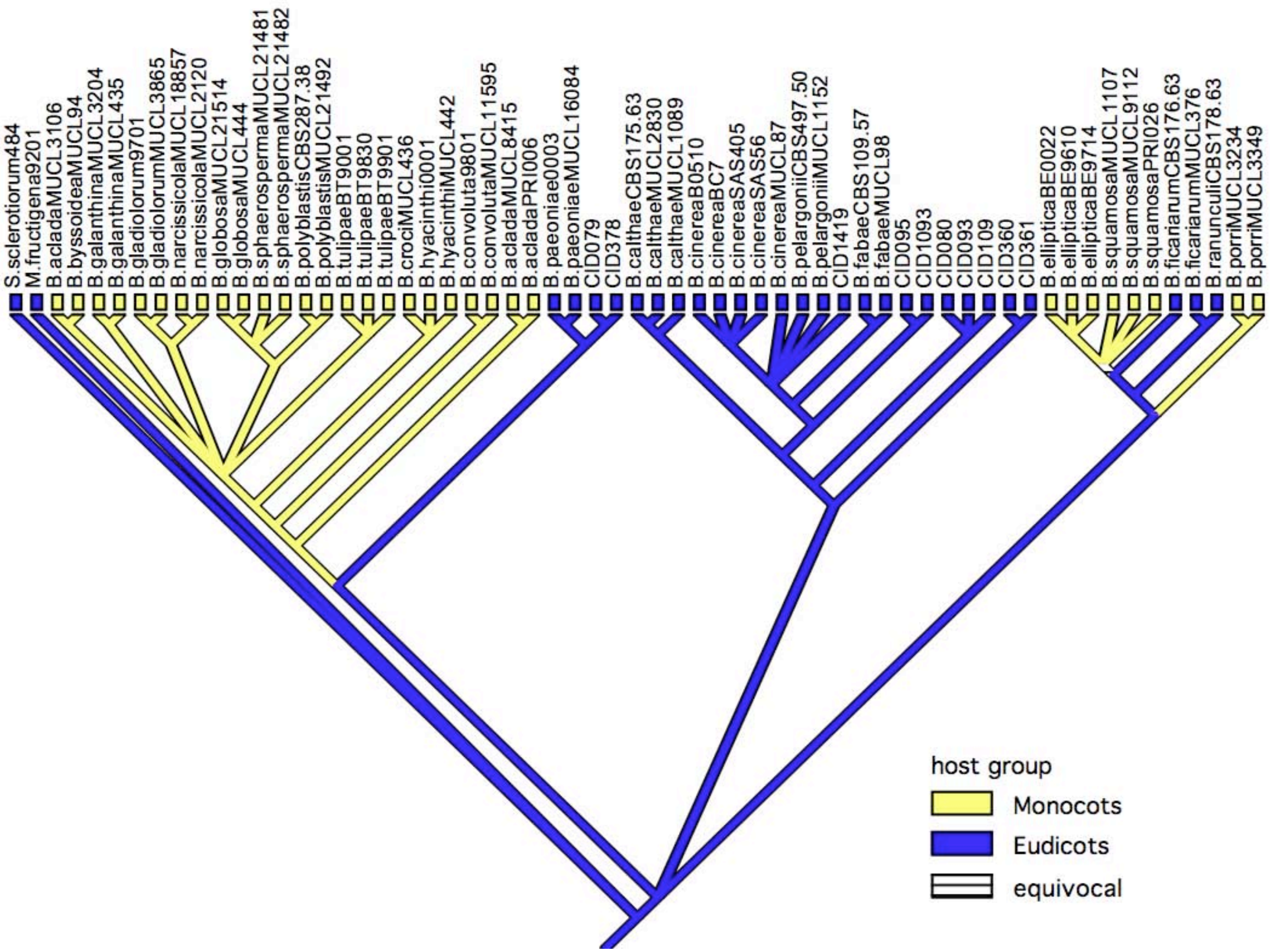


At least 6 new branches, 30% from **all** known diversity of *Botrytis*, extracted as endophytes from a **single plant species** (and there are 0.3 millions of them).

This is close to Hawksorth's (1991) 1:6 fungal:plant species score, but for a **single fungal genus**.

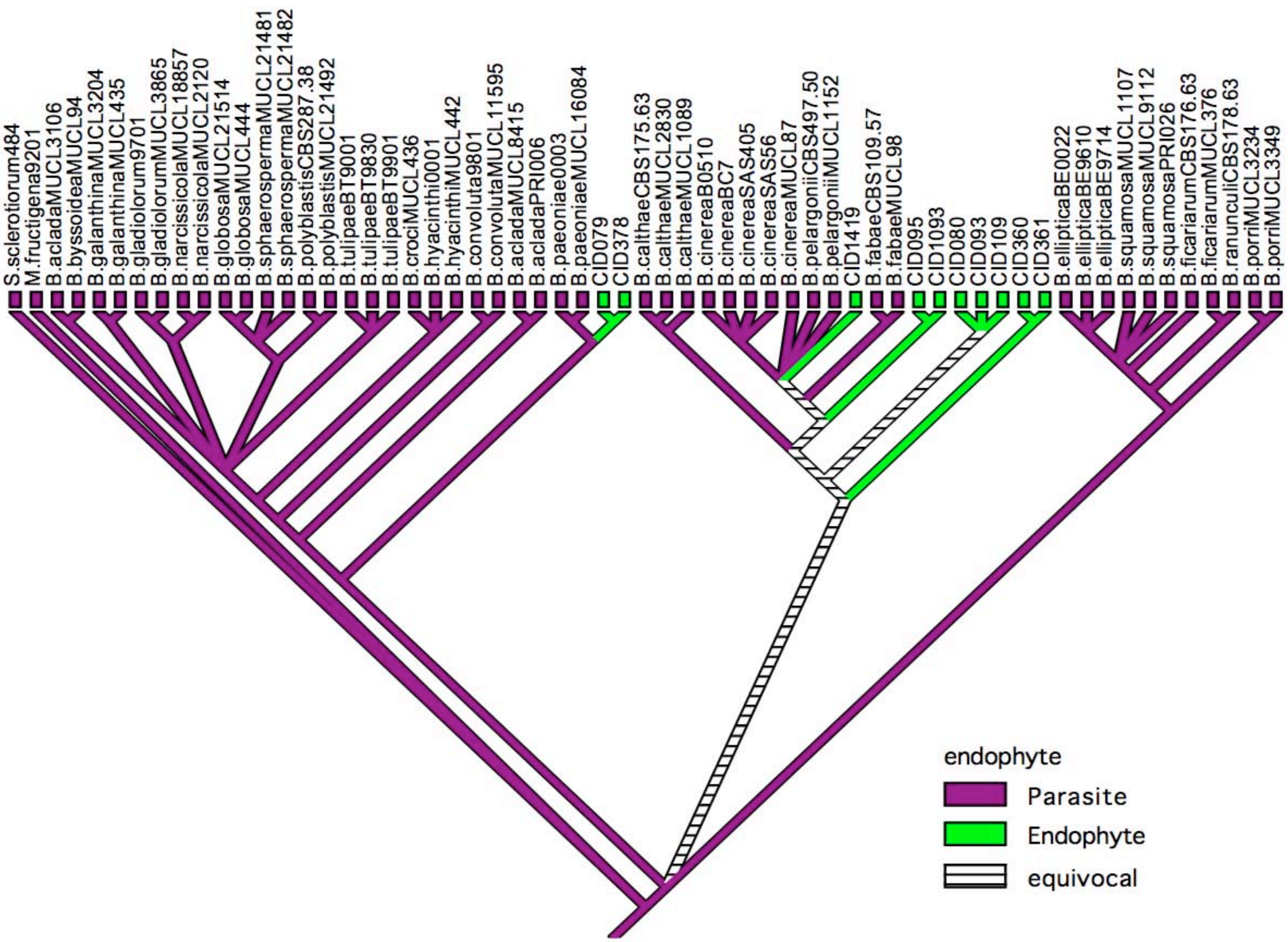
Therefore, we have a support for the least conservative predictions of real fungal biodiversity, like 1.5 millions (Hawksworth et al., 2001) or even 9.7 millions (Cannon, 1997).

Botrytis: evolution of host preference



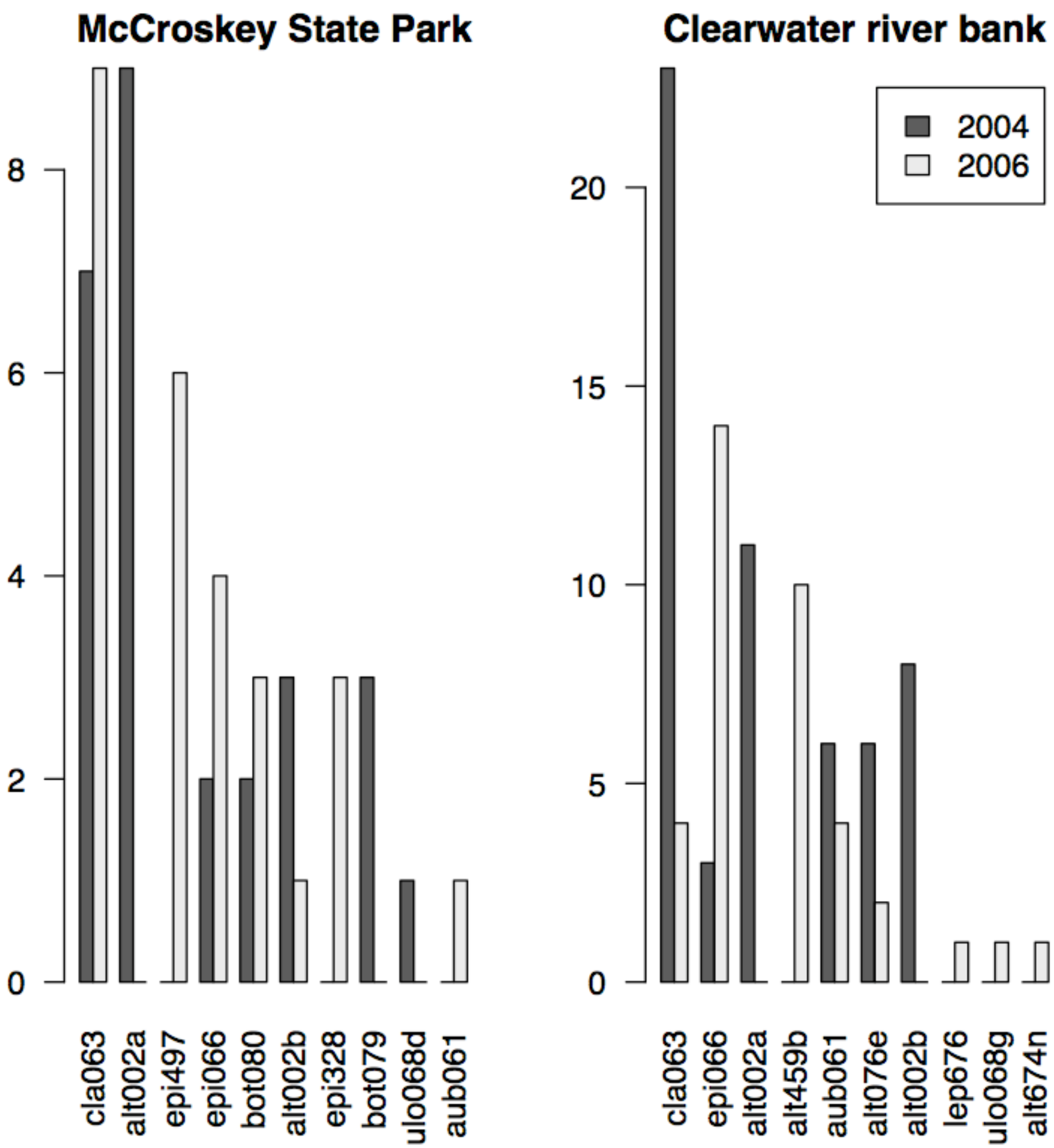
The discovery of endophytic *Botrytis* from knapweed changed the hypothesis of ancestral host: now it is clear that it was the eudicot, and shifting to monocots occurred several times

Botrytis: parasite roots of endophytes?



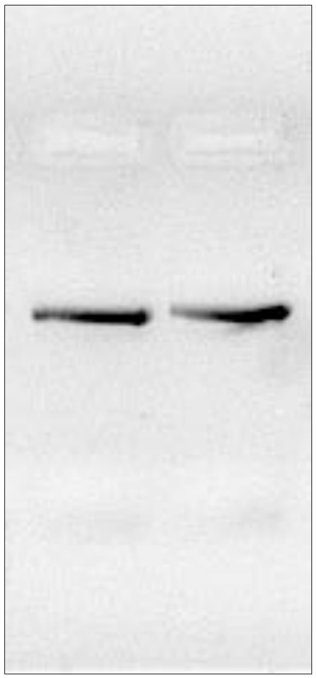
Even more striking is the evidence that endophytes in the genus are more likely evolved from parasites. Since *Botrytis* are necrotrophs, this can be due to the loss of toxins inducing host-cell death.

Instability of sampling



First time in the endophyte research, we conducted repeated sampling. The diversity and especially isolation frequency of endophytes showed to be unstable.

“Environmental” approach



With fungal-specific primers, ITS1-F and LR3, it is possible to amplify only fungal part of DNA extracted from knapweed plant. Our preliminary investigation showed that:

• The number of extracted haplotypes is significantly greater than from cultivated approach;

• Plants with zero isolation frequency from seeds have endophytes in leaves and roots;

• Root and aboveground endophyte floras are different;

• This approach is complementary to culture-based: some fungi which usually do not go to culture (like *Cryptococcus* basidiomycete yeast) obtained in relatively high proportions, and vice versa

−	**Spotted knapweed,** *Centaurea stoebe*	+
Delayed flowering: *Alternaria* 123 *Fusarium* 124 Reduced flowering extent: *Alternaria* 62		Protection from seedhead weevils: *Alternaria* 62 *Epicoccum* 66
Reduced aboveground biomass and increased generalist herbivory: *Fusarium* 107		Increase of aboveground biomass: *Alternaria* 73, *Alternaria* 432
Suppressed germination of *C. stoebe* itself: *Alternaria* 120 *Botrytis* 360 *Fusarium* 107 *Fusarium* 396		Suppression of germination of a competitor, assayed in the same manner as (-)-catechin: *Alternaria* 62 *Epicoccum* 66

“Novel weapons”: the idea

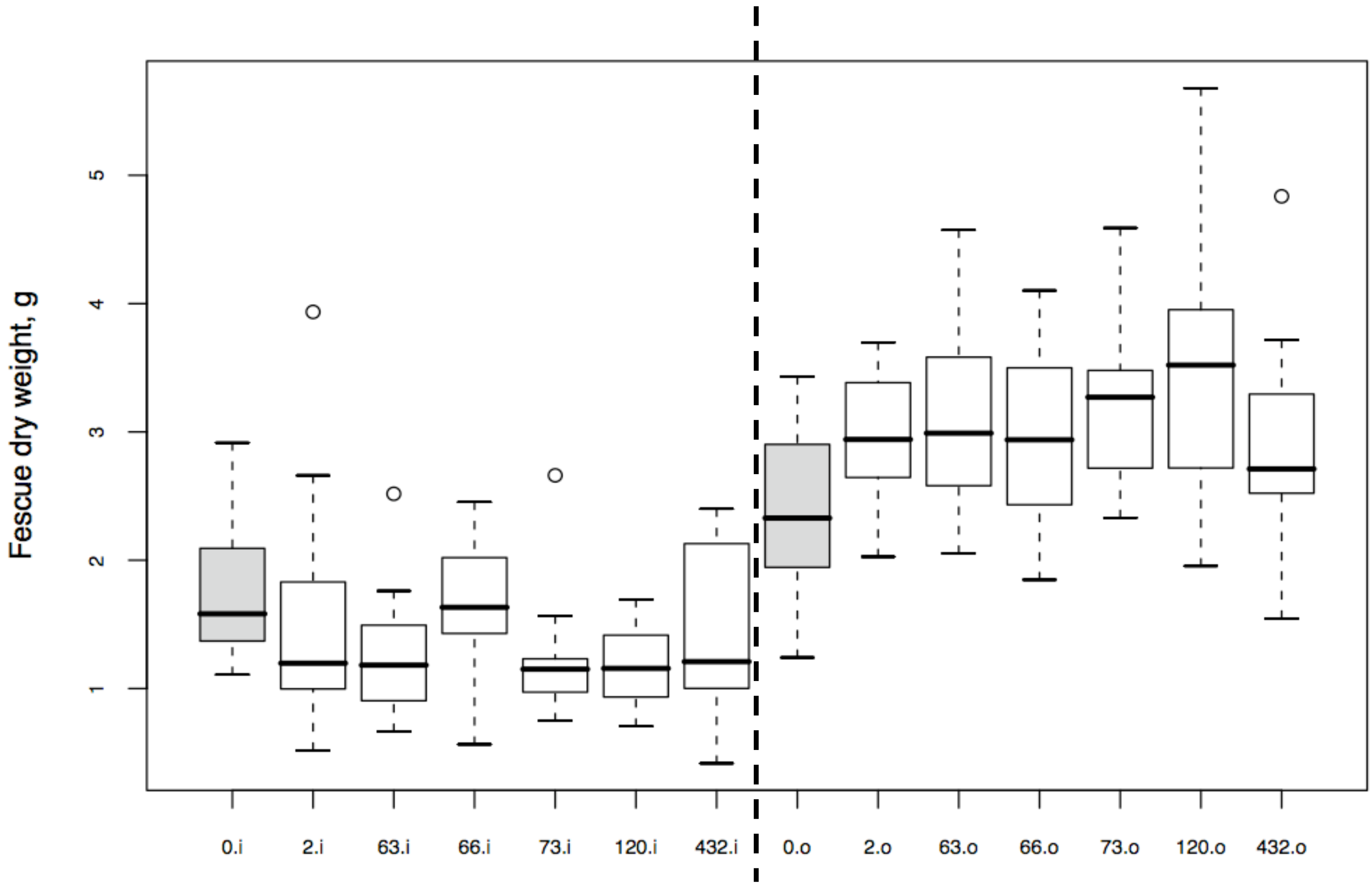
A Mongol warrior wielding the recurve bow, novel weapon of 13th century (illustration by W.M.Ridenour)

“Novel weapons” (Callaway & Ridenour, 2004) is the hypothesis of invasion, which tells that the success of invasive species may be due to the possession of biochemicals that naive native species never encountered.

Novel weapons are:

- the result of co-evolution of species in the native range;
- evolutionary challenge for native species
- the basis of competitive advantage of invasive species

“Novel weapons”: the support



Biomass of *Festuca idahoensis* (native species) decreased whereas biomass of *F. ovina* (Eurasian species) increased

Endophytes and invasion

Hypotheses of invasion	Our research supports
Enemy Release Hypothesis	**+**
Enhanced Mutualism Hypothesis	**+**
Novel Weapons Hypothesis	**+**

Acknowledgements

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- Cort Anderson
- Sanford Eigenbrode

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- Anil Kumar Raghavendra
- Hongjian Ding
- Maryse Crawford
- and more than 20 collectors

Web-site of the project

Knapweed project

Russian | English

Most of my materials are on the Russian Web-site (many of them are in English). Here I have put the information about my current project.

I am working now with Dr. George Newcombe and Dr. Cort Anderson in the Dept. of Forest Resources at the University of Idaho on investigating the ecology and systematics of endophytes in *Centaurea maculosa* (spotted knapweed) in its native and introduced ranges, including controlled greenhouse experiments to determine interactions among plants, endophytes, and insects and molecular systematics of endophytic fungi. I also coordinate the collaborative effort, involving faculty in ecology, entomology, mycology, and systematics (Dr. Sanford Eigenbrode, Dr. Mark Schwarzlaender, Dr. Tim Prather).

Specific objectives of the project [modified from grant proposal]:

1. Elucidation of the origin of the endophytes of *C. maculosa* (i.e., in either the native or the invaded range of *C. maculosa* itself) with sequence-based, phylogenetic tests. Origin is important because the «biogeographical source of the microbes» with which a plant interacts, can significantly affect the outcome of the host-symbiont interaction (Klironomos, 2002), and plant fitness (Callaway et al., 2004).
2. In planta determinations of interactions between endophytes of *C. maculosa* and insects, including biocontrol insects that have deliberately been released for the control of spotted knapweed.
3. In planta testing of the hypothesis of exclusive horizontal transmission of endophytes. Exclusive horizontal transmission of co-introduced fungi would have implications for plant quarantine policy and practice in the U.S. (Palm, 1999).
4. Evaluate the compositional similarity among symbiont communities from the native and invaded ranges, using a new statistical approach (Chao et al., 2005). Plant invasiveness may depend on the presence or absence, or relative abundance of key symbionts (Klironomos, 2002); host age may affect endophyte loading of *Centaurea* plants. We would employ a new aging technique for *Centaurea* (Dietz, 2002); patches have already been mapped across the Idaho landscape (Lass et al., 2002) and in eastern Washington (Roche and Roche, 1988).
5. In pursuit of generality, we would also research yellow starthistle, or *Centaurea solstitialis*, and cheatgrass, or *Bromus tectorum* (with respect to objectives 1, 3, and 4).

- Presentation of the first results (April 12, 2006), PDF file, 1.4 Mb
- Abstract to the Botany 2006 conference, PDF file, 90 kb
- Presentation to the Botany 2006 conference, PDF file, 1.4 Mb
- Presentation on College of Natural Resources seminar (November 3, 2006), PDF file, 2.1 Mb
- Presentation to the CRISSP advisory board (December 12, 2006), PDF file, 1.9 Mb
- Presentation on the Idaho weed conference (Nampa, ID, 01/31--02/01, 2007) PDF file, 2.1 Mb
- Abstract to the Botany 2007 conference
- Presentation to the Botany 2007 conference, PDF file, 1.2 Mb
- Presentation to the WSU Plant Pathology Seminar, September 17, 2007, PDF file, 2.7 Mb

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- Key for the description of plants from Centaurea stoebe/maculosa/diffusa group
- The sampling form for 2006, two additional protocols (Cynoglossum officinale and Chondrilla juncea)
- The bibliography database of the project in BibTeX format. BibTeX is the bibliography database format for TeX, you can open BibTeX files (for example) with JabRef (Mac, Linux or PC), this software could also convert BibTeX to Endnote format.

http://uidaho.edu/~shipunov