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Abstract

Stem rust on cereals is a large problem in parts of the world, and it may reduce the yields significantly. It is caused by *Puccinia graminis,* a heteroecious basidiomycete with 365 known telial host grass species. *Puccinia graminis* reproduces asexually on the grass host. The sexual reproduction is completed on the alternate hosts barberry or mahonia. In Sweden, due to harsh winters, sexual reproduction is crucial for maintaining the population and spreading the disease. This has lead to the Swedish population of *P. graminis* being very genetically diverse. The most efficient way to avoid infection of *P. graminis* is the use of resistant cultivars. In places with a high rate of sexual reproduction eradication of barberry is a good management strategy.

Puccinia graminis has undergone genetic differentiation in relation to their host species. This has given rise to the concept of *formae speciales* (f. sp.). A *forma specialis* is adapted to infect one or a few host species. The agronomically most important ones are *P. graminis* f. sp. *tritici* (Pgt)*, P. graminis* f. sp. *avenae* (Pga) and *P. graminis* f. sp. *secalis* (Pgs) who infect wheat, oats and rye respectively. Barley is susceptible to both Pgt and Pgs.

This thesis aims to examine which *formae speciales* are present in Sweden and also se if they can be distinguished using molecular techniques. The samples in the study were collected from barberry, cereals and grasses at different locations in Sweden.

According to established definitions on the host species of the *formae speciales* Pgt, Pga and Pgs are present in Sweden.

By sequencing the Internal Transcribed Spacer (ITS) region along with genes Elongation Factor 1 alpha (EF1-α) and Cytochrome Oxidase Subunit 1 (COI) the phylogenetic relationships of the different *formae speciales* was investigated. The sequences only distinguish between two phylogenetic clades; one containing samples collected from hosts susceptible to Pga and the other with hosts susceptible to Pgt and Pgs. Within Pga, a subclade of *P. graminis* f. sp. *dactylis* forms.

The role of barberry in the epidemiology of stem rust was investigated by using microsatellites (SSR) and examine if the same multilocus genotypes (MLG) are found on barberry and nearby grasses at the same location. The same MLG:s were not found but some of the populations from the respective locations proved to have a common ancestry. The SSR data was also used to try to confirm the results from the phylogenetic study. There were

statistically significant differences in genotype between the Pgt and Pga clades but not as consistent as for the phylogenetic study.

In addition to the common disease management strategies the use of crop rotations alternating between cereals susceptible to Pgt and Pga would decrease the risk of infection as well.

Future studies should focus on further investigate the differences between Pgt and Pga in order to determine if they could be regarded as two different species. The differentiation within Pga is also an interesting future study.

Keywords: Puccinia graminis, population biology, barberry/*Berberis* spp., phylogeny, small sequence repeats

Sammanfattning

Svartrost på stråsäd orsaker stora problem inom jordbruket världen över då sjukdomen kan orsaka stora skördeförluster. Svartrost orsakas av svampen *Puccinia graminis* som är en värdväxlande basidiomycet med både sexuell och asexuell förökning. Den asexuella förökningen sker på någon av dess 365 kända gräsvärdarter medan delar av den sexuella fasen av livscykeln är beroende av mellanvärden berberis.

I Sverige är sexuell reproduktion mycket viktig för *P. graminis* fortlevnad eftersom möjligheten för den att övervintra på växande gräs är mycket liten på grund av den långa och kalla vintern. Infektion av svartrost kan undvikas genom att odla resistenta sorter. I områden där sexuell reproduktion sker i hög utsträckning kan utrotning av berberis vara ett alternativ för sjukdomsbekämpning.

Inom arten har det skett en genetisk differentiering gentemot de olika gräsvärdarna och olika specialformer *formae speciales* (f. sp.) har utvecklats. En *forma specialis* är genetiskt anpassad till att infektera specifika värdar. De för jordbruket viktigaste *formae speciales* är de som infekterar vete (*P. graminis* f. sp. *tritici,* Pgt), råg (*P. graminis* f. sp. *secalis,* Pgs) och havre (*P. graminis* f. sp. *avenae,* Pga).

Denna uppsats syftar till att ta reda på vilka specialformer av *P. graminis* som finns i Sverige samt ta reda på om det går att skilja dem åt med molekylära metoder. Proverna som analyserats är insamlade från berberis, stråsäd samt olika gräsarter.

Baserat definitioner för vilka värdarter de respektive specialformerna har finns Pgt, Pgs och Pga alla i Sverige. Sekvensering av tre locus i genomet (ITS, EF1-α och COI) visade på att det går att dela upp *P. graminis* i två fylogenetiska grupper; en med prover med specialformen Pga och den andra med formerna Pgt och Pgs. Det bildas även en undergrupp som innehåller prover från *P. graminis* f. sp. *dactylis* inom Pga-gruppen.

Berberisens roll i spridningen av sjukdomen undersöktes med hjälp av mikrosatelliter. Detta gjordes genom att undersöka om samma multilocusgenotyp hittades på berberis och gräs som samlats in på samma plats. Identiska genotyper kunde inte hittas men på några av provtagningsplatserna fanns tecken på att proverna var mer lika varandra genetiskt än när de jämfördes med prover från andra platser.

Mikrosatellit-datan bekräftade den fylogenetiska studien, då även den visade på genetiska skillnader mellan Pgt och Pga.

Resultaten tyder på att en alternativ bekämpningsstrategi kan vara att i växtföljden växla mellan grödor som är mottagliga för antingen Pgt eller Pga för att minska sjukdomstrycket.

Framtida studier bör fokusera på att djupare undersöka de genetiska skillnaderna mellan Pgt och Pga för att kunna fastställa huruvida de kan betraktas vara olika arter eller inte. Det faktum att *P. graminis* f. sp. *dactylis* tycks vara genetiskt differentierad från Pga är också en intressant fråga att undersöka.

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Abbreviations

AMOVA	Analysis of molecular variance
BSA	Bovine serum albumin
COI	Mitochondrial cytochrome oxidase subunit I
DNA	Deoxyribonucleic acid
EF1-α	Elongation factor 1-α
f. sp.	*forma specialis* (pl. *formae speciales*)
ITS	Internal Transcribed Spacer
MLG	Multilocus genotype
MSN	Minimum spanning network
NJ	Neighbour joining
PCR	Polymerase chain reaction
Pga	*Puccinia graminis* f. sp. *avenae*
Pgs	*Puccinia graminis* f. sp. *secale*
Pgt	*Puccinia graminis* f. sp. *tritici*
SSR	Simple sequence repeats

1 Introduction

Crop diseases alone have the potential of reducing yields by up to 20 % annually (Oerke, 2006). This happens at the same time as the human population is growing and the arable land decreases. To adapt to the new conditions future agriculture will have to produce more high quality food per areal unit of land (Tilman *et al.*, 2002, 2011; Godfray *et al.*, 2010). This can partly be done by breeding for higher yielding crops in a changing climate but also by learning how to manage disease-causing organisms (pathogens) (Chakraborty & Newton, 2011).

Good agricultural management strategies cannot be established without knowing the biology and epidemiology of the pathogens. These questions can partially be answered by looking back into the evolution of the organism to find out how it has evolved into becoming the pathogen causing epidemics today.

Rust diseases can infect a wide variety of crops and can potentially cause large yield reductions and also considerably change the conditions for agricultural production. One famous example of change in agricultural practice is the outbreak of coffee rust, caused by *Hemileia vastratix* in Ceylon (present Sri Lanka) in the 1860's (McCook, 2006). The disease wiped out the entire production of coffee, forcing growers onto a different path, tea production. Nowadays, due to this huge epidemic, Sri Lanka is the fourth largest tea producer in the world (Chang, 2015).

Rusts on cereals have been a problem for farmers for thousands of years (Large, 1946). During this time people have tried to manage the disease with the methods available at hand. The romans sacrificed dogs in order to keep the disease at bay. Later, people started noticing an increased occurrence of stem rust, caused by *Puccinia graminis* (Pers.), in cereal fields with barberry bushes nearby than if barberry was not present. Now they moved on to eradicating the bushes and even legislating about it. The first law of eradication of barberry bushes was passed in France in 1660 (Large, 1946).

In present day, modern plant breeding has helped reduce the infestations of stem rust by breeding resistant cultivars. This has been a good strategy, especially in wheat (*Triticum aestivum*) (McIntosh *et al.*, 1995). In Uganda in 1998 though,

infections of stem rust were observed in previously resistant wheat varieties. Studies showed that they had been infected by a new and highly virulent race of stem rust, commonly known as Ug99 (Pretorius *et al.*, 2000). It's virulent to the long effective stem rust resistance gene *Sr31*. This race and its predecessors have continued to spread over large parts of Eastern Africa and into Southwestern Asia (Rust Tracker, 2016).

Predictions have been made that Ug99 could threaten the wheat production worldwide unless we are able to breed new resistant cultivars (Singh *et al.*, 2008, 2011). Such a serious development calls for increased research about this disease. In order to manage stem rust we need to understand its biology and epidemiology.

This thesis aims to examine the variation of *P graminis* in Sweden and answer the following questions:

- Which *formae speciales* of *Puccinia graminis* are present in Sweden?
- Can the *formae speciales* be distinguished by molecular methods?
- Can the same multi locus genotypes (MLG:s) found on barberry and grasses at a given location?

1.1 Background

Many of the rusts infecting cereals belong to the *Puccinia* genus, (kingdom Fungi, phylum Basidiomycota, class Urediniomycetes, order Uredinales). The rusts are biotrophic, meaning that they rely in a living host for survival and reproduction (Agrios, 2005). A major characteristic of rust fungi is their diverse types of spores; *P. graminis* that causes stem rust has five different spore stages. Another common feature of many of the *Puccinia* species is that they are heteroecious, meaning that they alternate hosts to complete their life cycle. In the case of *Puccinia graminis* barberry (*Berberis* spp.) is the most common alternate host. It is on barberry that the sexual reproduction is completed. *Puccinia* rusts not only infect domesticated cereals, but also wild grasses in the *Poa* genus (Anikster, 1984; Leonard & Szabo, 2005).

1.2 Puccinia graminis

1.2.1 Stem rust - the disease caused by *Puccinia graminis*

P. graminis causes the disease stem rust (or black rust) on cereals and grasses and cluster cup rust on barberry and mahonia. Characteristics of the disease are pustules of spores on the stem of the plants; in severe cases symptoms may also show on other parts of the plant (Leonard & Szabo, 2005). The pustules break the epidermis, causing the plants to lose water. The disease also decreases the photosyn-

thetic active surface of the plant, thus reducing its ability to photosynthesize. It can also make the plants more prone to lodging (Roelfs *et al.*, 1992; Agrios, 2005). All of this can lead to yield reductions.

1.2.2 Life cycle and epidemiology

Puccinia graminis has a complex life cycle consisting of both sexual and asexual reproduction on two different host plants. Overwintering occurs on cereal debris or wild grasses in the form of teliospores. In spring the teliospores germinate and form four basidiospores. They are spread by wind and can infect young leaves of barberry or mahonia where pycnia (sometimes referred to as spermagonia (Agrios, 2005)) are formed. *P graminis* requires that pycniospores of a different mating type meets the pycnium to be fertilized. Once the fertilization is completed, new structures called aecia are formed, usually on the bottom side of the leaf. These produce aeciospores that are spread by wind to nearby susceptible plants (wild grasses or cereal fields). An infection of an aeciospore results in the formation of uredinia and urediniospores. These are the structures that give rise to the striking symptoms of the stem rust. Urediniospores are clonally reproduced and in large numbers, and can re-infect the host during the growth season (Blumer, 1963; Leonard & Szabo, 2005). They can also spread over long distances (hundreds of kilometres) by wind (Aylor, 2003).

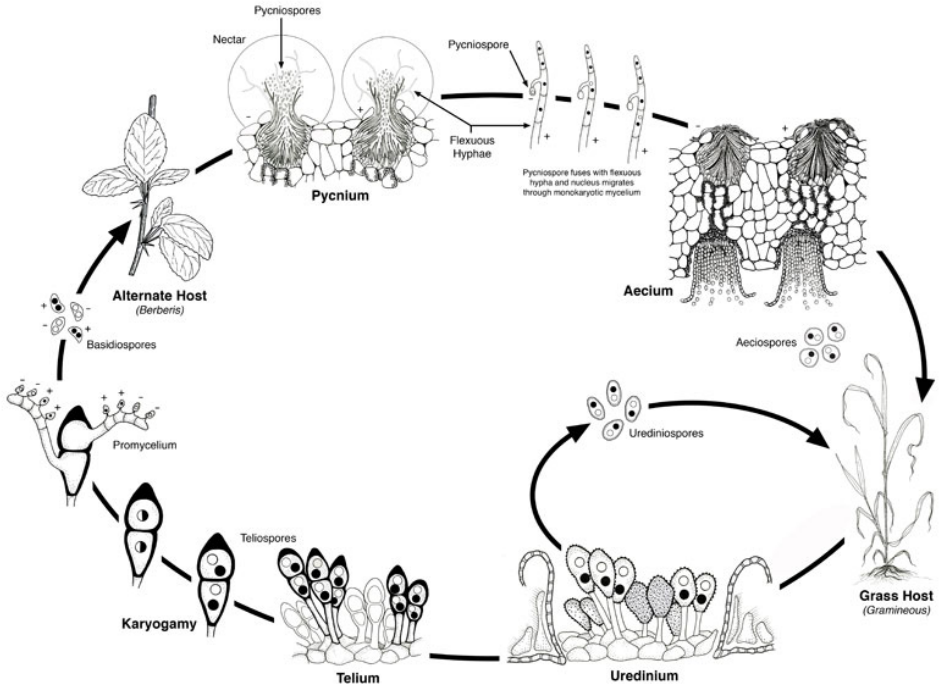

Figure 1. Life cycle of *P. graminis* (Leonard & Szabo, 2005).

1.2.3 Sexual or asexual populations?

Puccinia graminis is present all over the world but the population structure differs in different locations. In large parts of the world the populations show a low genetic diversity, indicating a high rate of clonal reproduction. Clonally reproduced urediniospores can infect new grass hosts over and over. If a grass host is always available it provides the fungus with what plant pathologists call a "green bridge". The spores will be able to reproduce asexually on the living grass host and then continue to cause new infections, both on the same and other plants. Since clonal reproduction occurs at a high rate and with a large number of new spores, the impact of sexually reproduced spores decreases under such conditions.

In Sweden, though, sexual reproduction is common and the populations are very diverse (Berlin *et al.*, 2013b). The reason the fungus cannot overwinter on a living host is likely due to our temperate climate with long, cold winters. Also, there are very few linages of *P. graminis* virulent to winter wheat, which is one of few overwintering crops in Sweden. Because of that, the chance of successfully overwintering as urediniospores on grasses and continue the asexual reproduction in spring is low. Therefore the sexual stages are important for maintaining and spreading the disease in Sweden. This means that the alternate host; barberry, plays an important role in the survival of the fungus.

Eradicating the alternate hosts does not guarantee that there will be no more outbreaks of stem rust. As many plant pathologic species *P. graminis* is capable to spread over large distances by wind, a phenomenon called long distance dispersal (LDD) (Brown & Hovmøller, 2002). It is mainly the urediniospores that manage to spread (Agrios, 2005). The spores follow atmospheric wind currents (one example is the so called *Puccinia*-pathway in North America) (Aylor, 2003). In Sweden such pathways may introduce inoculum from southern Europe, by carrying spores northwards (Nagarajan & Singh, 1990).

This means that although a farmer might have taken all precautions possible to avoid stem rust infection (resistant cultivars, removing barberry etc.), inoculum with virulence previously not present in Sweden might infect the crop. However, those kinds of infections are thought to have a delayed onset and therefore not cause as severe infections (Roelfs *et al.*, 1992).

1.2.4 The *formae speciales* concept

The concept of *formae speciales* (f. sp.) was introduced by Eriksson (1894). It is based on the hypothesis that different linages of the fungus are specialised in infecting a specific grass species or a group of host species. The *formae speciales* of rusts have adapted and coevolved with their respective hosts resulting in genetic differentiation. By doing inoculation experiments with different linages of *P. graminis* on different hosts, a number of different *formae speciales* and their host

range have been defined (Eriksson, 1898; Anikster, 1984). The *formae speciales* that Eriksson described in 1898 were: *P. graminis* f. sp. *tritici* (Pgt), *P. graminis* f. sp. *avenae* (Pga) and *P. graminis* f. sp. *secalis* (Pgs). Their respective host range is accounted for in Table 1.

Table 1. The *formae speciales* and their hosts. Adapted from Eriksson[1] (1898) and Stakman[2] (1916).

P. graminis f. sp. *secalis*	*P. graminis* s.fp. *avenae*	*P. graminis* f. sp. *tritici*
Rye (*Secale cereale*)	Oats (*Avena sativa*)[1]	Wheat (*Triticum vulgare*)[1]
Barley (*Hordeum vulgare*)[1]	*Dactylis glomerata*[1]	*Hordeum vulgare*[2]
Triticum repens (today *Elytrigia repens*)[1]	*Alopecurus pratensis*[1]	
Triticum caninum (close relative to *Elymus caninus*)[1]	*Avena elatior* (today *Arrhenatherum elatius*)[1]	

Later studies also showed that both Pgs and Pgt were virulent on barley (*Hordeum vulgare*) (Stakman, 1916 in Schumann & Leonard, 2000). Previous studies have shown that there are two or three subgroups of *P. graminis* that are phylogenetically similar to each other (Abbasi *et al.*, 2005; Berlin, 2012). In a study done on aceia on barberry it was evident that types that according to sequencing of the ITS region and species identification via GenBank belonged to either Pga or Pgt also showed morphological differences (Berlin *et al.*, 2013a). This further supports the theory about a differentiation between *formae speciales*; the differences can be seen both genetically and morphologically. There are inconsistencies between the phylogenetic groups and host plants though, (Abbasi *et al.*, 2005) raising questions about the "usefulness" of the *formae speciales* concept.

1.2.5 Managing stem rust

The most successful strategy to avoid disease has been the use of resistant cultivars. Stem rust resistance is in many cases regulated by single genes; R-genes, that prohibit the fungus from infecting the plants. This kind of resistance was discovered in the 1940s by H. H. Flor who did crossing experiments with a rust fungus infecting flax (Flor, 1971). The gene-for-gene hypothesis as it's called implies that a protein in the plant detects a protein (elicitor or effector) in the pathogen at the point of infection, resulting in a cascade of defence mechanisms that prevent further development of the disease (Jones & Dangl, 2006).

This type of resistance, often called vertical resistance, is quite easily overcome since the pathogen only has to mutate once at the specific site coding for the elicitor or effector to be able to infect the previously resistant host. In the 1930s the gene *Sr31,* originating from rye (*Secale cereale*), was introduced in wheat

(Bluthner & Mettin, 1973; Zeller & Baier, 1973) providing it with resistance that would last for many years. It took almost 40 years before *Sr31* had its real "breakthrough" as a resistance gene. When Norman Borlaug, known as the father of the "green revolution", among other things provided the world with stem rust resistant wheat cultivars that also weren't as tall as the previous varieties. This lead to significant reduction of yield losses due to stem rust and lodging and for this and other achievements, Borlaug was awarded the Nobel Prize for Peace in 1970 (Swaminathan, 2009). *Sr31* proved to maintain its efficiency as a resistance gene until the late 1990s. The first reports of stem rust overcoming the gene were in Uganda in 1998.

Another way to manage the disease has been to eradicate barberry bushes. The first time this method was used in larger scale was in France in the 17th century (Large, 1946). In 1918, the Swedish parliament passed a law stating that all barberry had to be eradicated (SFS 1976:451). The ban on barberry was lifted in 1994 and it has lead to increased prevalence of stem rust in Sweden (Berlin, 2012). The reason eradicating barberry is efficient as a measure of disease management in temperate regions is that it prohibits *P. graminis* from completing the sexual phase of its life cycle. The asexual spores can't survive the winter and the sexual spores will have no host to infect.

The two methods for disease management mentioned above target the different stages in the pathogen life cycle. Eradication of barberry stops the sexual reproduction and in turn the genotypic variation in the population will decrease since all reproduction is clonal. This in turn mean that plant breeders can focus on breeding varieties with specific resistance to the present races of the pathogen and thus prohibiting it from infecting the crops (Agrios, 2005).

Chemical control of the fungus is also an option. The Swedish board of agriculture (SJV) annually gives recommendations regarding the use of fungicides in agricultural production. They state that fungicide treatment against stem rust in wheat often is unnecessary due to the late emergence of stem rust symptoms. In oats, on the other hand fungicide application can be efficient if done before ear emergence. However, the most efficient way to avoid disease according to SJV is early sowing. That way the plants will be large enough to sustain infection when the inoculum reaches the oat fields (SJV, 2016). When using fungicides to manage a fungal disease you have to account for the risk of making the pathogen resistant to the chemical treatment. *Puccinia* sp. posses many of the characteristics attributed to fungi that are prone to develop fungicide resistance (Brent & Hollomon, 2007). They have sexual reproduction making it possible to overcome the effect of the fungicide by genetic recombination. The spores that survive fungicide application also grow in number very rapidly and can then infect new hosts. However, the

risk of resistance development is assumed to be low since none of the known fungicide resistance genes have been detected in the *P. graminis* genome.

1.3 History of taxonomic research, sequencing and microsatellites

For as long as there have been plant diseases people have tried to understand what is causing it and how to prevent disease from damaging their crop. In the early years of the existence of plant pathology, diseases were studied mainly on the basis of host plant and symptoms. Further along came the magnifying glasses and later microscopes, which enabled us to study the pathogens up close. Now knowledge about the morphology of the pathogens could be added to the previously known facts about them, making it easier to distinguish between different species.

Since the discovery of DNA and development of genetic analyses a lot of the taxonomic research has expanded from only studying phenotype and performing inoculation experiments to include genotype-based studies.

1.3.1 Morphological determination

When studying the morphology you simply look at the fungus and try to identify and quantify differences between the samples to confirm a species or *formae speciales.* By describing the shape and texture, and measuring spores or other structures, you can determine a species. Common measures for rust fungi are the length and the width of the spore as well as the area.

1.3.2 Gene sequencing

Gene sequencing techniques has provided us with tools to study differences between and within species or possibly *formae speciales* based on differences in only a few base pairs in a gene.

The genes used in this study

The internal transcribed spacer region (ITS) has been selected as the ”barcode” gene used to identify and compare fungal species (Schoch *et al.*, 2012). It is located between ribosomal genes and consists of exons: 18S, 5.8S and 28.S and introns ITS1 and ITS2. In this study, special primers developed for *Puccinia* were used (Barnes & Szabo, 2007).

Elongation factor 1-alpha (EF1-α) is a housekeeping gene that exists in basically all eukaryotes. It is present in two copies in the *P. graminis* genome (Schillberg *et al.*, 1995).

Mitochondrial cytochrome oxidase subunit 1 (COI) is another “barcoding” gene that can be used to examine the phylogenetic relationships between and within species. It is too, a housekeeping gene.

1.3.3 Microsatellites

A microsatellite is a section in the genome consisting of a repetitive sequence (a DNA motif) of usually two to five base pairs. The microsatellites are often referred to as SSR (simple sequence repeats). They are usually found in non-coding regions, introns, and don’t translate into proteins. Because introns have a higher mutation rate than exons, due to the polymerase slipping when copying the DNA motif, the microsatellites are highly variable in length both within and among populations. This makes them suitable for population studies (Bruford & Wayne, 1993; Ashley & Dow, 1994).

When making an analysis based on microsatellites a combination of several are used to detect multi locus genotypes (MLGs). Some of the SSR:s vary a lot, thus has a high number off different alleles while others vary less.

2 Material and methods

2.1 Sample collection

All the samples used in the study and information about fungal species, host species, assigned *forma specialis* is available in Appendix II.

2.1.1 Östergötland samples, 2014

In the spring of 2014, different lines of wheat, rye, barley and oat were planted in the edges of farm fields located close to barberry bushes at three locations (Å/Stenby, Bökestad and Skarpenberga) near Norrköping, Sweden. Aecia infected leaves from the barberry bushes were collected in early June. Later during the summer, uredinial samples from each line were collected at two separate occasions (mid July and end of July).

Information about the host lines in this collection

The cereal hosts (Table 2) used in these collection sites were provided by Dr. Yue Jin at CDL (Cereal Disease Laboratory, USDA, MN, USA). They were chosen since they are mainly susceptible to a single *forma specialis* of *P. graminis.*

Table 2. The cereals from the USA. * a Swedish cultivar included in the collection.

Species	Variety	Expected susceptibility
Wheat	Line E	Pgt, Pgs
Wheat	Rusty	Pgt
Rye	Prolific	Pgs
Barley	Hiproly	Pgt
Barley	Hypana	Pgt
Oats	Marvelous	Pga
Oats	Belinda*	Pga

The oat variety Belinda is a Swedish variety. It was not one of the grown lines but it is commonly grown in Sweden and samples from Belinda were collected from fields close to the barberry bushes at some of the sampling sites.

2.1.2 Uppsala samples, 2014

Additional samples from wild grasses were collected in the summer of 2014 in and around Uppsala, Sweden.

2.1.3 Östergötland and Småland samples, 2015

In summer 2015 samples were collected from the same barberry bushes as the Östergötland samples from 2014 along with samples from wild grasses in close approximate to the bushes. Additional samples were collected at other locations in Östergötland, Småland and Öland.

2.1.4 USA samples

A collection of four samples collected on grasses was provided by Dr. Yue Jin as a reference. These samples were collected in Michigan in 2015.

2.1.5 Storing the samples

All samples were air-dried stored in room temperature in envelopes or in folded sheets of paper from the time of collection until DNA-extraction.

2.2 DNA-extraction

One pustule of urediniospores were carefully cut out from infected grass straws and put in 2 ml tubes along with approximately 30 pieces of 2 mm glass beads and a knifepoint of diatomaceous earth. Similarly, single aceia were cut out from the barberry and mahonia leaves. In cases where the diameter if the aecia exceeded 3 mm, the aceia was divided in half and placed in two separate tubes. Two or three replicates were prepared for each grass host and five to seven were prepared for barberry or mahonia collections. When possible, the replicates were taken from different straws or leaves to secure that the sample collection captured the genetic diversity.

DNA-extractions were performed using the OmniPrep kit (G-Biosciences) according to the manufacturers instructions for fungal tissue. The nucleotide concentration for the extracted samples was measured using spectrophotometry (NanoDrop ND-1000).

2.3 Gene sequencing

Three genes were used in the phylogenetic study: ITS (internal transcribed spacer), EF1-α (elongation factor 1α) and COI (cytochrome oxidase subunit 1). They were amplified by a polymerase chain reaction (PCR) according to Table 2. The DNA template concentration in the reactions was 5 ng/µl for genes EF1-α and COI and 1 ng/µl for ITS. The PCR was performed in 20 µl reactions according to table 2 with the following cycling conditions: initial denaturation at 94 °C for 5 minutes; 35 cycles of denaturation at 94 °C for, primer annealing and primer elongation at 72 °C for 30 seconds each; final extension at 72 °C for 7 minutes. The different primers and annealing temperatures are accounted for in Table 3.

Table 3. *Contents of the PCR-reaction*

Component	Volume
MilliQ water	4,5 µl
DreamTaq (Fermentas, Helsingborg, Sweden) buffer	2,0 µl
DreamTaq (Fermentas, Helsingborg, Sweden) DNA polymerase	0,1 µl
dNTP	2,0 µl
Primer F	0,4 µl
Primer R	0,4 µl
$MgCl_2$	0,6 µl
DNA template	10,0 µl

Table 4. *PCR primers for genes ITS, EF1-α and COI.*

Gene	Primer	Annealing temperature	Reference
ITS	ITSrustF10d	55 °C	Barnes, 2007
	StdLSUR2a		Barnes, 2007
EF1-α	EFbasidR	57 °C	van der Merwe, 2007
	EF1		Berlin, 2012
COI	P360f	51 °C	Liu, 2010
	P360r		Lui, 2010

The PCR products were evaluated using 1% agarose TAE gel electrophoresis to see if the reactions had worked or not. The agarose gel was stained with 1 µl Nancy per 50 ml agarose solution to make DNA bands visible in the gel camera. The successfully amplified samples were purified using the Agencourt AMPure purification kit (Agencourt Bioscience Corporation) according to the manufacturers recommendations.

A few of the EF1-α-reactions displayed two or three visible bands on the agarose gel. Those samples were rerun on a 2 % agarose gel with a low voltage for two to three hours to separate the bands. The strongest visible band for each sample was cut out from the gel and purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research).

The PCR products were sent to Macrogen Inc. (Amsterdam, The Netherlands) for sequencing from both 5' and 3' ends using the same primers as above using the capillary electrophoresis sequencing method.

Initially, the ITS sequences were compared to sequences deposited in GenBank using the BlastN algorithm to identify the species. Samples with a match of 99 % or higher were regarded to be the same species as the match while samples with a lower match rate were marked "No ID" (Appendix II). Those samples were amplified and re-sequenced and a new attempt at determining their species was done.

The forward and reversed sequences of all three genes were assembled in SeqMan Pro (DNA Star Lasergene). Assembled contigs were aligned in Mega6 (Tamura *et al.*, 2013) by ClustalW alignment and then examined by eye to find and check ambiguous data.

Neighbour-joining (Saitou & Nei, 1987) phylogenetic trees were produced for ITS, EF1-α and COI alone as well as for the combination of ITS + EF1-α + COI and ITS + EF1-α; all with a bootstrap test of 1000 replicates. For ITS, the non-coding (introns) and coding (exons) parts of the sequences were also analysed. The location of the introns and exons were obtained by downloading a reference genome of *P. graminis* from GenBank (accession number DQ417383.1).

The trees of the different sequenced regions were checked against each other bye eye to make sure the samples clustered the same way for all the sequenced regions.

An additional comparison of the complete ITS-sequences to sequences in GenBank was done using the BlastN algorithm to see which *formae speciales* the samples could be assigned to based on the ITS sequence.

2.4 Microsatellites

SSR analysis were only performed on samples identified as *P. graminis* in the BLAST search based on the ITS sequence. Ten microsatellite (SSR) markers (Table 6) were analysed using PCR multiplex reactions with two primer pairs in each reaction.

In the PCR, a reaction volume of 15 µl was used for each sample. The PCR mix for each reaction was prepared according Table 4. The forward primers were either dyed with the fluorescence HEX or FAM to be able to distinguish the loci in later analyses. The cycling conditions were: initial denaturation at 98 °C for 5 minutes;

35 cycles of denaturation at 98 °C, primer annealing at 62 °C and primer elongation at 72 °C; final elongation for 10 minutes at 72 °C and hold at 4 °C.

Table 5. *Master mix for SRR reaction*

Component	Volume (µl)
MilliQ water	2,62
Phusion buffer	3
dNTP	0,6
Primer F	0,375
Primer R	0,375
BSA 2%	1,5
Taq-polymerase Phusion	0,15
DNA sample (10 ng/µl)	6
Tot volume	14,62

The success of the PCR reactions was evaluated on a TAE 1 % agarose gel electrophoresis. If the reaction had succeeded (visible bands for at least 2/3 of the samples), they were then sent for "GenScan" at SciLifeLab in Uppsala, Sweden (ABI 3730XL DNA Analyzer).

The length of the fragments was scored using the programme Gene Marker (Soft Genetics). The procedure is to look at the picture of the gene scan and identify peaks that indicate the length of the amplified sequence at the different alleles. When a sample displayed more than two clear peaks at a locus, the data for that particular locus was excluded. If a sample lacked more than three loci, the sample was removed from the data analysis.

Initial statistical analyses were performed using GenAlEx (Peakall & Smouse, 2012), a Microsoft Excel add-in. The following analysis were performed: observed heterozygosity H_o, expected heterozygosity H_e, number of alleles and number of private alleles for the different markers. An AMOVA (analysis of molecular variance) and pairwise F_{ST} was performed to investigate if the multi locus genotypes (MLG:s) from different hosts differed genetically. Additionally, principal coordinate analyses (PCoA) were performed on different populations to further illustrate the population differentiation. The populations can be defined differently depending on the question asked. In this study, the following definitions were used:

1. Species – is there a connection between MLG and species?
2. Location – are the same MLG:s found on the same location?
3. Clades A and B – can the phylogenetic trees from the gene sequencing be supported by SSR MLG:s?

PoppR (Kamvar *et al.*, 2014) is an R-package used for analysing population genetic data. It was used to evaluate the quality of the SSR data by looking at the gene accumulation curve. In addition, minimum spanning networks (MSN) that illustrate the genetic distances between the samples were also produced using PoppR.

Table 6. *The SSR primers used in the multiplex reactions. The primers included in the table were used in the analysis.*

SRR locus	Primer	Primer sequence (5'-3')	Flourophore	Repeat motif	N_a	Size range in this study	H_o	H_e
Pgestssr021	21AAGF	GTTTGCCTGATGATGGATGA	HEX	AAG	8	232-253	0,544	0,829
	21AGGR	CCGAATGCAGATTACCCTTG						
Pgetssr024	24F	TCATCGACCAAGAGCATCAG	HEX	ATG	14	118-157	0,747	0,882
	24R	TTCGGGAGTGAGTCTCTGCT						
Pgestssr279	279F	ATCGAAGAGCCGTTCACTGT	HEX	ATG	9	165-187	0,726	0,744
	279R	AGGGAATCCGATACCGGAGT						
Pgestssr368	368F	CATCTGATCACCGTCACAGC	FAM	AAC	11	219-249	0,415	0,790
	368F	AGCACAAGCTTCGTTTCTGAG						
PgtCAA53	CAA53F	AGGCTCAACACCACCCATAC	FAM	CAA	10	175-229	0,402	0,665
	CAA53R	AGGAGGAGGTGAAGGGGATA						
Pgestssr255	225F	CATCTGATCACCGTCACAGC	FAM	AAC	11	218-248	0,539	0,802
	225R	CCACAGCTTCGTTTCTGAGC						

N_a = number of alleles; H_o= observed heterozygosity; H_e= expected heterozyogsity

3 Results

3.1 DNA-amplification

A total of 244 sequences were successfully amplified and sequenced (79 for ITS, 95 for COI and 70 for EF1-α). The ITS sequences had an effective length between 417 and 1035 bp. The COI-sequences ranged between 280 and 362 bp and EF1-α between 308 and 699 bp.

3.2 Species determination

3.2.1 Genetic determination

Most of the samples could be assigned as *Puccinina graminis* by BlastN but other species were also found on the grasses. These included *P. coronata, P. striiformis, P. montenesis* and *P. triticina.* The *formae speciales* retrieved in the BlastN search were *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *avenae*.

3.2.2 Visual and genetical determination of Mahonia samples

Aecial samples collected from *Mahonia ssp*. didn't match any sequences deposited in GenBank and their species could therefore not be determined genetically. Instead a visual determination had to be done. The sample was collected by Dr Yue Jin and he did an initial visual examination of it and suggested that it could belong to the *Cumminsiella* genus.

To confirm this, aecio- and urediniospores were examined in a microscope (Figure 2). In addition to this the sequences were compared to known sequences of *Cumminsiealla mirabilissima* provided by Dr Les Szabo at CDL (Cereal Disease Laboratory, Agricultural Research Service, United Stated Department of Agriculture).

The visual determination failed due to the poor quality of the microscopy pictures but the samples could be determined to be *Cumminisella mirabilissima* by comparing the sequences of the ITS region to sequences of known origin.

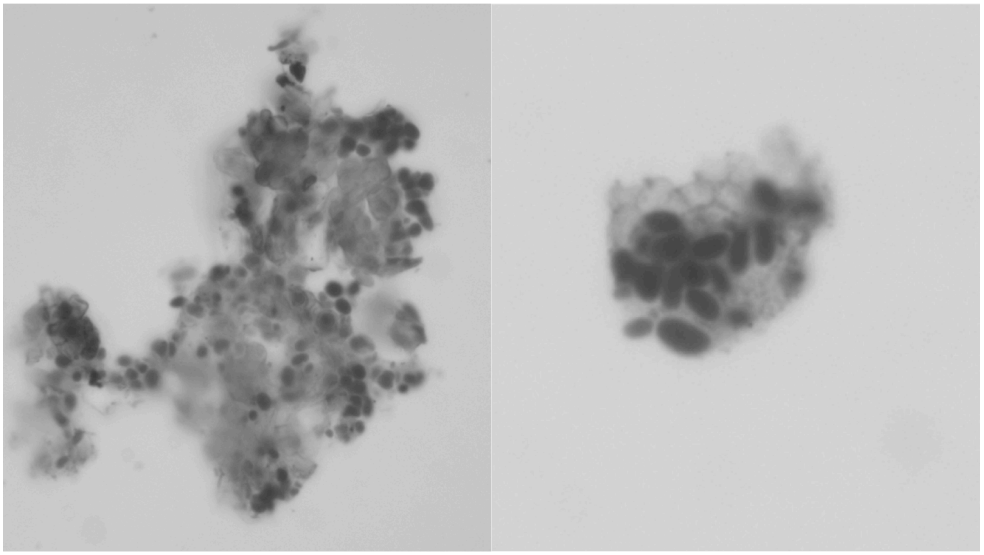

Figure 2. Microscope pictures of the *Cumminsiella mirabilissima* spores.

3.3 Formae speciales

When blasting the complete ITS sequences to GenBank they were assigned only two of the major *formae* speciales: Pgt and Pga. Some samples could be assigned as Pga or *P. graminis* f. sp. *lolii*, *P. graminis* f. sp. *dactylis* and *P. graminis* f. sp. *phlei-pratensis*. No sample identified as Pgs. The assigned *formae speciales* was used to investigate their phylogenetical relationship.

3.4 Phylogeny

The neighbour-joining (NJ) phylogenetic tree (Figure 3) based on the ITS region for samples collected on cereals and grasses shows a high supports of two clades (A and B). Clade A mainly consists of samples collected from *A. sativa, P. pratense, A. elatius, D. glomerata, Festuca* sp. and "unknown grass". Clade B consists of samples collected from *H. vulgare* and *E. caninus*. Samples from *S. cereal and E. repens* are present in both clades. Samples collected from *D. glomerata* form a subclade in Clade A together with one sample each of *A. elatius, Festuca* sp. and *E. repens.*

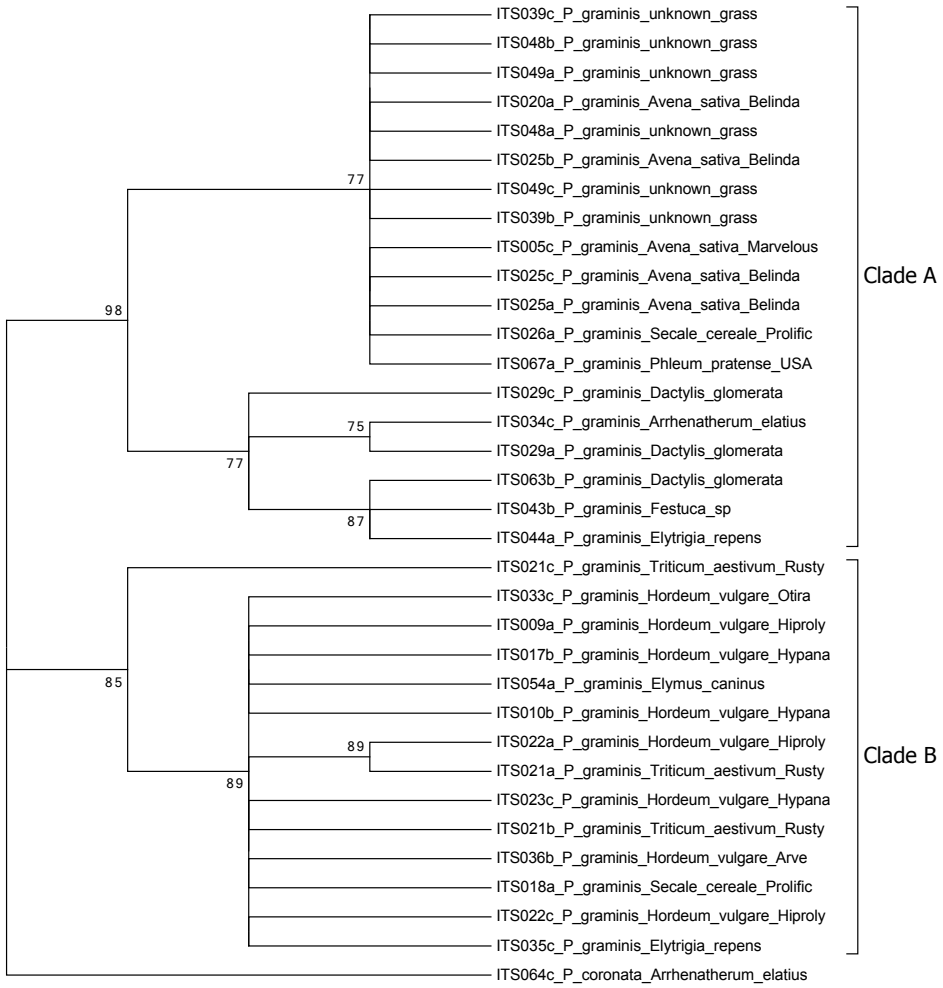

Figure 3. NJ phylogenetic tree based on the ITS region on samples collected from cereals and grasses. Samples are named according to: Gene+sample number_species_host_(variety). *P. coronata* is used as an out-group. Clade A is supported with a bootstrap value of 98 and Clade B with a value of 85.

When including the *formae speciale* assigned to the samples by GenBank, the NJ phylogenetic tree showed a clear distinction between Pga (and/or *P. graminis* f. sp. *lolii*, *P. graminis* f. sp. *dactylis* and *P. graminis* f. sp. *phlei-pratensis*) and Pgt (Figure 4).

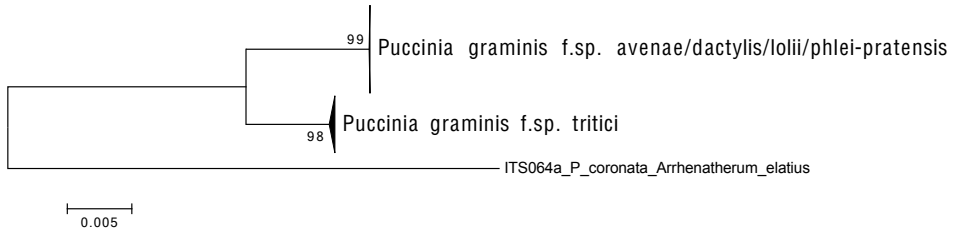

Figure 4. Collapsed NJ phylogetetic tree of the complete ITS region including assigned *formae speciales* according to GenBank. The *formae speciales* Pga and Pgt are supported by bootstrap values of 99 and 98 respectively.

The aecial samples collected from barberry grouped in different clades and could be assigned to either Pga (Clade A) or Pgt (Clade B). In some cases, samples taken from the same bush clustered in different clades (eg. Sample 104b in Clade A and sample 104d in Clade B). Samples that could be assigned either Pga or *P. graminis* f. sp. *dactylis* grouped with the Pga samples, and interestingly they formed a subgroup within Clade A (Figure 5).

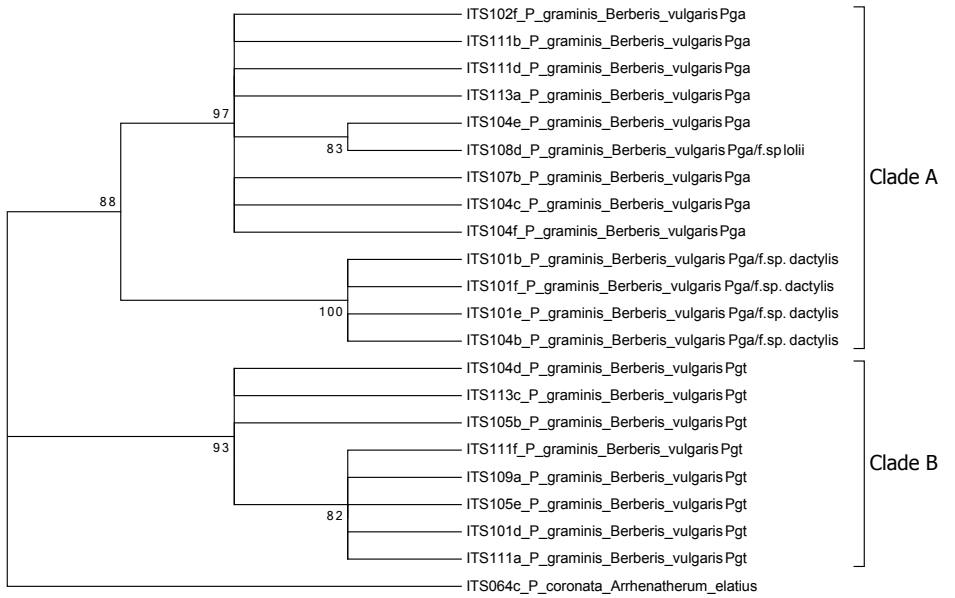

Figure 5. NJ phylogenetic tree based one the ITS region of samples collected from barberry. Clade A and B are supported by bootstrap values of 88 and 93 respectively.

The phylogeny based on EF1-α follows the same distinction between the clades. Samples collected from *A. sativa, D. glomerata, P. pratense* and an "unknown grass" cluster together in Clade A and samples collected from *H. vulgare, E. repens* and *E. caninus* cluster together in Clade B. Two replicates of the same sam-

ple collected from *Avena sativa* (EF024c1 and EF024c2) clustered in Clade B (Figure 6).

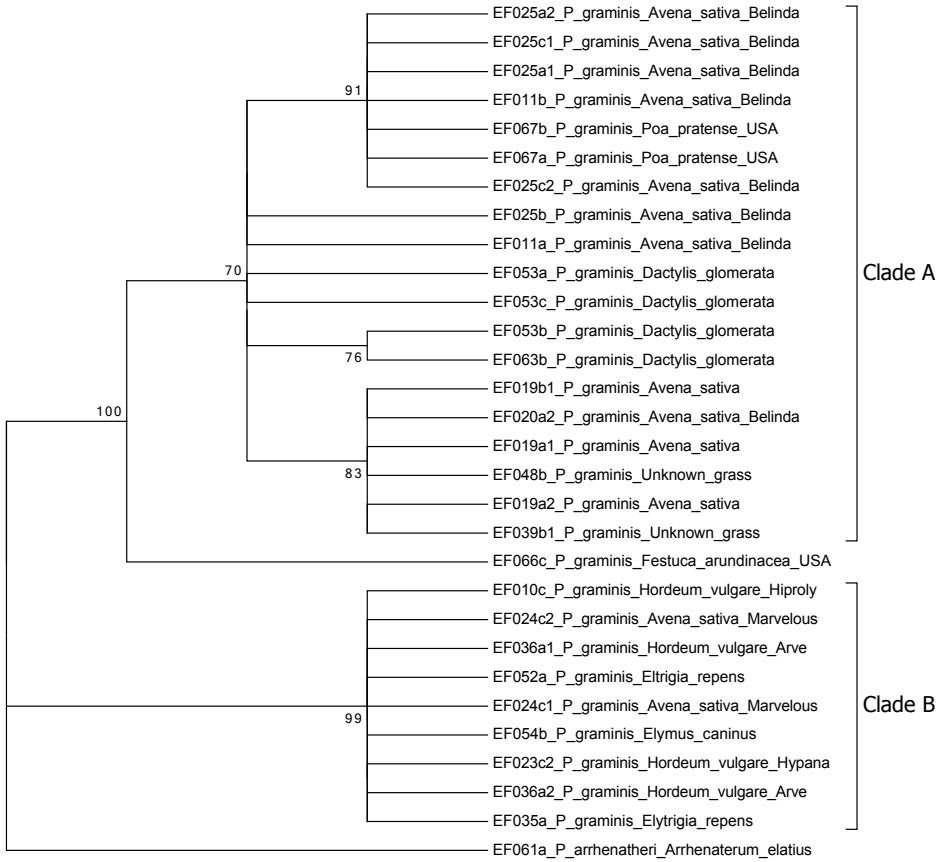

Figure 6. NJ phylogenetic tree based on EF1-α. Clade A is supported by a bootstrap value of 100 and Clade B by 99.

The phylogenies based on COI did not show any signs of differentiation between the assigned *formae speciales.*

3.5 Population genetic analysis

Out of the ten SSR markers used, two (PgtSSR21 and Pgestssr171) completely failed to amplify the samples in this study and they were immediately excluded. After analysis in Gene Marker, two additional markers (PgtCAA93, and Pgestssr109) were excluded from further analysis due to a large number of missing data. The remaining six markers were analysed in GenAlEx and PoppR. The geno-

type accumulation curve (Figure 8) shows that the variation within the samples can be displayed by including only five markers.

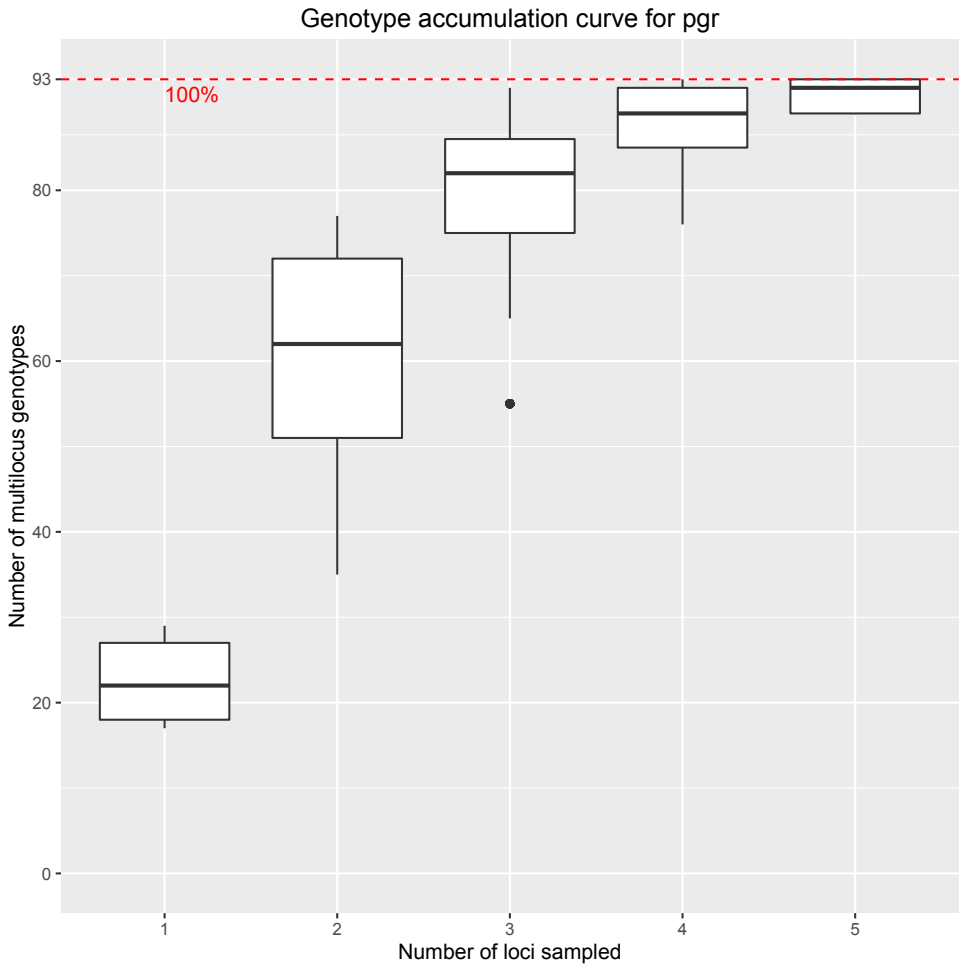

Figure 7. Genotype accumulation curve based on the data-set of MLG:s from six SSR markers

When viewing the allele frequencies with populations based on the clades from Figure 3 it is clear that the clades (indicated by colour; blue for Clade A and red for Clade B) differ genetically. Some markers show a high association between clade/*formae specialis* and allele, e.g. Clade A/Pga and the allele 184 for marker PgtCAA53.

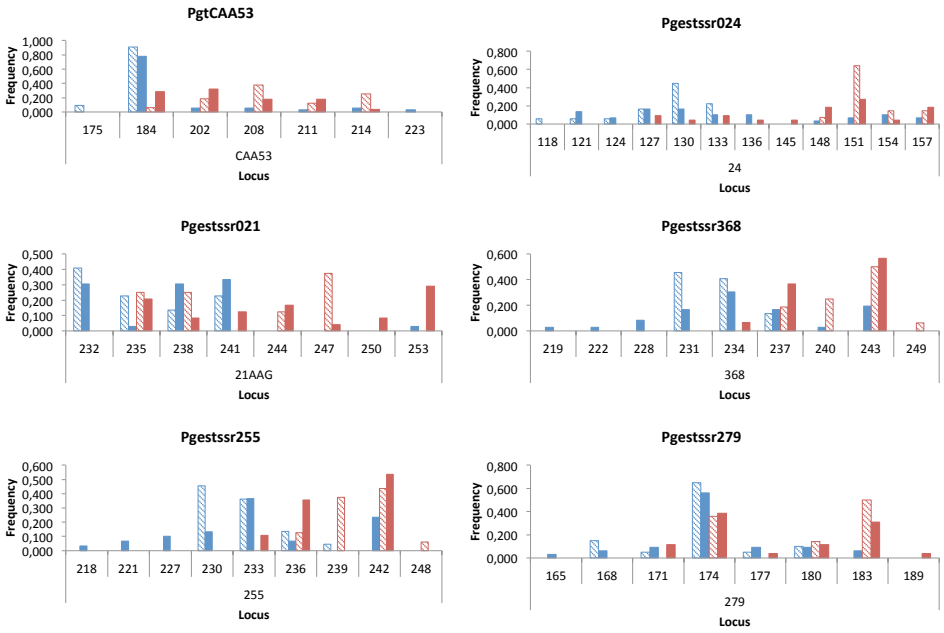

Figure 8. Allele frequencies for the six SSR markers used in the study. Populations are based on the phylogenetic species (clades) based on the ITS region (Figure 5). Clade A, samples from barberry (striped, blue bars); Clade A samples from cereals and wild grasses (blue bars); Clade B, samples from barberry (striped red bars); Clade B, samples from cereals and wild grasses (red bars).

3.5.1 Populations based on locations

The same MLG was not found on barberry and nearby grasses at any given location. Instead the MLGs differed a lot. Two samples had the same MLG but they were collected from barberry bushes at different sites and should therefore not be considered to be clones. When creating a MSN based the presumption that samples collected from the same location are one population, the large genetic variation among the samples becomes evident (Figure 9).

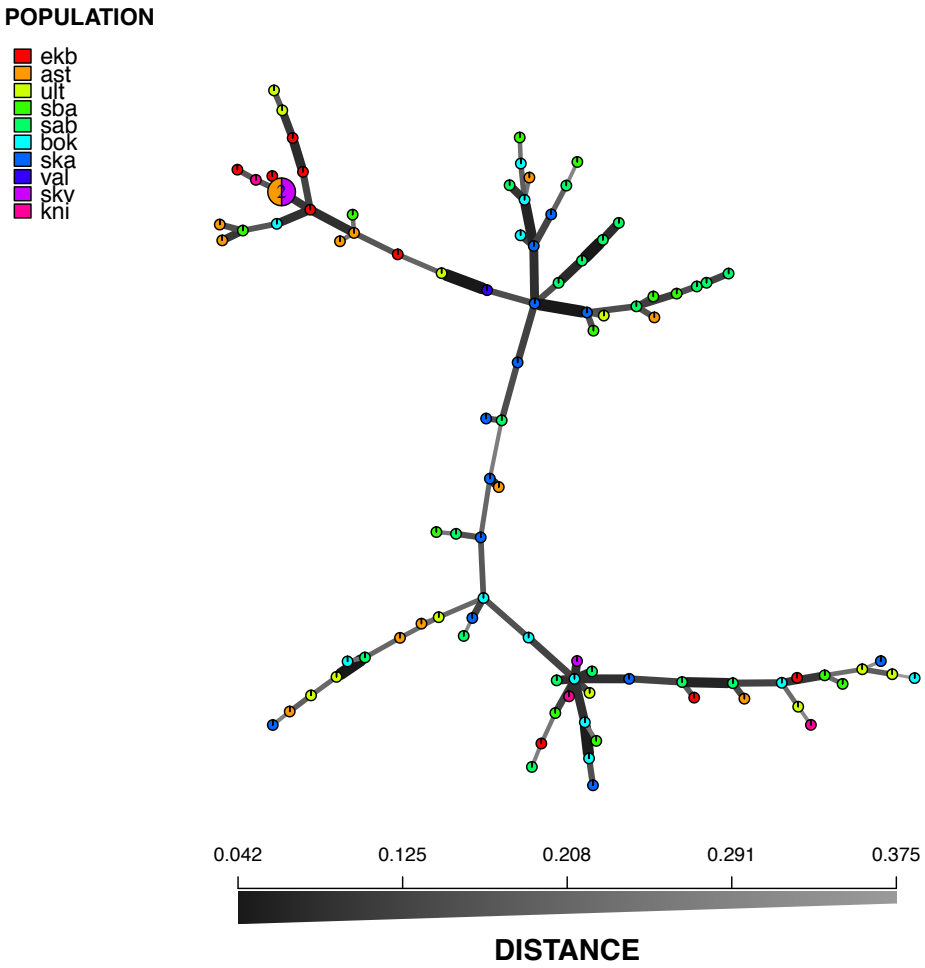

Figure 9. Bruvo distance MSN based on samples collected from barberry, cereals and grasses. The populations are based on the sampling site. ekb = Ekbacken; ast = Å Stenby; ult = Ultuna; sba = Södra Bäckmarken; sab = Säby; bok = Bökestad; ska = Skarpenberga; val = Vallby/Berg; skv = Skärva; kni = Knivsta.

The pairwise F_{ST} shows that there are significant differences between some of the populations (Table 7). The AMOVA showed that the largest variation was within each population (94 %) compared to among the populations (p-value 0,0579) (data not shown).

Table 7. Pairwise F_{ST}-table of the SSR data. The populations are based on the location they were collected. F_{ST} values are below the diagonal, bold text indicates significance. P-value is above diagonal, n.s. = non-significant F_{ST}

Bökestad	Ekbacken	Knivsta	S Bäckmarken	Skarpenberga	Skedbokvarn	Skärva	Säby	Ultuna	Å Stenby	
	**	n.s.	*	*	**	n.s.	*	n.s.	*	Bökestad
0,099		n.s.	*	***	n.s.	n.s.	***	***	n.s.	Ekbacken
0,000	0,000		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	Knivsta
0,031	**0,058**	0,000		n.s.	*	n.s.	n.s.	n.s.	n.s.	S Bäckmarken
0,033	**0,121**	0,028	0,000		**	n.s.	n.s.	n.s.	*	Skarpenberga
0,193	0,100	0,150	**0,128**	**0,168**		n.s.	*	**	*	Skedbokvarn
0,000	0,000	0,000	0,000	0,094	0,101		n.s.	n.s.	n.s.	Skärva
0,048	**0,137**	0,029	0,007	0,009	**0,142**	0,070		n.s.	n.s.	Säby
0,001	**0,139**	0,000	0,008	0,027	**0,240**	0,069	0,021		n.s.	Ultuna
0,030	0,034	0,000	0,000	**0,034**	**0,111**	0,000	0,022	0,025		Å Stenby

3.5.2 Populations based on host species

To se if any host specific differentiation could be detected based on the SSR data, the populations were divided by host species. The PCoA (Figure 10) reveals a large genetic variation within populations but there are still significant differences as shown by the pairwise F_{ST}-values (Table 8).

Table 8. Pariwise F_{ST}-values of the SSR data set. F_{ST} values are below the diagonal, bold text indicates significance. P-value is above diagonal, n.s. = non-significant F_{ST}.

Wheat	Unknown	Rye	Oat	*E. repens*	*E. caninus*	*D. glomerata*	Barley	
	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	Wheat
0,096	0,000	*	***	**	*	**	***	Unknown
0,000	**0,096**		n.s.	n.s.	n.s.	n.s.	n.s.	Rye
0,038	**0,132**	0,000		**	n.s.	**	**	Oat
0,003	**0,108**	0,009	**0,074**		*	n.s.	n.s.	*E. repens*
0,052	**0,144**	0,000	0,000	**0,102**		*	*	*E. caninus*
0,000	**0,166**	0,032	**0,102**	0,017	**0,213**		n.s.	*D. glomerata*
0,000	**0,143**	0,000	**0,109**	0,014	**0,131**	0,004		Barley

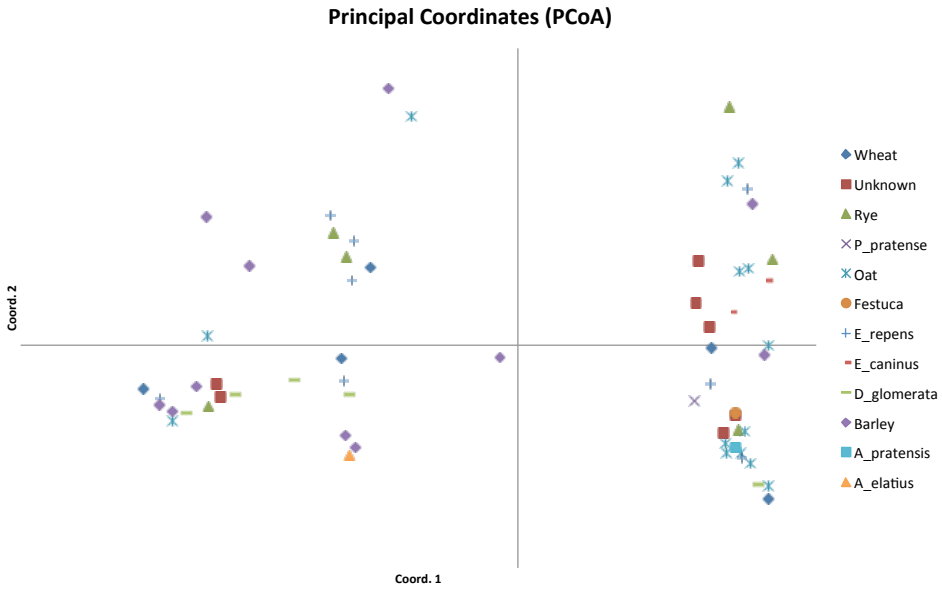

Figure 10. PCoA of samples collected from cereals (wheat, oats, rye, barley) and grasses (*D. glomerata, E. repens, E. caninus,* unknown). The first axis represents 30 % of the differentiation between samples and the second axis 10 %.

3.5.3 Populations based on clade/*formae specialis*

When basing the populations on the clades from the phylogenetic analysis (Figure 3), a clearer pattern appears (Figure 11). The samples are labelled according to clade (A/B) and if they are collected from barberry (b) or grasses/cereals (g). The pairwise F_{ST}-values support the differentiation between the clades (Table 9). The AMOVA shows that most of the variation is within the populations (83 %) compared to among the populations (p-value 0.001). There is a significant difference (p-value 0.05) between samples from barberry and grasses within Clade B. There are however no differences between samples collected from barberry and grasses in Clade A.

Table 9. Pairwise F_{ST}-table of the SSR data set. F_{ST} values below the diagonal, bold text indicates significance. P-value is above diagonal, n.s. = non-significant F_{ST}.

Clade A, barberry	Clade A, grass	Clade B, barberry	Clade B, grass	
	n.s.	***	***	Clade A, barberry
0,020		***	***	Clade A, grass
0,261	**0,159**		*	Clade B, barberry
0,197	**0,101**	**0,036**		Clade B, grass

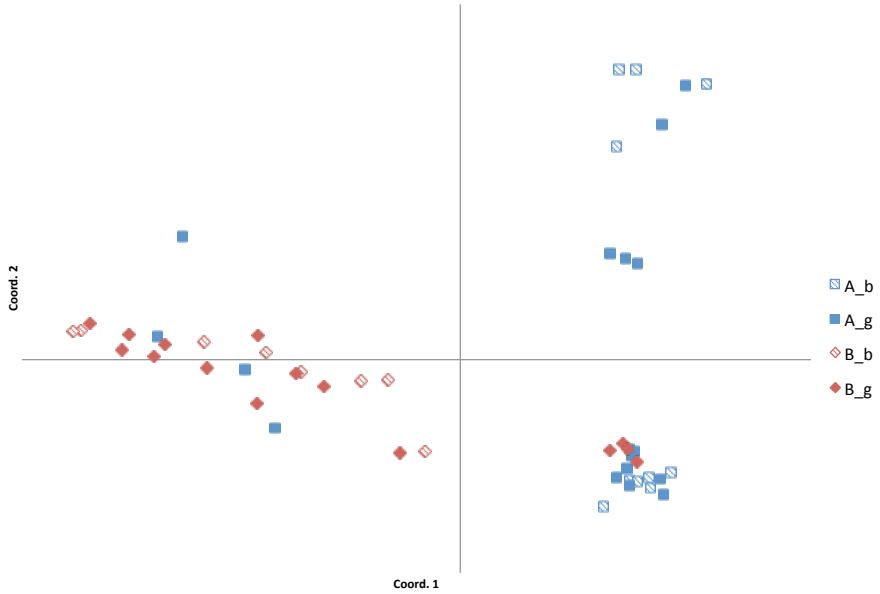

Figure 11. PCoA of samples collected from cereals, wild grasses and barberry. The populations are based on clades A and B from the phylogenetic analysis of the ITS region and stated if the samples were collected from barberry or grasses. A_b = Clade a, barberry; A_g = Clade A, grass, B_b = Clade B, barberry; B_g = Clade B, grass. Axis 1 represents 25 % of the total variation and axis 2 represents 13 %.

A similar pattern is shown in when creating a minimum spanning network (MSN) based on the same populations as above (Figure 12).

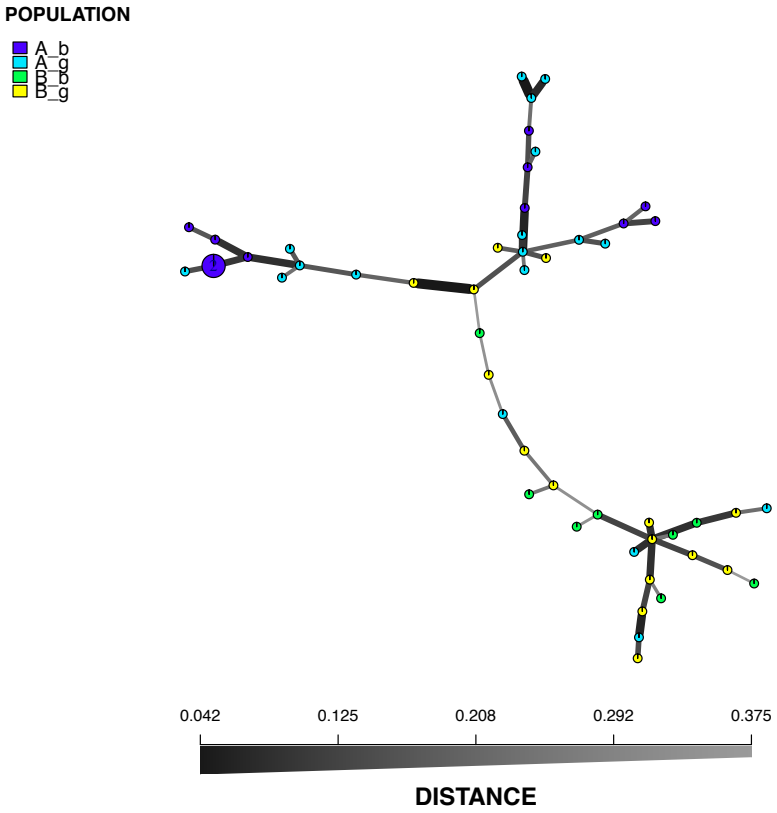

Figure 12. Bruvo distance MSN of samples collected on barberry, cereals and wild grasses. The population is defined by the clades formed when analysing the ITS region and describes if the samples were collected from barberry or grasses. A_b = Clade a, barberry; A_g = Clade A, grass, B_b = Clade B, barberry; B_g = Clade B, grass.

If this data is further divided into analysing barberry and grass samples separately it is clear that the barberry samples (Figure 13) separate into three even more distinct groups. Samples 101b, 101e and 101f identified as *P. graminis* f. sp. *dactylis*.

The grass samples show a larger extent of variation and the clades don't separate as clearly (Figure 14).

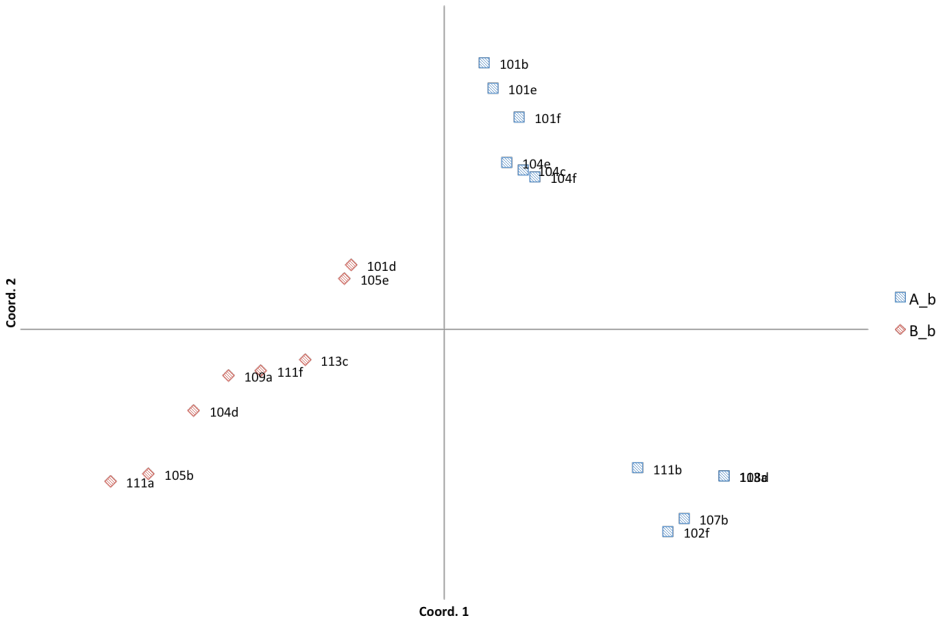

Figure 13. PCoA of barberry samples labelled according to the clade they belong to in the phylogenetic analysis based on the ITS region. The firs axis represents 27 % of the total variation and the second axis represents 19 %.

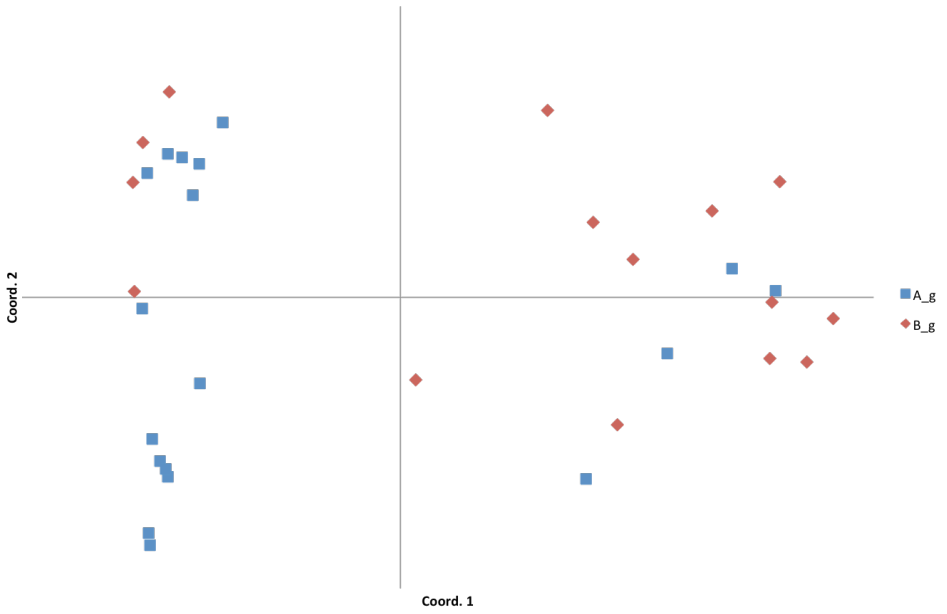

Figure 14. PCoA of samples collected from grass, labelled according to the clade they belong to in the phylogenetic analysis based on the ITS region. The first axis represents 30 % of the total variation and axis two represents 12 %.

4 Discussion

4.1 The *formae speciales* and the phylogenetic study

4.1.1 Which *formae speciales* are present in Sweden?

With Erikssons description of the *formae speciales* (Table 1), Pgt, Pga and Pgs were found among the sequenced samples. However, when comparing the ITS sequences of the samples to sequences in GenBank, they were only assigned two major *formae speciales*: Pgt and Pga, in most cases samples that "should" only be susceptible to Pgs matched Pgt sequences. Some samples also identified as *P. graminis* f. sp. *dactylis/lolii/phlei-pratensis*.

The reason why the three major *formae speciales* didn't show in the GenBank search has two possible explanations. One, GenBank is a database where scientists deposit sequenced genes, thus providing the scientific community with reference material. This means that the hits you get when searching the database depend on what kind of research has been made in your field before. Since stem rust is most severe in wheat and oats, a lot of research effort has gone into investigating Pgt and Pga. Pgs, on the other hand, is a *forma specialis* that needs more research.

Second, the sequenced part of the ITS region might not be able to distinguish between Pgt and Pgs. This means that although there might be a difference between them, it's not possible to detect it using the ITS region.

4.1.2 Did the *formae speciales* differ genetically?

The first phylogenetic tree (Figure 3) separates in two clades; A and B, indicating genetic differentiation between the two clades. Clade A consists of samples collected from *A. sativa, D. glomerata, P. pratense, F. arundinacea, E. repens, S. cereale* and an "unknown grass" while clade B consists of samples from *T. aestivum, S. cereale*, *H. vulgare, E. repens* and *E. caninus*. When using Eriksson's (1898) and Stakman's (1916) (in Schumann & Leonard, 2000) definitions for the host range of the different *formae speciales* that means that clade A consists of

hosts susceptible to Pga and in two cases Pgs and clade B consists of hosts susceptible to Pgt and Pgs. Thus, the connection between *formae specialis* based on host plant and clade is not fully consistent.

If instead the samples are given the *formae specialis* they are assigned by the GenBank search, there is a clear difference between Pgt and Pga (Figures 3 and 4). This means that the *E. repens* and *S. cereale* samples that end up in clade A are infected with Pga.

Berlin (2012) also had cases of *E. repens* being infected by Pga. It is possible that it is susceptible to more than one *forma specialis.* That is the case of barley (*H. vulgare*)*,* that according to Stakman (1916) is susceptible to both Pgt and Pgs.

The "out of place" sample collected from *S. cereale* in clade A is a bit more difficult to explain. Except for the possibility of it being mixed up with something else during lab work there are three explanations. The first one is that a *forma specialis* can infect "the wrong" host (i.e. a host that isn't normally within it's range) under the right circumstances. Such a circumstance might be a high disease pressure (Anikster, 1984). The second explanation is that the concept of *formae speciales* is a bad way to explain the biology of *P. graminis.* Previous studies have been done where the connection between *formae speciales* and hosts have proven not to exist (Abbasi *et al.*, 2005). According to Abbasi et al. (2005) three phylogenetic groups, based on the ITS region, were identified but there was no clear connection between host and clade.

The third explanation for both the sample collected from *E. repens* and *S. cereale* ending up in clade A is that what is considered to be the *forma specialis* is in fact two different *formae speciales;* one more genetically similar to Pga and one to Pgt. Since the fragments sequenced in this study couldn't distinguish even between Pgt and Pgs, this could only be further explored by finding other parts of the genome that can give an even deeper insight in the host specificity of *P. graminis.*

When basing the phylogenies on sequences of COI, no differentiation between the *formae speciales* was detected. This means that COI is highly conserved in the *Puccinia graminis* genome.

4.1.3 *P. graminis* f. sp. *dactylis* – does it exist?

The phylogenetic tree including samples collected from barberry (Figure 5) shown a subclade within Clade A, consisting of samples identified as *P. graminis* f. sp. *dactylis* in the GenBank search. The barberry samples cluster together with a bootstrap value of 100, indicating that they are indeed both genetically similar to each other and different from Pga. According to both Eriksson (1898) and Stakman (1916), *D. glomerata* is susceptible to Pga. However, *P. graminis* f. sp. *dactylis* is not listed in Index Fungorum and should therefore not be considered a "real" *forma specialis.*

Based on the sequences of samples included in this study, there is however a clear difference between the Pga samples in clade A and the ones identified *P. graminis* f. sp. *dactylis*. Thus, some kind of differentiation has occurred. To investigate whether this is a real *forma specialis* or not, more studies are required. A deeper genetic study, including more samples, should be performed to confirm this. An inoculation experiment can also be conducted to investigate if Pga and *P. graminis* f. fp. *dactylis* can infect the same hosts. In addition, crossing experiments between Pga and *P. graminis* s. fp. *dactylis* could be conducted to investigate if they can produce a viable offspring.

4.2 Microsatellites

4.2.1 Microsatellites as a method

Microsatellites are normally used to study the genetic differences or similarities within and between populations of the same species. The markers used are neutral and located in non-coding regions and therefore generally under a low selection pressure.

Microsatellites are good for displaying the complexity of *P. graminis* as a species. Although they too showed that there is a genetic differentiation between the *formae speciales* (Figures 11, 13 and 14), they did not show as distinct differences between the clades as the phylogenetic study did (Figures 4 and 5).

The markers that were used in this study are developed for Pgt and they were selected since they amplify fragments of Pga as well. Despite this, if markers developed for Pga would have been included, the result might have been different. Nonetheless, the genotype accumulation curve (Figure 7) and the allele frequencies (Figure 8) show that the markers used were sufficient to display the variation within the samples in this study.

The allele frequencies differ between the clades defined in the phylogenetic study, but they too do it in an inconclusive way. Some of the markers have alleles that seem highly associated with a *forma specialis;* an example is the connection between Pga and allele 184 for marker PgtCAA53.

4.2.2 Were the same MLG:s found on barberry and grasses?

The same MLG was not found on barberry bushes and nearby grasses at any location. This is probably due to the way the sampling was done. Sampling was made at many different locations but the number of samples per location was rather low, additionally at some locations, samples were collected from a number of different host plants. If we presume that the only source of inoculum for grasses are nearby barberry bushes, then the genotypes found on the grasses should also be

found on the barberry bushes. The reason this didn't happen in this study is probably that only about five aecial samples from each location were extracted and used in the molecular study. The chances of finding and genotyping the corresponding aecia are very slim. To some extent inoculum also reaches fields from further distance by wind, thus introducing even more genetic variation at a specific location.

It is still possible to examine the role of barberry as a source of inoculum by examining how genetically similar the populations collected from the different locations are. In theory; samples collected from a specific location should be more genetically alike thna when compared to a sample from another location. That is, if barberry is important for spreading the disease.

When using a MSN (Figure 9) to illustrate the genetic diversity from the different locations and looking at the pairwise F_{ST}-values (Table 7), some locations do prove to differ significantly from each other. This proposes that there are populations that are genotypically similar at some locations and that these samples share a genetic ancestry.

The reason why some locations differ and others don't probably has to do with the sampling. At some locations samples were taken from several different host species and in some locations they were only taken from a few. This means that the MLG:s do not only reflect the genotypic differences between locations but also between host species (should there be such a difference). To further investigate the role of barberry in the epidemiology of stem rust one should have to focus the sampling to only one or a few locations and collect many samples from barberry and grass species. Then more could be concluded about the relationship between the genotypes on barberry and the grass hosts while also examining the role that wind dispersed inoculum plays.

What I can conclude from analysing the MLG:s is that the genetic diversity of *P. graminis* is very high due to obligate sexual reproduction for maintaining the population in Sweden. This concurs with studies made by e.g. Berlin (2012).

4.2.3 How are the hosts connected in all of this?

When regarding samples from the respective host species as one population there were indeed some significant differences (Figure 10 and Table 8). Samples collected from wheat only differ significantly from samples from the "unknown" grass; in all other cases the difference was non-significant.

4.2.4 Are there differences between the *formae speciales?*

Part of this study focused on examining if the same patterns found in the phylogenetic study could bee seen also when using *P. graminis* specific microsatellite markers. That is, is there a clear distinction between clade A (Pga) and clade B (Pgt)? Both the PCoA (Figure 11) and the MSN (Figure 12) show that there is a

difference between the *formae speciales*, only not as conclusively as with the sequenced genes. Although the pairwise F_{ST}-values confirm the differences between the clades with high confidence, some samples seem to bee out of place and cluster with samples from the "wrong" clade.

The surprising thing is that the "out-of-place" samples are collected from grasses (Figure 14) rather than barberry (Figure 13). One should have expected that the largest genetic variation would be among samples collected from barberry since it's a host of all *formae speciales* of *P. graminis.* Even though quite many of the grass hosts were included in the sample collection, it's not even close to the 365 known ones. The full genetic diversity present on grasses could therefore not have been reflected in this study. As always the result can be dismissed by claiming that something went wrong when sampling. In this case though, I wouldn't go there quite yet. To fully understand this, future studies should increase the number of samples from both the grass host and barberry at different locations and possibly sample at several time points.

The cases of P. graminis *f. sp.* dactylis

Some of the samples that identified as *P. graminis* f. sp. *dactylis* in the phylogenetic study (Figure 5) are also included in the microsatellite study. These are samples 101b, 101e and 101f. They all group together in the PCoA (Figure 13), indicating that they not only have similar ITS sequences but also similar MLG:s. This is interesting since it shows that the samples are genetically different from many of the Pga samples. Had there only been those samples that grouped together in the PCoA, one could have assumed that there had been a differentiation between *P. graminis* f. sp. *dactylis* and Pga. However, there are other samples that identified as Pga that grouped together with the f. sp. *dactylis* samples (104c, 104e and 104f). This makes it hard to draw any conclusion regarding differentiation within Clade A. Although the f. sp. *dactylis* samples form a subclade of their own, they still have a MLG that resemble Pga.

On the other hand, there seems to be two separate groups formed from the Clade A samples (Figure 13). It would be interesting to perform a deeper study on these differences, using both more samples and possibly more markers.

4.2.5 Can *P. graminis* be divided in two species?

Speciation occurs due to some kind of selection pressure. Populations gets separates either by geographical or genetic barriers, during course of evolution these populations become so different they can be considered as different species. A species can be defined in a number of ways but a commonly adopted one has to do with the ability to produce a fertile offspring (Giraud *et al.,* 2010). Several studies have shown that it's possible to cross Pgt and Pgs and get a viable offspring (An-

ikster, 1984). So far only one case of a successful cross between Pgt and Pga has been reported (Johnson, 1949). That means that there is support for Pgt and Pga to be different species. What are the agricultural implications of this?
Based on this and others studies, the following conclusions can be drawn:

a) *P. graminis* can be divided into two major phylogenetic groups.
b) These groups seem to have evolved to infect only specific host species.

The agricultural implications of this would be that it's easier to predict eventual outbreaks of stem rust in cereal fields based on e.g. what grass species are infected near the fields. Early infections might also be avoided by eradicating barberry close to fields. In addition, using a crop rotation with cereals that are susceptible to different *formae speciales* of *P. graminis* would minimize the risk of total crop loss. If we assume that the cereal fields are the main source of infection of *P. graminis* on barberry then the same *forma specialis* will spread from the bushes as aeciospores the next year.

In Sweden, due to the climate and no viable grass host during the winter, *P. graminis* generally has to overwinter as teliospores and infect barberry before proceeding to infect cereals and grasses. That's why the barberry eradication was so successful. In warmer regions, such as eastern Africa where stem rust is a huge problem, the aecial stage isn't required for survival and maintenance of the local population since clonal reproduction is possible all year round. In these regions both cereals and wild/forage grasses act as a "green bridge" from which the disease can spread from field to field, and between different production areas. Under such conditions the knowledge of which *formae speciales* infect which hosts is important since it offers a better possibility for risk assessment.

The same knowledge might be applicable under Swedish conditions. Based on the results in this thesis farmers should be more worried if they find stem rust on *E. caninus* next to their wheat fields than if it's found on *D. glomerata.*

Unfortunately nearby infected grasses or barberry bushes aren't the sole source of inoculum. *P. graminis* is also to a large extent spread by wind. That means that although a farmer might have taken all necessary precautions to avoid disease (removing barberry bushes, using a good crop rotation etc) spores can still be introduced from afar. And even still, the fact remains that resistant varieties have been the most efficient way to avoid disease.

5 Conclusion

Three *formae speciales* of *P. graminis* were found in Sweden: Pga, Pgt, Pgs. They can be divided into two phylogenetic groups; one consisting mainly of Pga and the other of Pgt and Pgs. There is an association between Pga and host species: *A. sativa, P. pratense, A. elatius, D. glomerata, Festuca sp*., and the "unknown grass". Pgt/Pgs is associated with: *T. aestivum, H. vulgare, S. secale, E. caninus* and *E. repens*.

Within Clade A, which consists of samples assigned as Pga, a subclade of samples assigned as *P. graminis* f. sp. *dactylis* forms.

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Appendix I – Populärvetenskaplig sammanfattning

Evolutionen är en långsam process som skapat all den mångfald som finns i naturen. Ibland på ett tydligt sätt, som i exemplet med Darwins finkar och deras olika näbbar och ibland på ett mer subtilt sätt. Sådant är fallet för den växtpatogena svampen *Puccinia graminis* som denna uppsats handlar om. Den orsakar sjukdomen svartrost på totalt 365 olika gräsarter, däribland våra vanligaste sädesslag. Genom årtusendena har den evolutionära processen haft sin verkan på denna art och gjort att den har börjat dela upp sig i olika undergrupper. Man talar om sk. *'formae speciales'*, vilka är specialvarianter som är anpassade till att bara infektera vissa av de kända värdarterna. De tre specialformer som är av störst betydelse för lantbruket är de som infekterar havre, vete och råg. Alla dessa typer av svartrost finns i Sverige.

Ett grundantagande i arbetet med uppsatsen har varit att denna anpassning gentemot värdarten också borde reflekteras i svampens DNA. Genom att sekvensera tre olika sk. barcoding- regioner i *Puccinia graminis* genom har jag kunnat visa att det finns genetiska skillnader åtminstone mellan formerna som infekterar havre och vete. Havreformen av svartrost tycks förutom havre infektera hundäxing och olika svingelarter medan veteformen infekterar korn och vilda gräs som lundelm och kvickrot. Baserat på gensekvenserna infekteras även råg av veteformen. Det resultatet stämmer överens med resultat från tidigare studier. Förutom att de är olika genetiskt har tidigare även morfologiska skillnader, alltså skillnad i utseende, mellan havre- vetetypen påvisats. Detta tyder på att de två formerna i själva verket kan vara olika arter.

En annan fråga som undersökts är rollen hos *Puccinia graminis* alternativa värd berberis. För att kunna fullfölja sin sexuella reproduktionscykel infekteras berberisbuskarna på våren och från de infektionerna sprids sedan sporer till stråsäd och gräs. Hypotesen var att de infektioner som hittas i odlade åkrar eller på vilda gräs i fältkanter stammar från närliggande berberisbuskar och att de därför har samma genotyp som de infektioner som finns på berberis. Exakt samma genotyp gick inte att hitta på både berberis och gräs, däremot fanns ett tydligt släktskap mellan proverna från gräs och berberis på vissa av provtagningsplatserna. En till tydlig slutsats som kan dras är att den genetiska variationen inom *Puccinia graminis* är mycket stor. Sammantaget visade resultatet på att berberis spelar en viktig roll i spridandet av svartrost och den möjliggör också den sexuella förökningen som leder till den stora genetiska variationen.

Den praktiska nyttan av detta arbete är att vi fått en djupare förståelse för en växtpatogen som orsakar skördeförluster för lantbruket. I fallet med svartrost finns det tydliga bevis dels för att berberis spelar en viktig roll i spridandet av sjukdo-

men samt att de olika specialformerna har olika värdarter. Sedan tidigare är det känt att utrotning av berberis i odlingslandskapet radikalt kan minska förekomsten av svartrost både genom att frånta den möjligheten till sexuell reproduktion, något som minskar antalet raser av den.

Denna uppsats har bidragit till ökad förståelse för *Puccinina graminis* och öppnar upp för flera intressanta framtida frågor. Däribland huruvida arten egentligen kan delas upp i två och hur den genetiska differentieringen inom de möjliga nya arterna påverkar svampens möjlighet att infektera olika värdar.

Appendix II - Samples in the study

Sample	Host plant	Collection site	BlastN ID	Forma specialis	Reference	ITS	COI	EF	SRR
005c	Avena sativa, Marvelous	Å	P graminis	Pga	DQ460707.1	x	x		x
007b	Secale cereale, Prolific	Å	P graminis	Pgt	JX047470.1		x		x
009a	Hordeum vulgare, Hiproly	Skarpenberga	P graminis	Pgt	JX047461.1	x			x
010b	Hordeum vulgare, Hypana	Skarpenberga	P graminis	Pgt	JX047470.1	x		x	x
010c	Hordeum vulgare, Hypana	Skarpenberga	P graminis	Pgt	JX424529.1	x		x	x
011a	Avena sativa, Belinda	Skarpenberga	P graminis					x	
011b	Avena sativa, Belinda	Skarpenberga	P graminis					x	
011c	Avena sativa, Belinda	Skarpenberga	P graminis		KC853399.1		x		x
013a	Avena sativa, Marvelous	Skarpenberga	P graminis				x		x
013b	Avena sativa, Marvelous	Skarpenberga	P graminis				x		x
015b	Secale cereale, Prolific	Skarpenberga	P graminis				x		x
015c	Secale cereale, Prolific	Skarpenberga	P graminis		JX424533.1		x		x
017b	Hordeum vulgare, Hypana	Bökestad	P graminis	Pgt	JX047470.1	x			x
018a	Secale cereale, Prolific	Bökestad	P graminis	Pgt	JX047470.1	x			x
019a	Avena sativa	Bökestad	P graminis		JQ688988.1		x	x	
019b	Avena sativa	Bökestad	P graminis		JQ688988.1		x	x	
019c	Avena sativa	Bökestad	P graminis	Pga	JX047483.1	x			x
020a	Avena sativa, Belinda	Bökestad	P graminis	Pga	JX047475.1	x	x	x	x
020b	Avena sativa, Belinda	Bökestad	P graminis		KC853404.1		x		x
021a	Triticum aestivum, Rusty	Bökestad	P graminis	Pgt	JX047465.1	x	x		x
021b	Triticum aestivum, Rusty	Bökestad	P graminis	Pgt	JX047470.1	x	x		x
021c	Triticum aestivum, Rusty	Bökestad	P graminis	Pgt	JX047470.1	x			x
022a	Hordeum vulgare, Hiproly	Säby	P graminis	Pgt	JX047465.1	x			x
022b	Hordeum vulgare, Hiproly	Säby	P graminis		JQ688977.1		x		x
022c	Hordeum vulgare, Hiproly	Säby	P graminis	Pgt	JX047470.1	x	x		x
023b	Hordeum vulgare, Hypana	Säby	P graminis		JQ688984.1		x		
023c	Hordeum vulgare, Hypana	Säby	P graminis	Pgt	JX047470.1	x		x	x
024a	Avena sativa, Marvelous	Säby	P graminis	Pgt	JX424531.1	x			x
024b	Avena sativa, Marvelous	Säby	P graminis		JQ688965.1		x		
024c	Avena sativa, Marvelous	Säby	P graminis	Pgt	JX429532.1	x	x	x	x
025a	Avena sativa, Belinda	Säby	P graminis	Pga	DQ460727.1	x	x	x	x
025b	Avena sativa, Belinda	Säby	P graminis	Pga	JX047475.1	x	x	x	x
025c	Avena sativa, Belinda	Säby	P graminis	Pga	DQ460727.1	x	x	x	x

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026a	Secale cereale, Prolific	Säby	P graminis	Pga	DQ460727.1	x			x
027a	Triticum aestivum, Line E	Säby	P graminis		DQ460727.1		x		x
027c	Triticum aestivum, Line E	Säby	P graminis		JX047475.1				x
029a	Dactylis glomerata	Säby	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x			x
029c	Dactylis glomerata	Säby	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x	x	x	x
031a	Elytrigia repens	Ultuna, vid kornförsök	P graminis						x
031b	Elytrigia repens	Ultuna, vid kornförsök	P graminis		JQ688984.1		x		x
031c	Elytrigia repens	Ultuna, vid kornförsök	P graminis						x
033a	Hordeum vulgare, Otira	Ultuna, vid kornförsök	P graminis	Pgt	JX424529.1		x		x
033b	Hordeum vulgare, Otira	Ultuna, vid kornförsök	P graminis		JX424529.1		x		
033c	Hordeum vulgare, Otira	Ultuna, vid kornförsök	P graminis	Pgt	JX047461.1	x	x		x
034c	Arrhenatherum elatius	Ultuna, vid ån	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x			x
035a	Elytrigia repens	Ultuna, vid ån	P graminis	Pgt	JX424529.1			x	x
035b	Elytrigia repens	Ultuna, vid ån	P graminis		JQ688977.1		x		x
035c	Elytrigia repens	Ultuna, vid ån	P graminis	Pgt	JX047470.1	x	x		x
036a	Hordeum vulgare, Arve	Vallby/Berg	P graminis		JQ688977.1		x	x	
036b	Hordeum vulgare, Arve	Vallby/Berg	P graminis	Pgt	JX047470.1	x	x		x
038a	A sativa, havre	Ultuna, vid kornförsök	P graminis		KC853413.1	x	x		x
038b	A sativa, havre	Ultuna, vid kornförsök	P graminis		KC853399.1	x	x		x
039b	Unknown grass	Å Stenby	P graminis	Pga	JX047480.1	x	x	x	x
039c	Unknown grass	Å Stenby	P graminis	Pga	JX047480.1	x			x
040c	Poa pratensis	Å stenby	P striiformis		KC305497.1		x		
041a	Elytrigia repens	Å stenby	P triticina		DQ460721.1		x	x	
041b	Elytrigia repens	Å stenby	P triticina		DQ417418.1			x	
041c	Elytrigia repens	Å stenby	P triticina					x	
043b	Festuca sp	Å stenby	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x			x
044a	Elytrigia repens	Ekbacken	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x			
045c	A pratensis, ängskavle	Ekbacken	P graminis		JQ688944.1		x	x	x
046c	P pratense, timotej	Ekbacken	P graminis		JX047483.1				x
047a	Arrhenatherum elatius	Ekbacken	P coronata		DQ355444.1		x	x	
048a	Unknown grass	Ekbacken	P graminis	Pga	JX047480.1	x		x	x
048b	Unknown grass	Ekbacken	P graminis	Pga	JX047480.1	x		x	x
049a	Unknown grass	Ekbacken	P graminis	Pga	JX047480.1	x			x
049b	Unknown grass	Ekbacken	P graminis		KC853411.1		x	x	x
049c	Unknown grass	Ekbacken	P graminis	Pga/Pg lolii	JX047480.1/DQ417384.1	x			x
050a	Arrhenatherum elatius	S Bäckmarken	P arrhenatheri				x		
051a	Elymus caninus	S Bäckmarken	P striiformis		KC305497.1		x	x	
052a	Elytrigia repens	S Bäckmarken	P graminis		KC853411.1		x	x	x
052c	Elytrigia repens	S Bäckmarken	P graminis				x	x	x
053a	Dactylis glomerata	S Bäckmarken	P graminis		KC853411.1		x	x	x
053b	Dactylis glomerata	S Bäckmarken	P graminis		JQ688900.1		x	x	x
053c	Dactylis glomerata	S Bäckmarken	P graminis		JQ688900.1			x	x
054a	Elymus caninus	S Bäckmarken	P graminis	Pgt	JX424529.1	x	x		x
054b	Elymus caninus	S Bäckmarken	P graminis		KC853411.1		x	x	x
055a	Arrhenatherum elatius	Skärva	P arrhenatheri		JX047494.1	x			
056a	Unknown grass	Skärva	P striiformis		KC305497.1		x	x	

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057a	Unknown grass	Romneby	P coronata		DQ355444.1	x	x	x	
057b	Unknown grass	Romneby	P coronata		JX047496.1	x	x	x	
057c	Unknown grass	Romneby	P coronata		JX047496.1	x			
058a	Unknown grass	Romneby	P striiformis		KC305497.1		x		
059a	Unknown grass	Elleholm	P striiformis		KC305497.1		x	x	
061a	Arrhenatherum elatius	Djurön	P arrhenatheri		JX047494.1		x	x	
061b	Arrhenatherum elatius	Djurön	P arrhenatheri		JX047494.1	x			
063a	Dactylis glomerata	Högstrum, Öland	P graminis		KC853411.1		x		
063b	Dactylis glomerata	Högstrum, Öland	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x		x	
064a	Arrhenatherum elatius	Kellog Bio Station, Michigan	P coronata		AB693935.1	x	x	x	
064c	Arrhenatherum elatius	Kellog Bio Station, Michigan	P coronata		AB693935.1	x			
065b	Arrhenatherum elatius	Åby	P arrhenatheri		JX047494.1	x	x		
066a	Festuca arundinacea	USA	P graminis				x		
066b	Festuca arundinacea	USA	P graminis				x		
066c	Festuca arundinacea	USA	P graminis				x	x	
067a	Phleum pratense	USA	P graminis	Pga/Pg phlei-pratense	DQ460727.1/DQ417392.1	x	x	x	
067b	Phleum pratense	USA	P graminis	Pga	JX047483.1	x	x	x	
067c	Phleum pratense	USA	P graminis				x		
068a	Arrhenatherum elatius	USA	P coronata		JX047496.1	x	x		
069a	Elymus virginicus	USA	P montenesis				x		
069b	Elymus virginicus	USA	P montenesis				x		
101b	Berberis sp	Säby	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x		x	x
101d	Berberis sp	Säby	P graminis	Pgt	JX047470.1	x			x
101e	Berberis sp	Säby	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x	x		x
101f	Berberis sp	Säby	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x	x	x	x
102a	Berberis sp	Bökestad	P graminis		JQ688977.1		x	x	x
102b	Berberis sp	Bökestad	P graminis			x		x	x
102c	Berberis sp	Bökestad	P graminis		JQ688977.1			x	x
102f	Berberis sp	Bökestad	P graminis	Pga	JX047480.1	x	x	x	x
104a	Berberis sp	Skarpenberga	P graminis	Pga	JX047482.1	x	x		x
104b	Berberis sp	Skarpenberga	P graminis	Pga/Pg dactylis	JX047475.1/DQ417390.1	x	x	x	
104c	Berberis sp	Skarpenberga	P graminis	Pga	DQ460727.1	x	x		x
104d	Berberis sp	Skarpenberga	P graminis	Pgt	JX047470.1	x			x
104e	Berberis sp	Skarpenberga	P graminis	Pga	JX047475.1	x		x	x
104f	Berberis sp	Skarpenberga	P graminis	Pga	DQ460727.1	x	x	x	x

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105b	Berberis sp	Knivsta	P graminis	Pgt	JX047456.1	x	x		x
105e	Berberis sp	Knivsta	P graminis	Pgt	JX047470.1	x	x		x
105f	Berberis sp	Knivsta	P graminis		JQ688944.1		x	x	
107b	Berberis sp	Ekbacken	P graminis	Pga	JX047480.1	x	x	x	x
107f	Berberis sp	Ekbacken	P graminis	Pgt	JX424529.1	x	x	x	x
108b	Berberis sp	Skärva	P graminis		JQ688977.1		x	x	x
108d	Berberis sp	Skärva	P graminis	Pga/Pg lolii	JX047475.1/DQ417384.1	x	x	x	x
109a	Berberis sp	Å Stenby	P graminis	Pgt	JX047470.1	x	x		x
109b	Berberis sp	Å Stenby	P graminis		JQ688977.1		x	x	x
111a	Berberis sp	S Bäckmarken	P graminis	Pgt	JX047470.1	x	x		x
111b	Berberis sp	S Bäckmarken	P graminis	Pga	JX047480.1	x	x	x	x
111d	Berberis sp	S Bäckmarken	P graminis	Pga	JX047480.1	x			
111e	Berberis sp	S Bäckmarken	P graminis	Pgt	JX424529.1	x	x		x
111f	Berberis sp	S Bäckmarken	P graminis	Pgt	JX047470.1	x		x	x
111g	Berberis sp	S Bäckmarken	P graminis		KC853399.1			x	x
112a	Mahonia sp	Norrköping	C. mirabilissima			x	x		
112g	Mahonia sp	Norrköping	C. mirabilissima			x	x		
112h	Mahonia sp	Norrköping	C. mirabilissima			x	x		
113a	Berberis sp	Å stenby	P graminis	Pga	JX047480.1	x	x	x	x
113b	Berberis sp	Å stenby	P graminis		JX424520.1		x		x
113c	Berberis sp	Å stenby	P graminis	Pgt	JX047470.1	x	x	x	x
113d	Berberis sp	Å stenby	P graminis		KC853399.1			x	x
113f	Berberis sp	Å stenby	P graminis		JQ688994.1			x	x