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Uppsala 2011

Taxonomic and phylogenetic study of rust fungi forming aecia on *Berberis* spp. in Sweden

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Master' thesis, 30 hec
Ecology Master's programme

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Credits: 30 hp
Level: E
Subject: Biology
Course title: Independent project in Biology
Course code: EX0565
Online publication: http://stud.epsilon.slu.se

Key words: rust fungi, aecia, aeciospores, morphology, barberry, DNA sequence analysis, phylogenetic analysis

Front-page picture: Barberry bush infected by *Puccinia* spp., outside Trosa, Sweden. Photo: Anna Berlin

Content

Abstract

Rust fungi are important plant pathogens that have been studied for a long time. They are of great economic importance due to the severe damage they cause on agricultural crops. Rust fungi are very interesting organisms in terms of biology because their life cycle is quite complex: it alternates between two hosts and consists of up to five distinct spore stages. This makes these organisms difficult to study.

The barberry plant was known to be associated with stem rust (*Puccinia graminis*) from the Middle Ages but only recently these plants were identified as an alternative hosts for stripe rust of important cereal crops and grasses *Puccinia striiformis* (Jin *et al*. 2010). Since 1994 barberry eradication is no longer taking place in Sweden so these plants can be found around the country. The aecial stage in the rust fungi life cycles have got less attention because it is not economically important compared with uredinial and telial stages that have been studied a lot for the species infecting cereal crops. That is why the main objective of this study was to identify aecia of rusts species that may be found on *Berberis* spp. collected in different parts of Sweden.

To answer this question spore measurement and aecia description as well as DNA sequences analysis (using ITS region, EF1-α and β-tubulin partial genes) were performed. Also phylogenetic analysis of obtained sequences was conducted.

According to spore and aecia morphology four different species were distinguished. DNA sequences analysis (by the comparison of obtained sequences with BLAST library) identified four different species *P. graminis* f.sp. *avenae*, *P. graminis* f.sp. *tritici*, *P. poae-nemoralis* and *P. striiformis* that fitted well to the morphology data. The identity of the *Berberis* spp. was also checked.

Phylogenetic analysis showed that all obtained species and formae speciales are distinct from each other and form separate clades with high branch support.

1 Introduction

Rust fungi (kingdom Fungi, phylum Basidiomycota, class Urediniomycetes, order Uredinales) are important plant pathogens. Rusts are obligate parasites (depend upon living host in order to complete its life cycle) and biotrophic (invade living plant tissue and with the help of haustoria drag nutrients from the host cell without killing it) parasites (Deacon, 1997). This type of parasitism cause serious crop losses and in the case of favorable condition (viable spores, susceptible or moderately susceptible host plants, dew on the leaves and favorable temperatures) may lead to epidemics (McMullen *et al.* 2010). The number of recognized rust species in different sources varies from 5000 (Cummins and Hiratsuka, 2003) to 7000 species (Hawksworth *et al*. 1995; Maier et al. 2003; Ono and Aime, 2006). Rusts fungi parasitize angiosperms, conifers, and ferns. At the same time, rust species have narrow and specific host ranges (Cummins and Hiratsuka, 2003).

The rust fungi are very interesting organisms in terms of biology. Their life cycle is complex and consists of up to five distinct spore stages (macrocyclic rust taxa). Some rust fungi have all of these stages but others lack uredial stage (demicyclic rust taxa) or both aecial and uredial stages (microcyclic rust taxa). All these types of life cycle may lack pycnia stage (Cummins and Hiratsuka, 2003). In addition there are rust fungi that require two unrelated hosts to complete their life cycle (heteroecious fungi) and those who can complete it on a single host species (autoecious fungi). In rust fungi three distinct nuclear stages in the life cycle may be identified: the haploid monokaryon, the dikaryon, and the diploid (Petersen, 1974).

In terms of evolutionary biology it is generally concidered that the species with a reduced life cycle (microcyclic) is a descendent of the species with the all spore stages (macrocyclic) (Shattock and Preece, 2000). In microcyclic rust taxa the telia simulate the habit of the aecia of the parental macrocyclic rust taxa and occur on the aecial host originally colonised by the ancestral heteroecious species (Shattock and Preece, 2000). This was used in the Tranzschel' method of identifying the alternate host of the rust assumed to be heteroecious. According to this method one should look for aecial stages of microcyclic species that have morphologically similar telia and teliospores to the suspected heteroecious rust. Such species that exhibit similar telia and teliospore morphology, and share a common host but with different life cycles are called "correlated species" (Cummins and Hiratsuka, 1983).

Rust fungi are well known and very successful plant pathogens that have been studied for a long time, but because they are obligate parasites and due to their complicated life cycle they are very difficult to work with. One can find a lot of historical records about rust attack on cereals. This is mainly due to the economic losses from rust pathogens and also because of its clearly distinguishable features. Aristotle (384 - 322 BC) as well as Theophrastus (371 - 287 BC) described years with a heavy damage of cereals crops caused by rust fungi induced by "warm vapors" (Roelfs *et al.* 1992). The purpose of the Roman festival of Robigalia was to pray to the rust god Robigo to reduce the damage caused by cereal rust (Chester, 1946), which indicates that rust was a serious problem for wheat crops in Italy at that time. Only in 1767 Fontana and Tozzetti provided the first report on stem rust as a parasite on cereals. This organism was named *Puccinia graminis* by Person in 1797 (Roelfs *et al.* 1992).

1.1 Life cycle

Most rust fungi have complex life cycle that consists of five spore stages and require two hosts. This macrocyclic heteroecious life cycle of rust fungi (Figure 1) starts in the end of the growing season by formation of two-celled teliospore with two nuclei in each cell (Leonard and Szabo, 2005). In some genera instead of teliospores, lactospores are formed; they usually have thinner walls and germinate without a resting period (Petersen, 1974). Teliospores that form dark pustules (telia) on dead host tissue usually have thick walls and serve as overwintering and resting stage (Kolmer et al 2009). When the uredinial host starts to get old the shift in the type of spore production is occurring: teliospores are formed in the uredinium as a second type of spores,

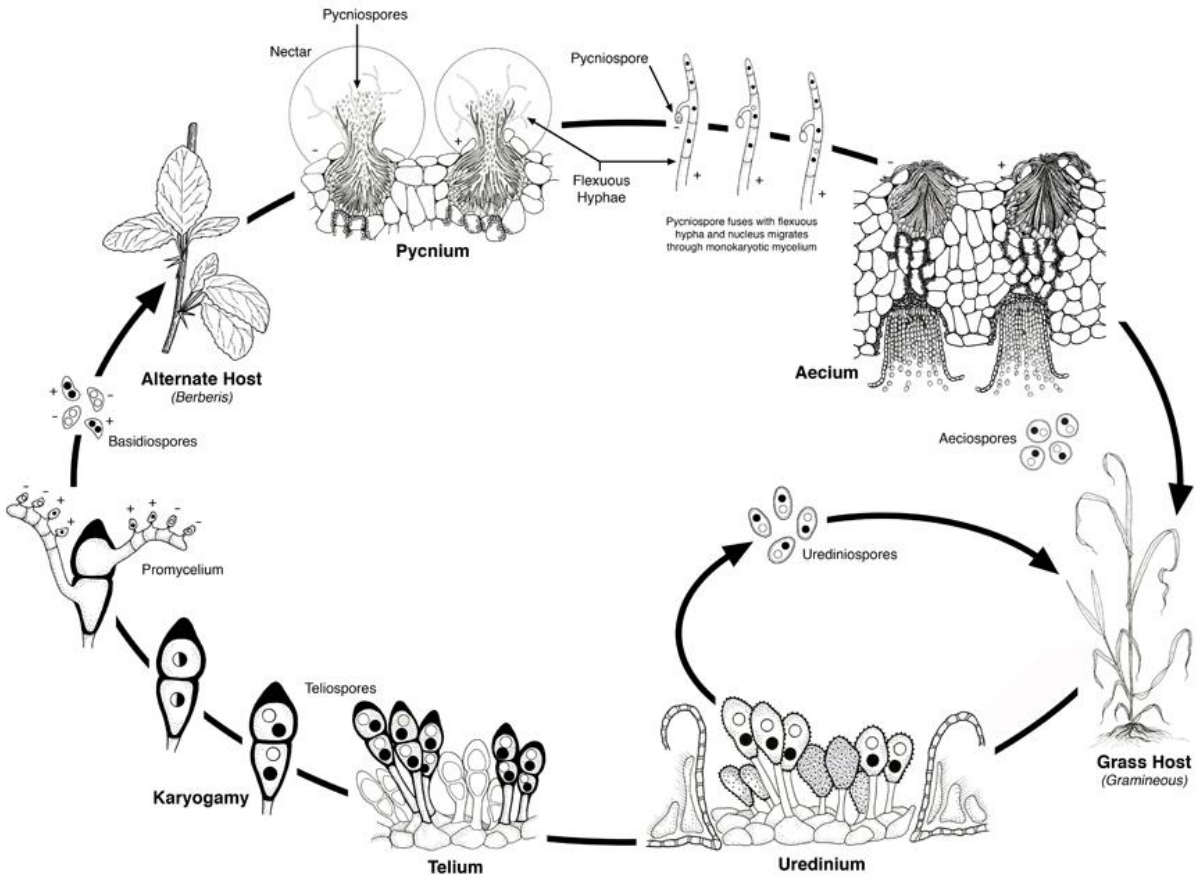


Figure 1. *Life cycle of rust fungi* (Leonard and Szabo, 2005).

following after urediospore production (Mendgen, 1984). The uredium transforms into telium or telial sorus (Mendgen, 1984). Telia can have different form and location. In some genera (*Uredinipsos*) teliospores remain on telia enclosed with the host plant epidermis; in *Melampsora* teliospores form a monolayer while in *Phakopsora* they can be found in more than one rank (Petersen, 1974). According to Anikster (1986) teliospores together with basidia are two of the most important stages in the life cycle of rust fungi. Teliospores are important for overwintering for at least half of all *Uredinales.* Often is an after-ripening period of the teliospores necessary for promoting germination (Cumminis and Hiratsuka, 1983). In addition, mainly teliospores

contribute to formation of new physiological races of the rust fungi (Anikster and Wahl, 1979). In each cell of the teliospore, karyogamy occurs and right after this, the teliospores germinates into a short tube, the promycelium (or basidium) (Petersen, 1974). The diploid nucleus migrates into the promycelium and goes through two meiotic divisions (Petersen, 1974). The four haploid nuclei are separated by three transverse septa and from each promycelium cell projecting sterigma are formed (Leonard and Szabo, 2005). Through these sterigma the haploid nuclei migrates into the newly formed basidiospores at the tips of each sterigma (Roelfs, 1985). Each mature basidiospore contains two haploid nuclei as a result of a mitoic division (Leonard and Szabo, 2005). In the study of 27 rust species of the genera *Puccinia*, *Uromyces*, *Tranzschelia*, *Frommea (Phragmidium)*, and *Melampsora* the optimal temperature for teliospore germination was determined (Anikster, 1986). It varied between 16 and 18°C with the limits between 12 and 25°C (Anikster, 1986). Teliospores lose their ability to germinate if they are exposed to sunlight or kept in shade for one year, while under favorable condition (at 5°C in partial vacuum and dry conditions) teliospores can maintain vitality for more than 14 years (Anikster, 1986).

Mature basidiospores of heteroecius rusts are actively discharged into the air and carried by wind to their alternative host (Leonard and Szabo, 2005). Basidiospores infect their host by direct penetration by the germ tube through the intact wall of the epidermal cell (Longo *et al.* 2006). As a result, infection structures such as vesicles and infection hypha develop inside the plant tissue under the epidermis (Bushnell and Roelfs, 1984). Apparently basidiospores are not able to infect older plants because a thick cuticle on the leaf surface will not allow the germ tube to penetrate into the leaf tissue (Leonard and Szabo, 2005). Basidiospores are fragile and cannot tolerate dry conditions and are mainly released during nights and during moisture periods (Kolmer *et al.* 2009). Infection of leaf tissue by basidiospores results in the production of haploid hyphal colonies from which a flask-shaped pycnia arise (Leonard and Szabo, 2005; Kolmer *et al.* 2009; Bushnell and Roelfs, 1984). There are studies that show that some rusts have a defect in basidiospore formation (Pavgi, 1975; Anikster *et al.* 1980) which can lead to production of two basidiospores with nuclei of both mating type that gives a possibility to skip formation of pycnia and produce aecia straightforward (*Puccinia sorghi* and *Uromyces* spp.).

Within the pycnium, small, simple pycniospores are produced. The pycnia are surrounded by a cap of insect-attracting nectar (Leonard and Szabo, 2005; Kolmer *et al.* 2009). Insects as well as rain drops scatters the pycniospores among pycnia on one plant or between surrounding plants (Kolmer *et al.* 2009). In this stage of the rusts life cycle, mating takes place during which pycniospores of one mating type (+) fertilize pycnia of another (-) mating type (Anikster, 1999). In this process, pycniospores represent male gametes and flexuous hypha that grows from the top of the pycnia represents the female gametes (Leonard and Szabo, 2005). For successful mating e.g. production of aecia, formation of pycniospore caps is essential (Anikster, 1999). The main component of this cap consists of a protein that probably serves as fungal mating-type specific pheromones (Anikster, 1999). After the fusion of haploid nucleus of pycniospore and a flexuous hypha, the dikariotic stage is established (Leonard and Szabo, 2005) and the fungus forms an aecium below the pycnium (Kolmer *et al.* 2009).

The aecium is the dikaryotic cup-like fruiting structure on the leaf surface containing multiple aecia (Bushnell and Roelfs, 1984). The location of the pustule may vary in different rust species. In cereal rusts, it usually reflects the pycnial clusters on the opposite side of the leaf while in

other rust species aecia can appear at the periphery of pycnia or even in the same place where the pycnia were formed (Kolmer *et al.* 2009). Sato and Sato (1985) distinguish 14 morphological types of aecia: eridermia, roestelia, aecidium, 3 types of peridermium, 6 types of caeoma and 3 types of uraecium. This classification is based on a number of morphological characteristics such as spore ontogeny, hymenium, peridium, aeciospores morphology, and position of aecia in the leaf tissue, and some others. Inside each aecium, numerous dikaryotic aeciospores are formed. Aeciospores may travel long distances since they serve as infective agents to the other host in heteroecious fungi (Petersen, 1974). Aeciospores are unicellular (Cummins and Hiratsuka, 2003) and usually produced in chains (Kolmer *et al.* 2009). Once a suitable host is infected, aeciospores germinate by producing a dense net of hyphae (Leonard and Szabo, 2005), which invade the host tissue through stomata openings (Petersen, 1974). Dikaryotic mycelium is formed and as a result of the infection, pustules known as uredinium (containing single-celled urediniospores) are formed (Leonard and Szabo, 2005).

Urediniospores are dikaryotic and produced singly on stalks (Kolmer *et al.* 2009). They are dispersed by wind and serve as re-infecting agent of the gramineous host (Petersen, 1974; Leonard and Szabo, 2005). At the end of the growing season, the production of the teliospores begins. Transformation into the telium can occur from both original uredium or from uredial sori produced by re-infecting mycelium (Petersen, 1974). The uredinial and telial stages in the life cycle of the rust fungi have had the most research interest because of its obvious economic importance (Leonard and Szabo, 2005).

1.2 Hyphae and haustoria

The hyphae of the rusts are intercellular and septate (Cummins and Hiratsuka, 2003). Rusts have two major types of septa: the typical hyphal septa that are formed after conjugate nuclear division (Harder 1984), and so called "pseudosepta" (Ehrlich *et al.* 1968) that is characterized by the absence of a pore apparatus and occur in hyphae of axenic growth or in hyphae near the leading edge of colonies (Harder 1984).

It is generally assumed that the haustorium (the structure that formed by parasitic fungi in host's tissue) is involved in the uptake of nutrients from the living host's cells (Cummins and Hiratsuka, 2003) by increasing relative surface area of contact with the host (Jennings and Lysek, 1996). However, there is as yet no direct evidence for this role. Depending on which sexual stage rust fungi have (monokaryotic or dikaryotic), haustoria of different morphology are produced (Staples, 2001). D-haustoria are produced from dikaryotic hyphae and M-haustoria from monokaryotic hyphae (Cummins and Hiratsuka, 2003). For example, among the cereal rusts, the M-haustoria produced by the monokaryon differ from the D-haustoria formed by the dikaryon because growth of the M-haustorium is filamentous and the neck ring, which seals the haustorial membrane from the apoplast, does not form (Staples, 2001), although each type has an extrahaustorial matrix (Harder and Chong, 1984). For some group of rust fungi shape and size of D-haustoria are considered to be a useful morphological character for species systematics (Berndt and Oberwinkler, 1995, 1997).

1.3 Rust taxonomy

According to Cummins and Hiratsuka (1983) the *Uredinales* consist of 14 families and of 120 (160 according to Ono and Aime (2006)) holomorph genera but later these authors (Cummins and Hiratsuka, 2003) reduced the number of families to 13 (*Chaconiaceae, Coleosporiaceae, Cronartiaceae, Melampsoraceae, Mikronegeriaceae, Phakopsoraceae, Phragmidiaceae, Pileolariaceae, Pucciniaceae, Pucciniastraceae, Pucciniosiraceae, Raveneliaceae,* and *Uropyxidaceae*) by merging *Sphaerophragmiaceae* with *Raveneliaceae*.

Most classifications of rust fungi are based on teliospore morphology (Cummins and Hiratsuka, 2003). At the same time, it has been demonstrated that the morphology of life stages other than teliospore also have significant characters for taxonomical and phylogenetical studies (Maier *et al.* 2003). It includes aecia morphology (Sato and Sato, 1985), urediniospore germ pore arrangement and spore shape (Cummins, 1936), uredinia morphology (Kenney 1970), and morphology of pucnia (spermogonia) (Hiratsuka and Cummins 1963).

1.3.1 Formae specialis

The concept of *forma specialis* has been reviewed by Anikster (1984) and is used to classify varieties or subspecies of rust fungi. This concept is applied for the cereal rusts, like *Puccinia graminis*. In general, a *forma specialis* designation is given according to the most common genus on which the particular rust fungi causes diseases (Anikster, 1984), like *Puccinia graminis* f.sp. *tritici* causing stem rust on wheat. This classification emphasizes that *Triticum* spp. is the most important host (Staples 2000). Another study (Abbasi *et al.* 2005) shows that *P. graminis* is a complex species and that subspecific classification and *formae specialis* do not represent natural monophyletic groups. The authors claim that a new taxonomic concept for this species is needed. Nevertheless, the awareness that many rust species, and especially economically important cereal pathogens exist as highly adapted and specialized forms to specific host, have greatly improved the breeding of rust-resistant crops (Staples 2000).

1.4 Economic importance

Rust fungi are of great economic importance due to their potential to cause severe damages on crops. Rust pathogens on agricultural crops have got special interest in research.

Rust on cereal crops include rust on wheat (stem rust *Puccinia graminis* f.sp. *tritici* Erikss. and Henn., leaf rust *P. triticina* Erikss., and stripe rust *P. striiformis* f.sp. *tritici* Erikss.), oats (crown rust *P. coronata* f.sp. *avenae* P. Syd & Syd., stem rust *P. graminis* f.sp. *avenae* Erikss. and Henn.), barley (stem rust *P. graminis* f.sp. *tritici* Erikss. and Henn., leaf rust *P. hordei* G. H. Otth, stripe rust *P. striiformis* f. sp. *hordei* Erikss., crown rust *P. coronata* Corda), rye (*P. recondita* Roberge), and corn (*P. sorghi* Schwein).

Furthermore, other important crop plants that may be infected by rust fungi are: coffee (coffee leaf rust *Hemileia vastatrix* Berk. & Broome), soybean (soy bean rust *Phakopsora pachyrhizi* Syd. & P. Syd., *Phakopsora meibomiae* Arthur), beans (common bean rust *Uromyces appendiculatus* F. Strauss, *Uromyces phaseoli* (Pers.) Wint. var. *typica* Arth., broad bean rust

Uromyces viciae-fabae (Pers.) J. Schröt., faba bean rust *Uromyces viciae-fabae* var. *viciae-fabae* (Pers.) J. Schröt.), cowpea (cowpea rust *Uromyces phaseoli* (Pers.) Wint. var. *vignae* (Barel.) Arth.), chickpea (chickpea rust *Uromyces ciceris-arietini* (Grognot) Jacz. & Boyd), alfalfa (alfalfa rust *Uromyces striatus* Schrot.), pea (pea rust *Uromyces pisi-sativi* (Pers.) Liro), flax (flax rust *Melampsora lini* (Pers.) Lév.), mint (mint rust *Puccinia menthae* Pers.), sugarcane (sugarcane rust *Puccinia Melanocephela* Syd.), chives, garlic, leek and onion (*Puccinia allii* (DC.) F. Rudolphi), and many others.

A number of fruit and ornamental species are also attacked by rusts. It has been reported that rust fungi occur on 46 % of the species of European *Rosaceae* (Helfer, 2005) including leaf rust on plum (*Tranzschelia pruni-spinosa* (Pers.) Dietel var. *discolor*), rust on peach (*Tranzschelia discolor* (Fuckel) Tranzschel & Litwinow f.sp. *persica* Bolkan, J.M. Ogawa, Michailides & Kable), blackberry cane and leaf rust (*Kuehneola uredinis* (Link) Arthur), rusts on roses (*Phragmidium tuberculatum* Jul. Müll., *Phragmidium mucronatum* (Pers.) Schltdl., and *Phragmidium rosae-multiflorae* Dietel), and cedar-apple rust (*Gymnosporangium juniperi-virginianae* Schwein). Rusts fungi can be also found on several tree species such as eucalyptus (*Puccinia psidii* G. Winter); birch (*Blastospora smilacis* Dietel); mulberry (*Aecidium mori* Barclay); willow and poplar (*Melampsora* spp.).

The most important rust pathogens on conifers are white pine blister rust (*Cronartium ribicola* J.C. Fisch.), western gall rust (*Endocronartium harknessii* J.P. Moore), pine gall rust (*Cronartium quercuum* f.sp. *fusiforme* (Hedgc. & N. Hunt) Burdsall & G. Snow), pine needle rusts (*Coleosporium asterum* (Dietel) Syd. & P. Syd.), spruce needle rust (*Chrysomyxa ledicola* Lagerh.), and chrysomyxa rust of spruce (*Chrysomyxa ledi* (Alb. & Schwein.) de Bary).

According to Arthur (1924) ferns have the most ancient lineage of rust fungi, and there are only three fern families *(Osmundaceae, Polypodiaceae* and *Schizaeaceae)* that may be infected by rusts. On the fern species from the family *Osmundaceae,* only one rust species occurs **(***Uredinopsis osmundae* Magn.), on *Polypodiaceae* spp. *Hyalopsora aspidiotus* (Magn.) Magn., *Milesia magnusiana* (Jaap) Faull, *Calidion lindsaeae* (Henn.) Syd. & P. Syd. and *Calidion dumontii* Buriticá occur, and *Schizaeaceae* spp. are bearing rust species from the genus *Dicaeoma* (Arthur, 1924).

Summarizing all that was mentioned above, one could say that rust fungi have been a well-known group of organism for a long time due to their distinct features and economic importance. At the same time, they exhibit rather complicated life cycles that make them difficult to study. A good example of this is the recent identification of barberry (*Berberis* spp.) an alternative hosts for *P. striiformis* (Jin *et al.* 2010). Even though barberry plants were known to be associated with cereal rusts since the Middle Ages and eradication of barberry bushes near cereals fields was established by law in many countries (Zadoks and Bouwman 1985). Barberry bushes in Sweden were also eradicated until 1994, when the law was repealed (Berberislag, SFS 1976:451, SFS 1994:103). Uredinial and telial stages in the life cycle of the rust fungi are the two stages that mostly have been studied due to their ability to cause damage on economically important crops (Leonard and Szabo 2005), while others like the aecial stage have got less attention. For example, the morphological study of aecia of some rust species was made by Sato and Sato in 1985 and the review of the grass fungi that have *Berberis* and *Mahonia* spp. as uredinial and

aecail host was made by Cummins and Greene in 1966. In addition to morphological description of rust species, nowadays various PCR technologies are available that makes it possible to detect and effectively amplify even small parts of fungal DNA for sequencing. But at the same time, one cannot rely only on DNA sequence data, morphological features also give useful information about species and their biology (Abbasi *et al.* 2005).

Considering this, the main aim of this study was to examine which species of rust fungi that form aecia on *Berberis* spp. collected in different parts of Sweden by using molecular techniques and spore and aecia morphology description.

Images of barberry bushes from several locations are presented in Figure 2.

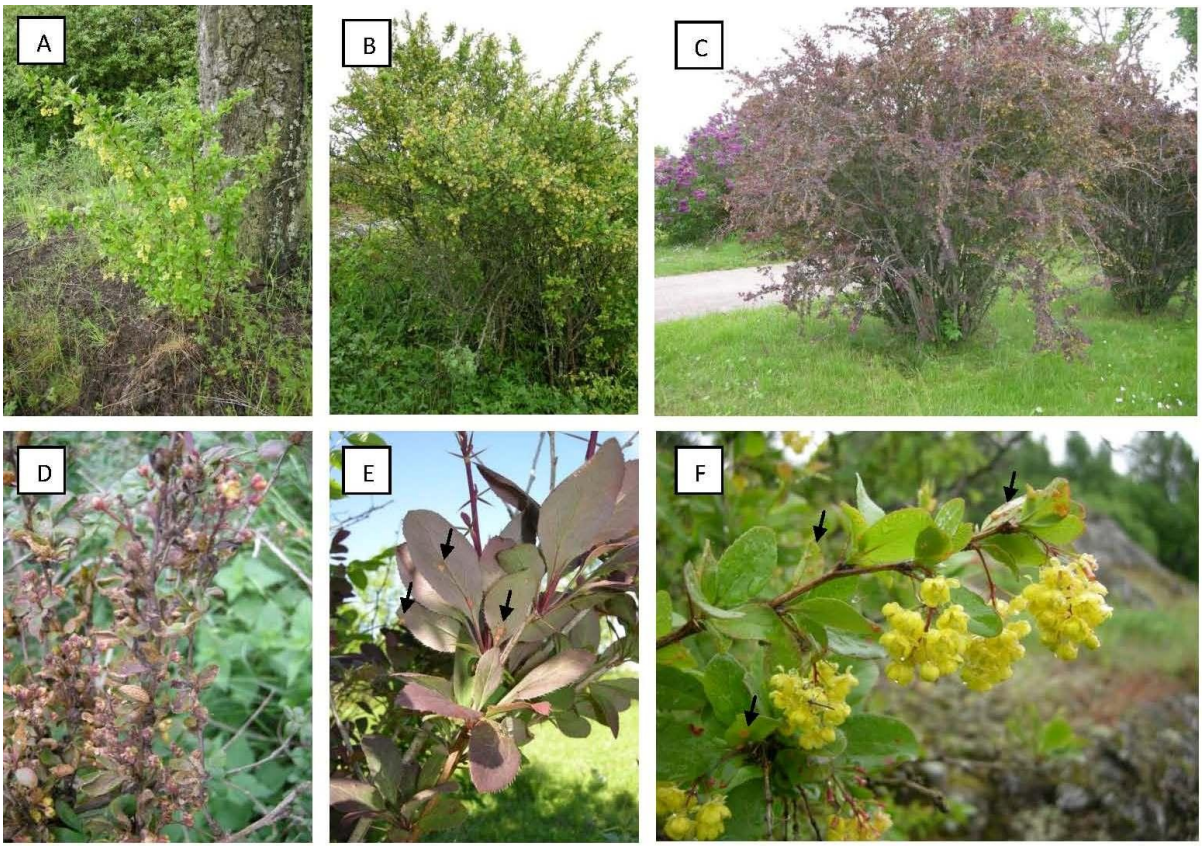


Figure 2. *Infected barberry bushes from different locations: 52 (A, F), 55 (B), 59 (C), 61 (D) and 69 (E). Arrows indicates where aecia are found on the leaves. Photo: Anna Berlin.*

Three different genes were used in DNA sequencing: the internal transcribed spacer (ITS) region, β-tubulin and elongation factor 1-α partial (EF1-α). The ITS region is the most popular locus for species identification and phylogenetic inference in sequence-based mycological research (Roose-Amsaleg *et al.* 2002; Nilsson *et al.* 2008) and is the most frequently sequenced region among fungi (Wang *et al.* 2009). This region is situated between the 18S and 28S ribosomal genes, which are found in multiple copy number in eukaryotes (Weider *et al.* 2005). Another two genes (β-tubulin and EF1-α) were selected because they have been successfully used in fungal systematics. A number of fungal phylogenies have been inferred from a

combination of three gene regions (de Jong *et al*., 2001; Slipper *et al*., 2004; Frøslev *et al*., 2005; Hansen *et al*., 2005). As an example, Slippers *et al*. (2004) in their study have used combination of ITS, β-tubulin and EF1-α to reidentify several fungi species. Moreover EF1-α, β-tubulin, and mitochondrial ATPase 6 genes, have been used in many recent multi-locus phylogenetic studies (Chaverri *et al*., 2003; Reeb *et al*., 2004; Tanabe *et al*., 2004; Thell *et al*., 2004; Cai *et al*., 2005). The main advantage of using these protein-coding genes is that they are strictly single copies in fungi and thus avoid the pitfalls of paralogous comparisons. In addition these genes (ITS region, β-tubulin and EF1-α) have previously been successfully used in phylogenetic studies of rust fungi (van der Merve *et al*. 2007; M. van der Merve *et al.* 2008; Liu & Hambelton, 2010).

2 Materials and methods

2.1 Rust and barberry collection

The rust and barberries specimens were collected by Anna Berlin during 11th - 15th of June 2010 in south-eastern Sweden (Figure 3). All collected samples were kept as herbarium samples.

2.2 Genomic DNA extraction

For DNA extraction as well as for image analysis the same aecia were investigated. For each sample one aecium was cut in to two pieces; one half was used for DNA extraction and the other one for morphological study of the spores and aecia. For DNA extraction, rust samples were taken from infected herbarium specimens and put in 2 ml test tubes with the screw cap together with 6 cm of dried oat leaf, 20 pieces of 2 mm glass beads and a knife point of diamateous earth. The samples were shaken in a FastPrep shaker (Precellys24-Dual, Bertin technologies) at 5,000 rpm for 2×20 seconds to a fine powder. Genomic DNA was isolated using the *OmniPrep*™ kit (G-Biosciences, St Luis, MO) according to the manufacture's protocol for fungal tissue. The DNA concentration was estimated using a spectrometer (ND-1000 Nano Drop®, Saveen Warner) and each sample was diluted to 20 ng /µL.

Genomic DNA of barberry leaf samples were isolated as described above (without adding dried oat leaf) with the *OmniPrep*™ kit (G-Biosciences, St Luis, MO) according to the manufacture's protocol for solid tissue.

2.3 PCR amplification and sequencing

In this study the ITS region, β-tubulin and elongation factor 1-α partial (EF1-α) genes were sequenced.

To produce the ITS sequences, a 50 µl PCR reaction solution was used. It contained 80 ng of template DNA, 5 µl 10× Dream Taq™ Buffer (Fermentas, Helsingborg, Sweden), 5µl 0,2 mM dNTP (Larova, GmbH), 0,8 µM of each primer: forward TS1rustF10d and reverse StdLSUR2a (Barnes and Szabo 2007), 1,25 units Dream Taq™ DNA Polymerase (Fermentas, Helsinborg, Sweden) and 1,5 µl $MgCl_2$.

Figure 3*. Map representing locations from where samples were collected.*

The PCR 2720 Thermal Cycler (Applied Biosystems) was programmed to implement following cycling conditions: 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; 1 cycle of 72°C for 7 min and holding at 4°C. Part of the samples (532, 551, 552, 571, 612, 613, 621, 701, 702, 703 and 704) did not produce amplifications with primers mentioned above. For them, the forward primer ITS3 (White *et al*, 1990) was used.

For amplification of the ITS region of the nuclear ribosomal DNA of barberry samples, a PCR reaction using 50 µl was conducted. The reaction mixture contained 80 ng of template DNA, 10 µl 5× Phusion® HF Buffer (Finnzymes OY), 2 µl 10 mM dNTP (Larova, GmbH), 1,3 µl each of the universal ITS1 (White *et al*, 1990) and ITS2 primers (White *et al*, 1990), 1 unit Phusion® DNA Polymerase (Finnzymes OY), 1,5 µl $MgCl_2$ and 1,5 µl DMSO. The PCR Thermal Cycler was programmed to implement following cycling conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 30 s, 62°C for 30 s and 72°C for 30 s; 1 cycle of 72°C for 7 min and holding at 4°C. The PCR products evaluation, purification and sequencing were performed as described above.

EF1-α DNA sequences amplification of rust samples was performed in 50 µl reaction mixtures using the same protocol as for ITS but without $MgCl_2$ (more water was added instead) and with forward primer EF1 (Anne-Marie Justesen, unpublished) and reverse primer EfbasidR (van der Merve *et al* 2007). The following cycling conditions were used: 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s; 1 cycle of 72°C for 7 min and holding at 4°C. The PCR products evaluation, purification and sequencing were performed using the same procedure as for the ITS sequences.

The amplification of β-tubulin DNA sequences was conducted using the same protocol as for ITS amplification with the following thermal conditions: 1 cycle of 94°C for 5 min; 38 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s; 1 cycle of 2°C for 7 min and holding at 4°C. About 77 % of the samples were amplified using the Tub1 (Anne-Marie Justesen, unpublished) and Tub2 (Anne-Marie Justesen, unpublished) primers. Two samples (582 and 672) were amplified using a nested PCR. During the first PCR reaction, a 20 µl mixture containing 80 ng of template, 2µl 10× Dream Taq™ Buffer (Fermentas, Helsingborg, Sweden), 2µl 0,2 mM dNTP (Larova, GmbH), 0,4 µM of each primer β-tub 1317F (van der Merve *et al* 2007) and β-tub 2662R (van der Merve *et al* 2007), 0,6 units Dream Taq™DNA Polymerase (Fermentas, Helsinborg, Sweden) and 0,7 µl $MgCl_2$. The thermal cycling conditions were: 1 cycle of 94°C for 5 min; 38 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s; 1 cycle of 72°C for 7 min and holding at 4°C. The second PCR reaction was performed analogically with the 1317F and 2662R primers. For successfully amplified samples, a 50 µl reaction was conducted using the PCR products from the first cycle.

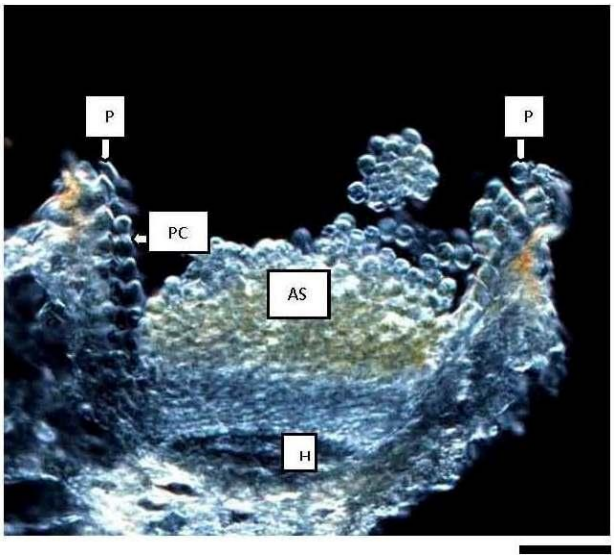
For all reactions, the success of the PCR reactions was evaluated using 1 % agarose gel electrophoresis. Successfully amplified samples were purified with Agencourt® AMPure® PCR Purification kit (Backman Coulter Inc.). All purified PCR products were sent to Macrogen Inc. (Seoul, South Korea) for sequencing from both directions using the same primers as for the PCR reactions respectively.

2.4 Phylogenetic analysis

All sequences were checked using SeqMan™II 5.07 (1989-2003 DNASTAR Inc.) and a consensus sequence was generated. Sequences of each gene were edited using the program MEGA 5 (Tamura *et al.* 2011) and all sequences for each gene were aligned separately using CLUSTAL W (Thompson *et al.* 1994). In order to check single apparent misalignment, the three sequence alignments were checked by eye and compared with the primary sequences. Even though this process is time consuming it was important to get reliable sequence alignment since following analysis will be carried out on their basis. For species identification, all aligned and checked sequences were compared with the available sequences in the BLAST database (Altschul *et al.* 1997). Three analyses were conducted. In the first analysis for all obtained sequences phylogenetic tree was made in MEGA 5 using neighbor-joining analysis (Saitou and Nei, 1987), which was conducted using a bootstrap method with 1000 replicates (Felsenstein, 1985). Transitions and transversions (with the equal ratio) were included in the analysis. A maximum composite likelihood method was used in the substitution model. Cut-off values of the condensed trees were set to 75 %. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*

2004) and were in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. In the second analysis, only controversial sequences were included; for each gene the alignments were compounded by 44 sequences downloaded from GenBank (33 for ITS, 1 for EF1-α and 12 for β-tubulin) (Appendix A-C). Bayesian Markov chain Monte Carlo analyses (MCMC) were performed using cross-platform BEAST 1.4. Using BEAUti graphical software, the Hasegawa-Kishino-Yano substitution model and strict molecular clock (assumes a global clock rate with no variation among lineages in a tree) models were conducted. The starting trees in the MCMC run were randomly generated and the MCMC algorithm was run for 100,000,000 generations. The information from a sample of trees produced by BEAUti/BEAST package was summarized using the program TreeAnnotator. Posterior probability limit was set to 0,5 and the target trees were set as Maximum clade credibility tree for each gene separately.

2.5 Spore collection and morphology



Cross-sections of aecium were done by cryostat using a Leica CM 1850 (Leica Microsystems Nussloch Gmb H). Jung Tissue Freezing Medium® for frozen tissue specimens (Leica Microsystems Nussloch Gmb H) was used in order to prepare samples for cross-section. The cross-sections were examined only for samples that visually differed from others, eight in total (Table 2). All samples were put in lactophenol. For obtaining digital images of cross-sections, the same equipment was used as for aeciospores.

Figure 4. Aecia (aecidium type). Hymenia (H), peridia (P), aeciospores (AS), peridial cells (PC). Bar = 75 µm.

The morphological type of aecia obtained from cross-sections was analyzed according to Sato and Sato (1985). For each aecium characteristics such as type of hymenia, peridia and peridial cells were evaluated (Figure 4).

In order to get aeciospores for analysis, different methods of sample rehydration were tested (with water, 70% ethanol and KOH). The shapes of rehydrated aeciospores were compared with spore samples that had been deep frozen (-70 °C) within two days after collection, which were considered to be fresh. Rehydration was conducted in Petri dishes filed with water for at least 1 hour at room temperature (Liu and Hambleton, 2010). Aeciospores were scratched from the leaf material using a single-edged blade and put under cover slips on slides in lactophenol to avoid accumulation of air bubbles around their hydrophobic surface (Anikster *et al*. 2005).

For description of aeciospore morphology length, width and spore projection area was measured (Anikster *et al.* 2005). An observation field under the microscope was selected randomly and all

spores within this field were measured except those touching field boundaries, other spores or plant debris. The best fitting ellipse was used for spore projection area measurement; the larger axis was assumed as length and the smaller axis as width (Anikster *et al.* 2005). At least 20 spores were measured within each sample. More spores were measured if the obtained data was not normally distributed. Spore images were produced using differential interference contrast (DIC) and bright field (BF) microscopy (Figure 2) with Leica DM 5500 B Digital Microscopes (Leica Microsystems CMS Gmb H) and Leica Application Suite Advanced Fluorescence 235 build 5371 software. Length, width and spore area was analyzed for significance in differences by one-way ANOVA and Student's t-test. Using these statistical analyses all samples were put in different groups. Samples from one group differ significantly from other groups, whereas the difference between the samples within each group was not significant. For length, width and projection area, separate analyses were conducted. This grouping was then compared with the BLAST results and arranged in tables 4-6.

3 Results

This study investigated the rusts forming aecia on barberry in Sweden. The aeciospores and morphology of the aecia as well as DNA sequences of ITS, β-tubulin and EF1-α from 45 aecial samples collected form 18 barberry bushes were analyzed.

3.1 DNA sequences analysis

In total 138 sequences were successfully amplified and sequenced (18 for barberry plants, 44 for ITS, 44 for EF1-α and 32 for β-tubulin). The effective length of the ITS region fragment varied between 782 and 1056 bp, between 411 and 638 bp for EF1-α, and from 659 to 1180 bp for β-tubulin. The effective length of the ITS region for barberry varied between 206 and 368 bp.

In accordance with sequences in the BLAST reference database, five different rust species were distinguished: *P. graminis* f.sp. *avenae*, *P. graminis* f.sp. *tritici*, *P.coronata*, *P. striiformis* and *P. poae-nemoralis* (Table 1). The presence of species *P. graminis* f.sp. *avenae*, *P. graminis* f.sp. *tritici* and *P. coronata* was confirmed by all three genes with high level of maximum identity (97 to 100%).

The rest of the 12 samples were identified differently: *P. striiformis* (according to the sequences from ITS region) and *P. poae-nemoralis* (according to β-tubulin and EF1-α partial gene). For the most ITS sequences maximum identity was 94 %, sample number 701 had maximum identity 96% and sample number 703 – 97%. For most EF1-α sequences maximum identity was the lowest (from 90 to 91 %) except sample number 704 that had maximum identity of 93 %. The highest maximum identity was obtained for β-tubulin sequences (98 %) except 97 % for samples number 632 and 704.

DNA sequences analysis gave no clear results about species identity of the collected barberry plants and indicated with the same maximum identity for three different species for each sample. The following species were identified: *Berberis aetnensis, Berberis croatica, Berberis vulgaris* and as out-group *Rhamnus cathartica* was identified for location 72 (Table 2).

Table 1. *Species name identified according to sequences from BLAST database for ITS region, EF1-α and β-tubulin genes. Maximum identity indicates similarity of obtained sequence to a database sequence. Sample identity: first two numbers specify location, the last one – the number of replication; -, sequences were not amplified.*

Sample	ITS		EF1-α		β-tubulin	
	Species name	**Maximum identity, %**	**Species name**	**Maximum identity, %**	**Species name**	**Maximum identity, %**
521	*P. graminis* f.sp. *tritici*	100	-	-	-	-
522	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	96	*P. graminis* f. sp. a*venae*	99
523	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	99
531	*P. graminis* f.sp. *tritici*	100	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	99
532	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	99
533	*P. graminis* f.sp. *tritici*	100	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	99
551	*P. graminis* f.sp. *avenae*	100	*P. graminis* f.sp. *avenae*	98	-	-
552	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	99
553	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	98
571	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	96	*P. graminis* f.sp. *tritici*	99
572	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	97	-	-
581	*P. graminis* f.sp. *avenae*	100	*P. graminis* f.sp. *avenae*	98	*P. graminis* f.sp. *avenae*	99
582	*P. coronata*	99	*P. coronata*	96	*P. graminis* f.sp. *avenae*	99
591	*P. graminis* f.sp. *tritici*	100	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	99
592	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	99
601	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	98	*P. graminis* f.sp. *avenae*	99
602	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	97	*P. graminis* f.sp. *tritici*	98
603	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	97	*P. graminis* f.sp. *tritici*	99
604	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	98	*P. graminis* f.sp. *avenae*	99
611	*P. striiformis*	94	*P. poae-nemoralis*	90	*P. poae-nemoralis*	98
612	*P. striiformis*	94	*P. poae-nemoralis*	90	-	-
613	*P. striiformis*	94	*P. poae-nemoralis*	90	*P. poae-nemoralis*	98
614	*P. striiformis*	94	*P. poae-nemoralis*	90	-	-
621	*P. graminis* f.sp. *tritici*	99	*P. graminis* f. sp. *tritici*	98	*P. graminis* f.sp. *tritici*	98
622	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	97	*P. graminis* f.sp. *avenae*	99
631	*P. striiformis*	94	*P. poae-nemoralis*	90	*P. poae-nemoralis*	98

Sample	ITS		EF1-α		β-tubulin	
	Species name	**Maximum identity, %**	**Species name**	**Maximum identity, %**	**Species name**	**Maximum identity, %**
632	*P. striiformis*	94	*P. poae-nemoralis*	91	*P. poae-nemoralis*	97
633	*P. striiformis*	94	*P. poae-nemoralis*	91	*P. poae-nemoralis*	98
634	*P. striiformis*	94	*P. poae-nemoralis*	91	*P. poae-nemoralis*	98
641	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	98	*P. graminis* f.sp. *avenae*	99
642	*P. graminis* f.sp. *avenae*	98	*P. graminis* f.sp. *avenae*	90	*P. graminis* f.sp. *avenae*	98
661	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	98
662	-	-	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	99
663	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	98	-	-
664	*P. coronata*	99	*P. coronata*	98	-	-
671	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	98	*P. graminis* f.sp. *avenae*	99
672	*P. graminis* f.sp. *avenae*	99	*P. graminis* f.sp. *avenae*	99	-	-
691	*P. graminis* f.sp. *tritici*	99	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	99
692	*P. graminis* f.sp. *tritici*	100	*P. graminis* f.sp. *tritici*	98	*P. graminis* f.sp. *tritici*	99
701	*P. striiformis*	96	*P. poae-nemoralis*	91	-	-
702	*P. graminis* f.sp. *tritici*	100	*P. graminis* f.sp. *tritici*	98	-	-
703	*P. striiformis*	97	*P. poae-nemoralis*	91	-	-
704	*P. striiformis*	94	*P. poae-nemoralis*	93	*P. poae-nemoralis*	97
721	*P. coronata*	99	*P. coronata*	99	-	-
722	*P. coronata*	100	*P. coronata*	98	-	-

Table 2. *Species name identified according to similarity to sequences from the BLAST database for the ITS region. Maximum identity indicates similarity of obtained sequence to a database sequence. The numbers specifies the locations from where the samples were collected*

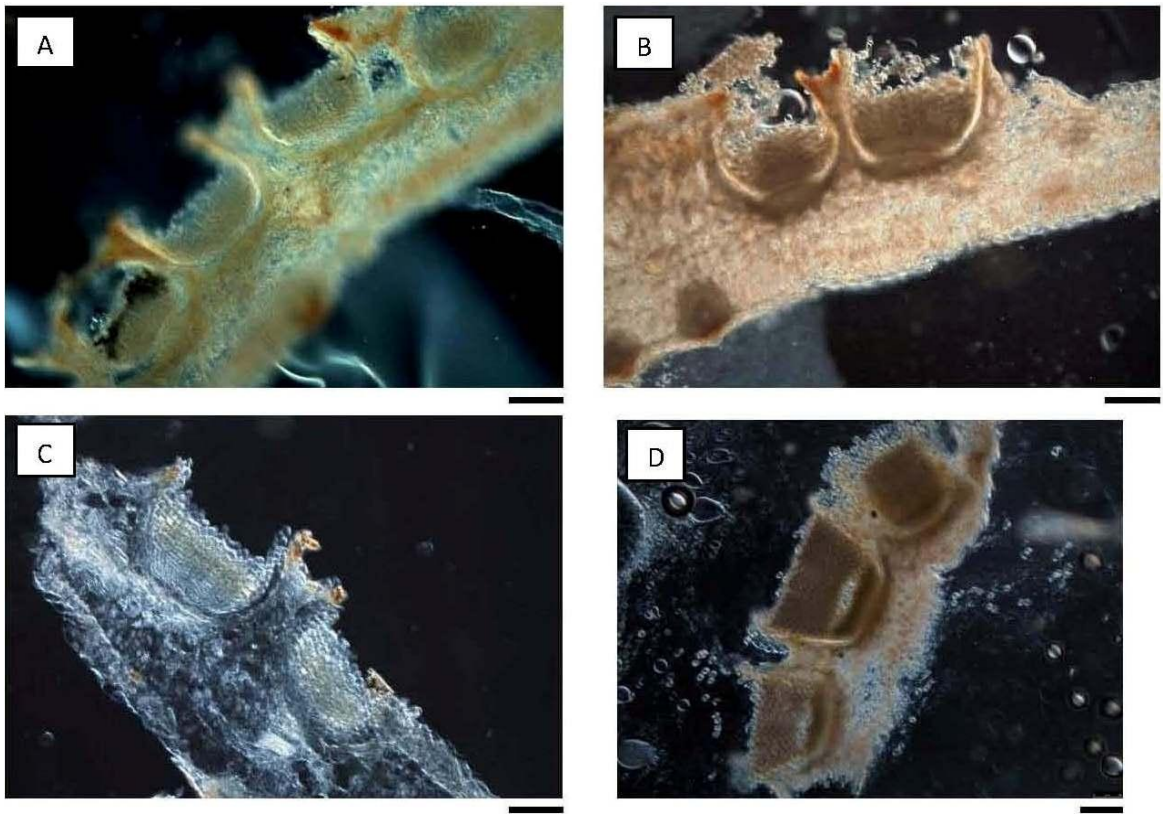
Sample	Species name	Max identity, %	Sample	Species name	Max identity, %
52	*B. aetnensis*	100	**63**	*B. aetnensis*	100
	B. croatica	100		*B. croatica*	100
	B. vulgaris	100		*B. vulgaris*	100
53	*B. aetnensis*	100	**64**	*B. aetnensis*	100
	B. croatica	100		*B. croatica*	100
	B. vulgaris	100		*B. vulgaris*	100
55	*B. aetnensis*	98	**66**	*B. aetnensis*	98
	B. croatica	98		*B. croatica*	98
	B. vulgaris	98		*B. vulgaris*	98
57	*B. aetnensis*	99	**67**	*B. aetnensis*	99
	B. croatica	99		*B. croatica*	99
	B. vulgaris	99		*B. vulgaris*	99
58	*B. aetnensis*	100	**68**	*B. aetnensis*	100
	B. croatica	100		*B. croatica*	100
	B. vulgaris	100		*B. vulgaris*	100
59	*B. aetnensis*	88	**69**	*B. aetnensis*	92
	B. croatica	88		*B. croatica*	92
	B. vulgaris	88		*B. vulgaris*	92
60	*B. aetnensis*	100	**70**	*B. aetnensis*	97
	B. croatica	100		*B. croatica*	97
	B. vulgaris	100		*B. vulgaris*	97
61	*B. aetnensis*	100	**71**	*B. aetnensis*	99
	B. croatica	100		*B. croatica*	99
	B. vulgaris	100		*B. vulgaris*	99
62	*B. aetnensis*	97	**72**	*R. cathartica*	99
	B. croatica	97			

3.2 Morphology

Between the samples, there were two distinct patterns of aecia appearance on the leaves. Mostly (in 72 % or 31 samples) aecia on leaf appeared in yellow, light orange, cup-like, gregarious spots. On the rest of the samples, aecia resembled from bright yellow to brownish powder that covered the whole surface of the leaf, which were stunted and curled (Figure 5). These two types of aecia were denominated spot like (S) and powder like (P) respectively and specified for each sample in table 3.

Cross-sections for 4 different rust species were obtained (Figure 6).

Figure 5. *Aecia on barberry leaf. Spot like (S) (left) and powder like (P) (right).*



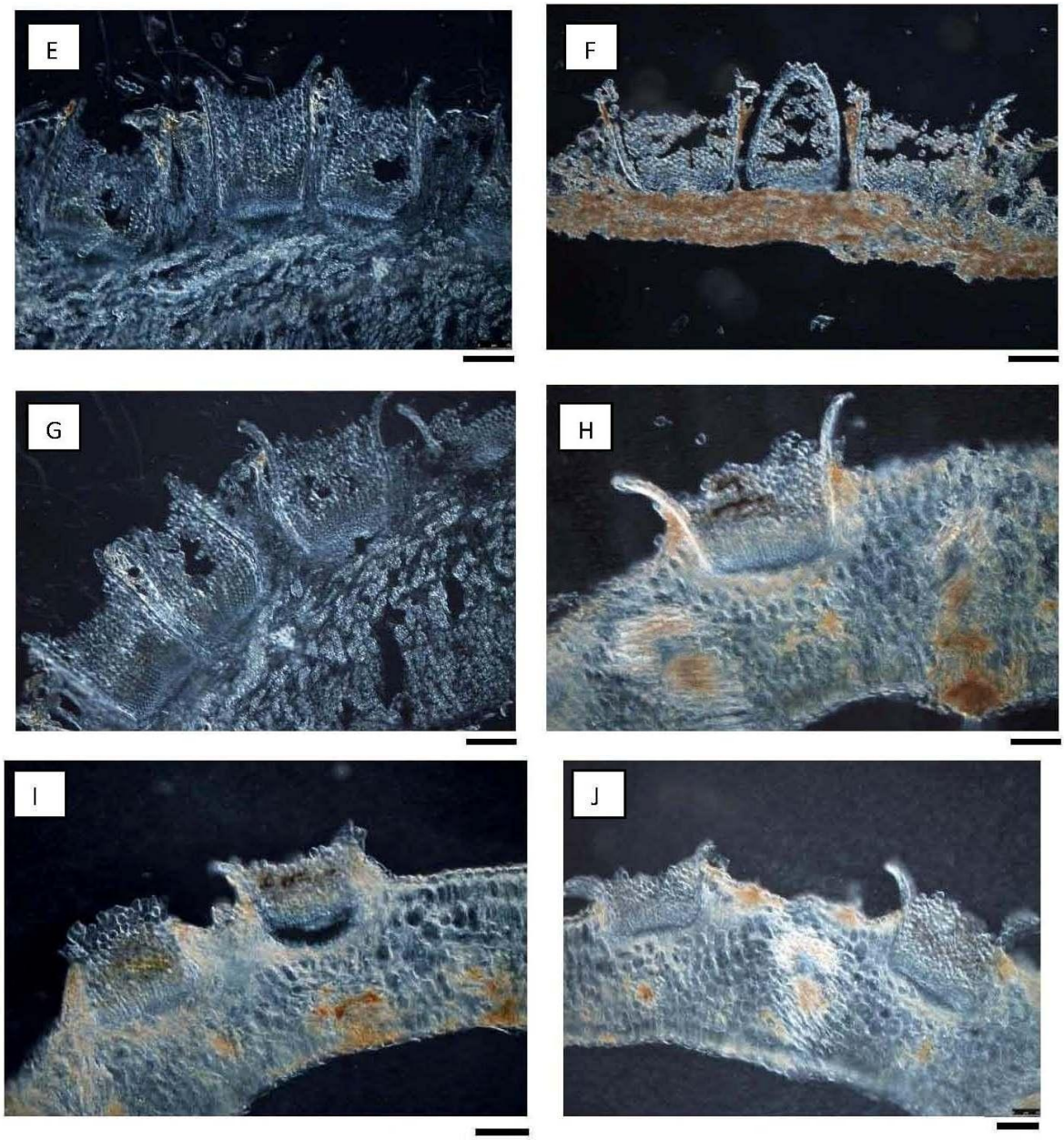


Figure 6. *Cross-section of aecium. P. graminis* f.sp. *tritici (A, B, C, D), P. graminis* f.sp. *avenae (E, F, G) and P. striiformis/P. poae-nemoralis (H, I, J). Bar = 100 µm.*

According to Sato and Sato (1985), all aecia obtained from cross-sections in this study have morphological type *aecidium.* It is cupulate, gregarious, yellowish, and erumpent with elliptical or oval peridial cells, intramesophyllic, flat hymenium, peridiate sori with single-layered, fragile peridium (Sato and Sato, 1985).

Even though all obtained aecia belong to the same morphological type by visual evaluation of images from figure 6, some obvious dissimilarity between aecia of different species was found. Aecia of *P. graminis* f.sp. *tritici* are deep-seated into leaf tissue, have roundish shape with close disposition to each other. The ending of the peridium is bent inside the aecia cup. Aecia of *P. graminis* f.sp. *avenae* differ from others and have oblong shape. The ending of the peridium is

slightly bent outside the aecia cups, which are densely located. Finally, samples identified as *P. striiformis/P. poae-nemoralis* have wide aecia with peridium ends fairly folded outside. Aecia are placed deeply into the leaf tissue and on a relatively large distance from each other compared with the other species.

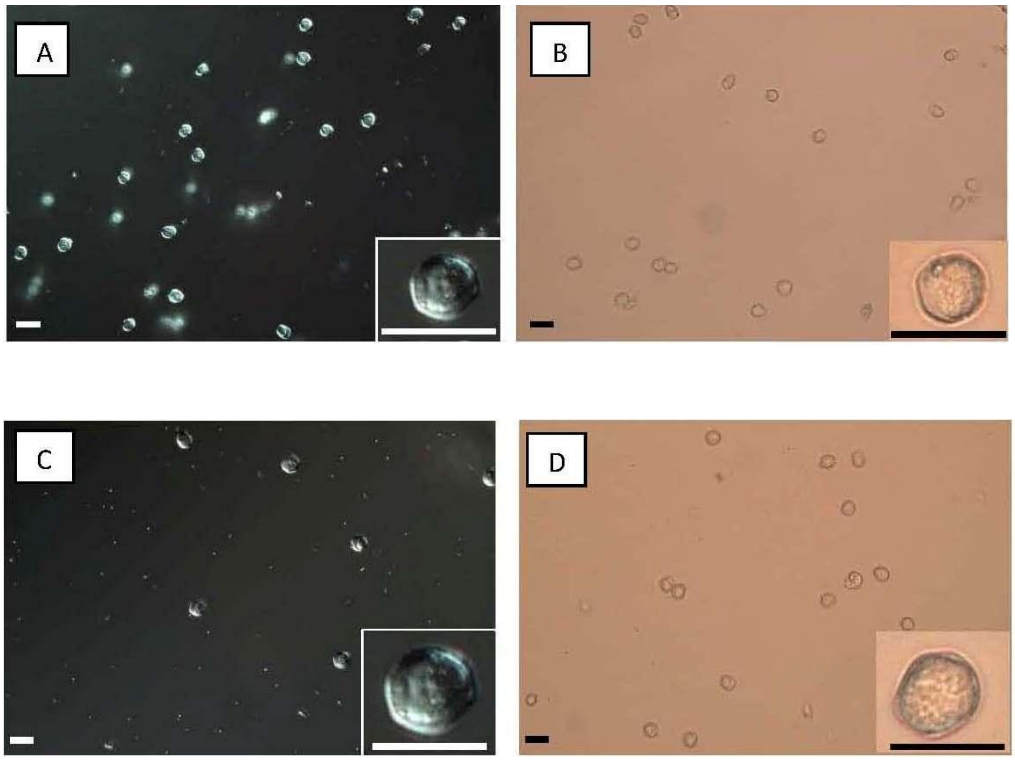
In total, 984 spores were measured. Table 3 summarizes all obtained measurements. For samples 521, 531, 553, 572 and 722 spore measurements are missing because no spores were found in those samples.

Spore images of five different species are presented in figure 7.

Table 3. *Mean values and standard deviation (SD) of measured length, width and projection area by sample. Sample identity: first two numbers denote location, the last one the number of repetition, letters specify type of aecium on barberry leaves: spot like (S) or powder like (P); "*" marks the samples from which cross-sections of aecia were taken. Number of spores – number of spores measured for each sample*

Sample	Number of spores	Mean length, µm	SD	Mean width, µm	SD	Mean projection area, μm^2	SD
521S	22	16,98	1,09	15,56	1,14	207,15	20,58
522S	21	16,73	0,87	15,06	1,02	198,53	20,69
531S	22	17,70	1,68	15,11	1,46	209,48	36,38
532S	23	18,32	1,44	15,57	1,00	222,92	18,13
551S*	36	17,49	0,85	16,14	0,74	222,96	17,13
552S	22	16,41	1,36	13,78	0,86	177,95	19,75
571S	20	20,76	1,68	16,63	0,98	265,38	26,00
581S	20	16,37	1,26	15,25	0,86	196,68	21,40
582S	30	21,96	0,93	18,68	0,94	322,82	13,83
591S	20	18,88	1,32	16,98	1,27	252,66	32,40
592S	37	18,51	1,42	16,12	1,00	236,10	23,31
601S*	26	18,45	1,33	15,84	1,37	228,64	30,36
602S	34	18,52	1,61	16,47	1,02	239,62	30,54
603S	20	18,25	1,73	15,84	1,07	227,27	32,10
604S	21	16,65	1,21	14,49	1,06	189,34	21,13
611P*	36	24,52	2,58	20,43	2,04	396,61	63,88
612P	21	23,42	1,85	19,64	1,76	364,94	54,20
613P	21	23,82	1,41	19,55	1,39	366,19	38,52
614P	29	24,47	1,29	20,80	1,47	400,95	27,87
621S	20	18,20	1,17	16,34	0,98	240,36	22,56
622S	25	18,22	0,86	15,83	1,08	229,12	17,84
631P*	36	24,54	1,92	20,71	1,44	400,21	47,29
632P	25	24,09	1,53	20,59	1,75	397,43	35,60

633P	21	23,75	1,58	19,91	1,26	371,90	38,90
634P	24	23,77	2,15	19,78	1,18	373,23	42,66
641S	21	18,29	1,63	15,82	1,05	227,68	27,63
642S	20	17,68	1,22	15,51	0,79	221,56	18,45
661S*	20	19,58	1,18	16,98	1,08	263,65	25,55
662S	23	19,69	1,53	17,02	1,06	265,93	24,76
663S	21	18,42	1,14	16,00	0,96	230,64	24,05
664S	30	20,10	1,83	16,89	0,87	265,68	30,21
671S	26	19,50	1,57	15,78	0,98	244,46	28,26
672S	20	18,06	1,33	15,94	0,89	227,18	24,52
691S	25	18,24	1,19	16,12	0,92	231,42	22,79
692S	21	18,58	1,15	16,13	0,85	236,06	21,37
701P	27	26,94	2,44	19,64	1,75	414,13	43,22
702S*	22	20,44	0,93	18,55	0,82	297,26	17,73
703P	23	28,10	2,62	20,79	1,28	458,60	37,79
704P	27	24,70	2,14	20,36	1,50	393,32	46,21
721S	26	19,55	1,53	18,68	0,93	337,31	28,10



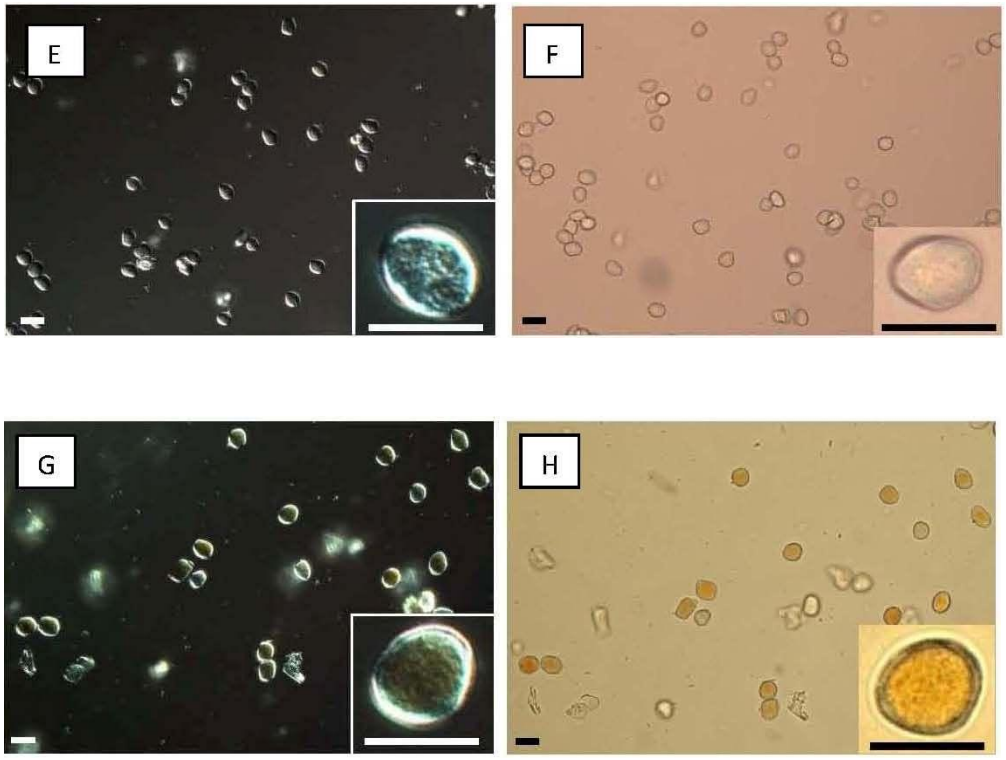


Figure 7. *Spore images obtained by DIC (left) and BF (right) microscopy. P. graminis* f.sp. *avenae (A, B), P. graminis* f.sp. *tritici (C, D), P.coronata (E, F) and P. striiformis/P. poae-nemoralis (G, H). Bar = 25 µm.*

P. graminis f.sp. *avenae* and *P. graminis* f.sp. *tritici* have roundish spores compared with *P.coronata* and *P. striiformis/P. poae-nemoralis* which have spores with more prolonged shape. The mean length, width and projection area for all detected species are presented in table 4-6. Samples *P. striiformis* and *P. poae-nemoralis* are written in quotation marks because of the inconsistency in the species names based on the different genes. According to ITS *P. striiformis* was obtained and according to EF1-α and β-tubulin *P. poae-nemoralis* while morphological data analysis indicates that it is the same species.

Table 4. *Species obtained from ITS sequence (according to the BLAST database) and one-way ANOVA analysis of spore dimensions (P-value < 0,0001)*

ITS	Number of spores	Mean length, µm	SD	Mean width, µm	SD	Mean projection area, µm^2	SD
P. graminis f.sp. *avenae*	259	17,69	1,54	15,50	1,19	216,95	29,33
P. graminis f.sp. *tritici*	326	18,63	1,64	16,27	1,30	239,14	33,97
P. coronata	86	20,58	1,79	17,32	1,37	280,51	40,32
'P. striiformis'	290	24,76	2,40	20,25	1,61	395,58	50,38

Table 5. *Species obtained from EF1-α sequence (according to the BLAST database) and one-way ANOVA analysis of spore dimensions (P-value < 0,0001)*

EF1-α	Number of spores	Mean length, µm	SD	Mean width, µm	SD	Mean projection area, µm^2	SD
P. graminis f.sp. *avenae*	259	17,69	1,54	15,50	1,19	216,95	29,33
P. graminis f.sp. *tritici*	349	18,70	1,65	16,32	1,30	241,02	33,91
P. coronata	86	20,58	1,79	17,32	1,37	280,51	40,32
'P. poae-nemoralis'	290	24,76	2,40	20,25	1,61	395,58	50,38

Table 6. *Species obtained from β-tubulin sequence (according to the BLAST database) and one-way ANOVA analysis of spore dimensions (P-value < 0,0001)*

β-tubulin	Number of spores	Mean length, µm	SD	Mean width, µm	SD	Mean projection area, µm^2	SD
P. graminis f.sp. *avenae*	233	18,24	2,13	15,77	1,64	228,78	46,70
P. graminis f.sp. *tritici*	327	18,58	1,62	16,17	1,19	237,24	31,30
'P. poae-nemoralis'	190	24,23	2,00	20,26	1,60	387,88	48,28

3.3 Phylogenetic analysis

The phylogenetic analysis involved 44 nucleotide sequences with the total of 1147 positions in the final dataset (for ITS region sequences); 44 nucleotide sequences with the total of 738 position (for EF1-α gene sequences), and 32 nucleotide sequences with the total of 1219 positions (for β-tubulin gene sequences). The number of position resembles the longest sequences in each alignment. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Felsenstein, 1985).

Evolutionary relationship between species for different genes is presented in figure 8 to10.

For better visualization, well-distinguishable clusters were joined together with a brace and named as group A for *P. graminis* f.sp. *tritici*, group B for *P. graminis* f.sp. *avenae*, group C for '*P. striiformis'* or *'P. poae-nemoralis'* and group D for *P. coronata,* respectively. The main tree branches was marked by arrows and named as Clade 1, 2 and 3. Also within each group, tree branches with support value more than 75 % were pointed by arrows and named according to the group (e.g. A1 or B2).

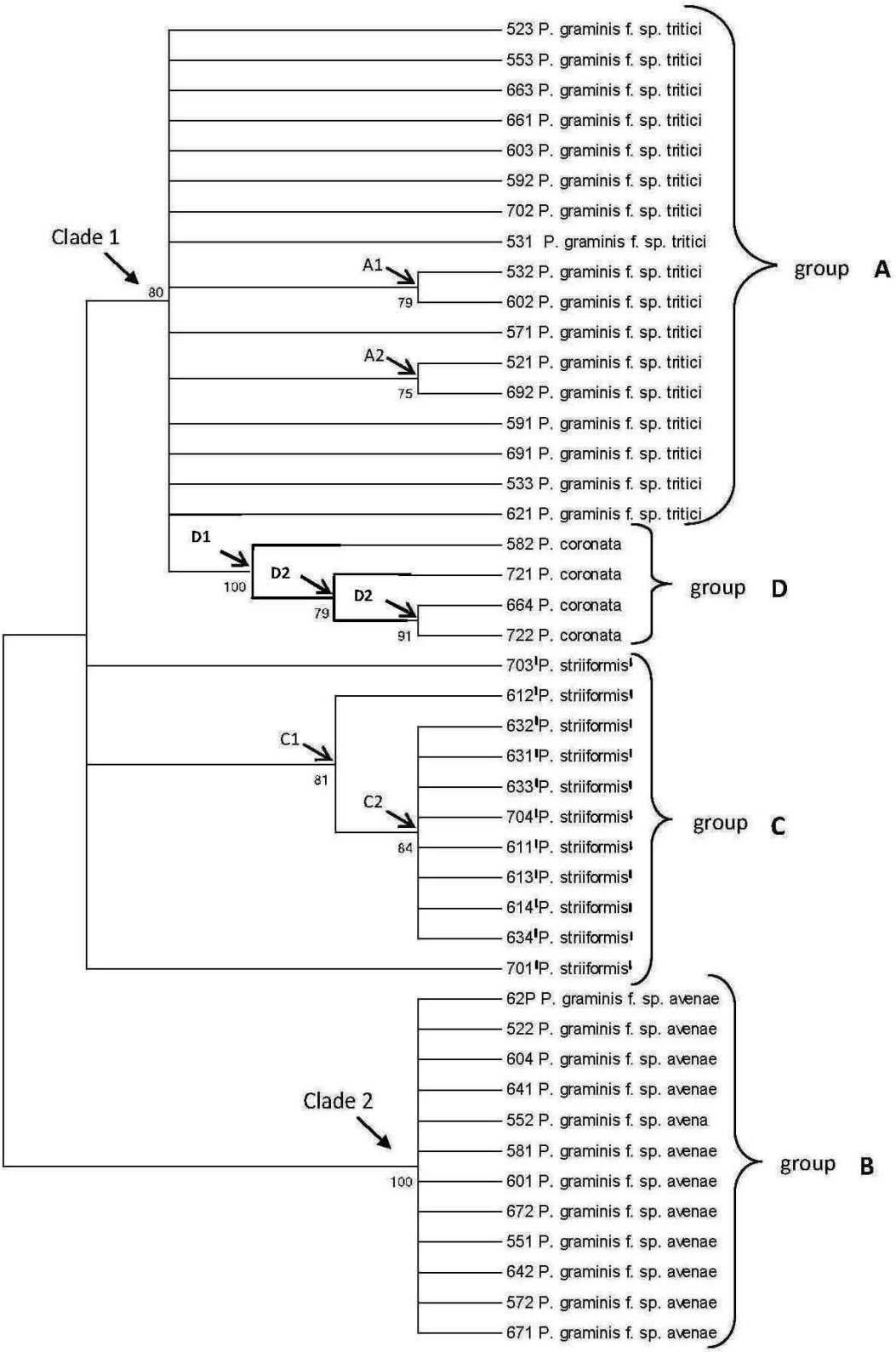


Figure 8. *Evolutionary relationships of taxa obtained using partial gene sequence of the ITS region. Arrows indicate nodes linking taxa into sub-clades. The optimal tree with the sum of branch length = 1.16307078 is shown. Species identity is based on sequence similarities to sequences deposited in the BLAST database.*

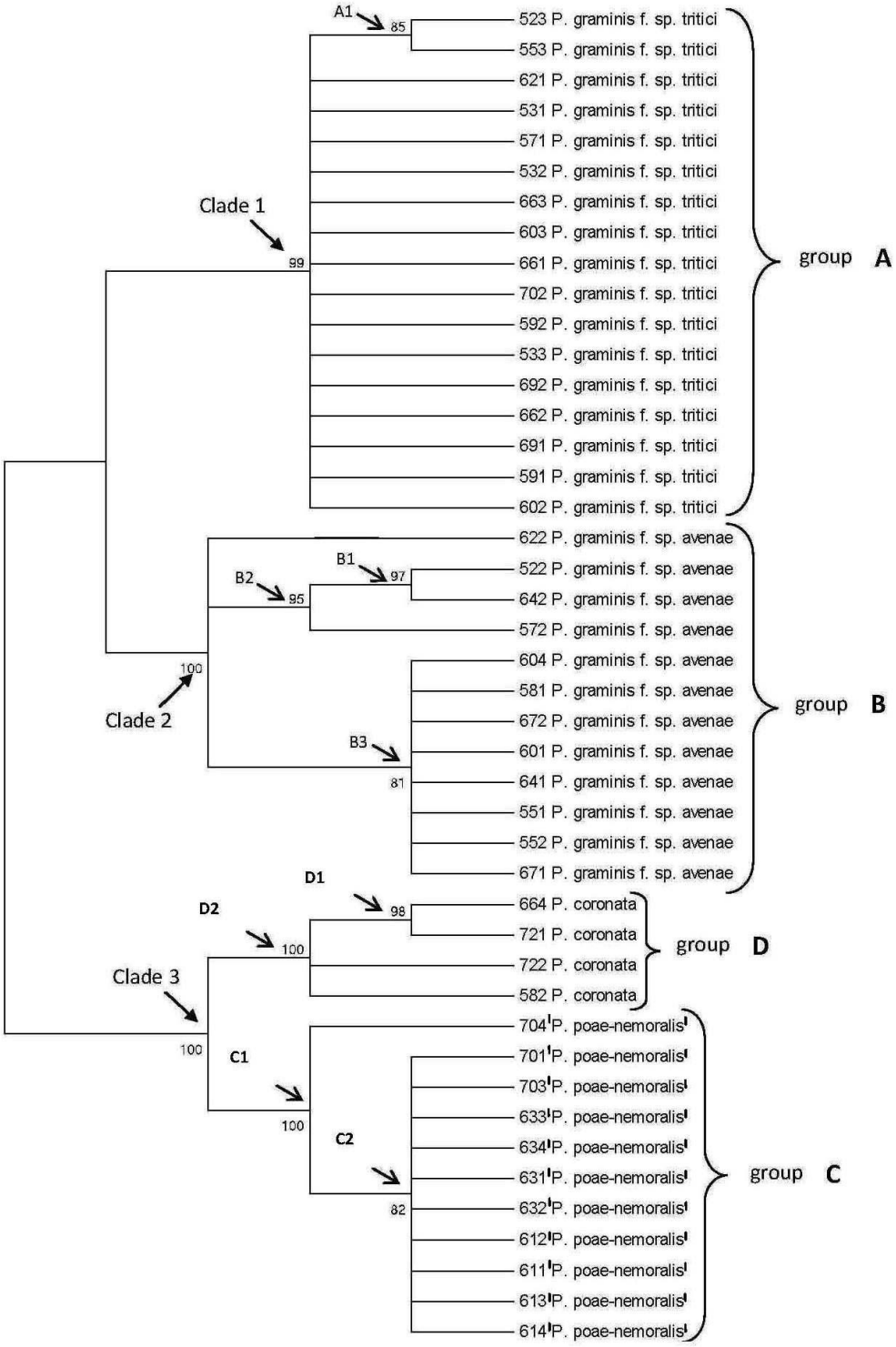


Figure 9. *Evolutionary relationships of taxa obtained using partial gene sequence of the EF1-α gene. Arrows indicate nodes linking taxa into sub-clades. The optimal tree with the sum of branch length = 0.74639005 is shown. Species identity is based on sample sequence similarities to sequences deposited in the BLAST database.*

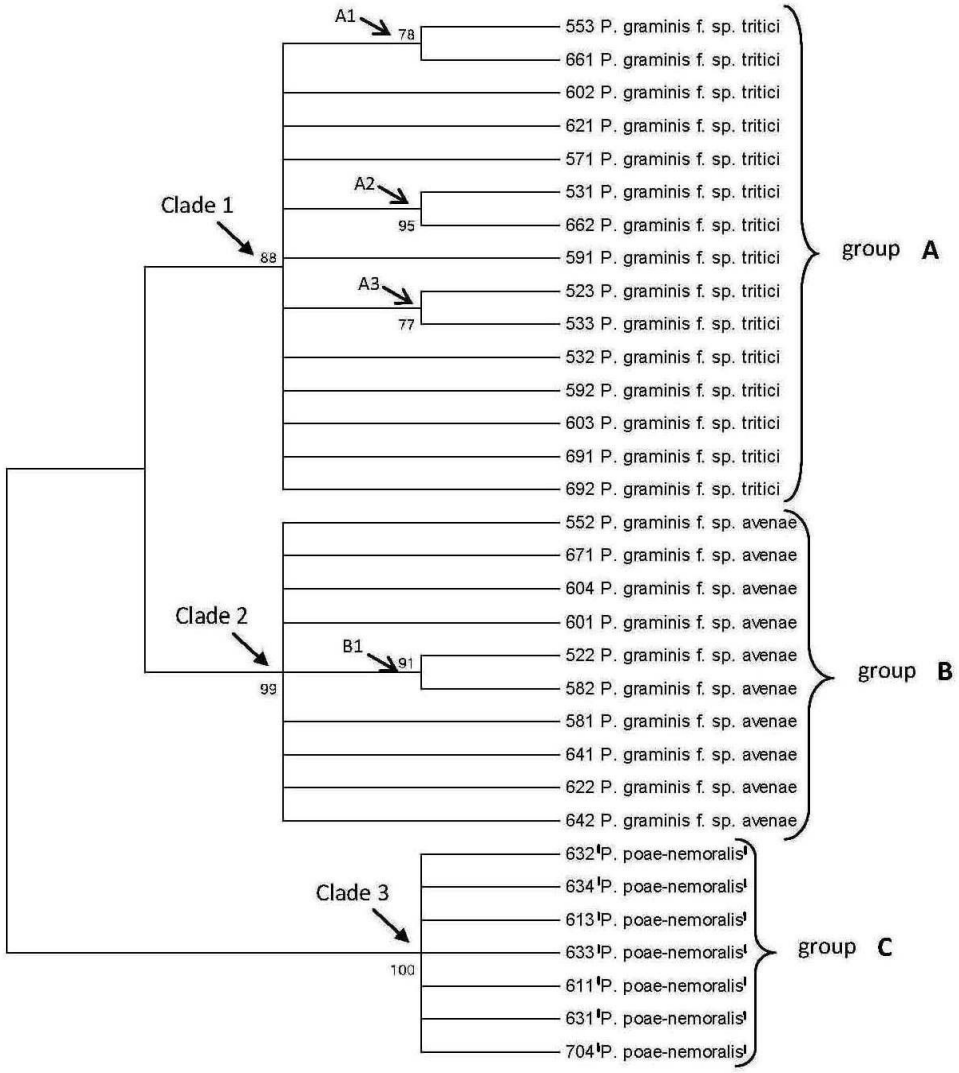


Figure 10*. Evolutionary relationships of taxa obtained using partial gene sequence of the β-tubulin gene. Arrows indicate nodes linking taxa into sub-clades. The optimal tree with the sum of branch length = 0,23053799 is shown. Species identity is based on sequence similarities to sequences deposited in the BLAST database.*

The phylogenetic tree shown in figure 8 indicates two distinct clades (with 80% and 100% support respectively). Clade 2 represents only one species (*P. graminis* f.sp. *avenae*) and within group B, *P. graminis* f.sp. *tritici* is placed in Clade 1 and has two sub-clades with the 75 % and 79% of support. *Puccinia striiformis* forms a separate group (with two rather well supported sub-clades) within Clade 1. Group D within Clade 1 represents *P. coronata* and is characterized by well-supported sub-clades.

In figure 9, three clades within this phylogenetic tree can be distinguished. The EF1-α gene phylogeny (compared with ITS) indicates that Clade 1 consists of only one group (group A), which represents *P. graminis* f.sp. *tritici.* This clade has one sub-clade, which divides samples 523 and 553, with a support of 85%. Clade 2 also consists of one group, group B. This group represents *P. graminis* f.sp. *avenae*, which is similar to the ITS region tree phylogeny. Within

this group, three sub-clades are present which is not the case for ITS. The third clade contains two groups: group C (*P. poae-nemoralis*) and group D (*P. coronata*). Group C forms two sub-clades, one of which (D1) has 100 % support and separates sample 704 from the others. Group D consists of two sub-clades, which separate samples 664 and 721 from two other samples (722 and 582).

Figure 10 shows the phylogenetic tree for the β-tubulin gene with three clades. Clade 1 contains group A, which represents *P. graminis* f.sp. *tritici* which is also confirmed by the EF1-α gene phylogeny. This group has three sub-clades, but none of them correspond to any of the sub-clades in the comparable group in the EF1-α gene phylogeny. Clade 2 consists of group B (*P. graminis* f.sp. *avenae*) which is the same for the two other genes. Finally, clade 3 represents *P. poae-nemoralis* (group C) without any sub-clades. *P. coronata* was not identified due to failing in PCR amplification.

Figure 11-13 represent trees that consist of controversial sequences and sequences of the same species downloaded from GenBank. *Puccinia striiformis* (identified according to ITS sequences) and *P. poae-nemoralis* (identified according to EF1-α and β-tubulin sequences) belong to the controversial sequences because morphological data indicates that it is the same species.

Figure 11 shows that sequences obtained using the ITS region sequences and were identified as *P. striiformis* are clustered separately from the other *P. striiformis* sequences. They form a separate clade that has a rather strong support and consists of four well supported sub-clades. These sub-clades separate samples 631-634, 611, 613, 614 and 704 from the other three samples. At the same time, these three sequences (612, 701 and 703) are clustered together with five of the downloaded sequences identified as *P. poae-nemoralis* and *P. brachypodii* f.sp. *poae-nemoralis*.

The tree that represents sequences obtained using the EF-1α gene sequences (Figure 12) contains only one downloaded sequence of *P. poae-nemoralis* because no other sequences for this gene was found in the GenBank database. Nevertheless, all our sequences except one (704) have formed an individual clade with 99% support. Within this clade, sample 633 and 634 has formed a sub-clade, which is similar to the previous tree. The sample 704 is cluster separately both from the downloaded and from the sequences obtained in this study.

In the tree with sequences obtained using the β-tubulin gene sequences (Figure 13), all sequences from this study clustered separately from the downloaded ones. This clade consists of three well supported sub-clades that isolate samples 631, 632 and 704 from 633 and 634, and from 611 as well as from 613. The reference sequences formed individual clades for *P. striiformis*, *P. striiformides*, *P. pseudostriiformis* and *P. poae-nemoralis*.

Figures 14-16 represent Bayesian phylogeny trees for the three genes that have been computed in the software BEAST.

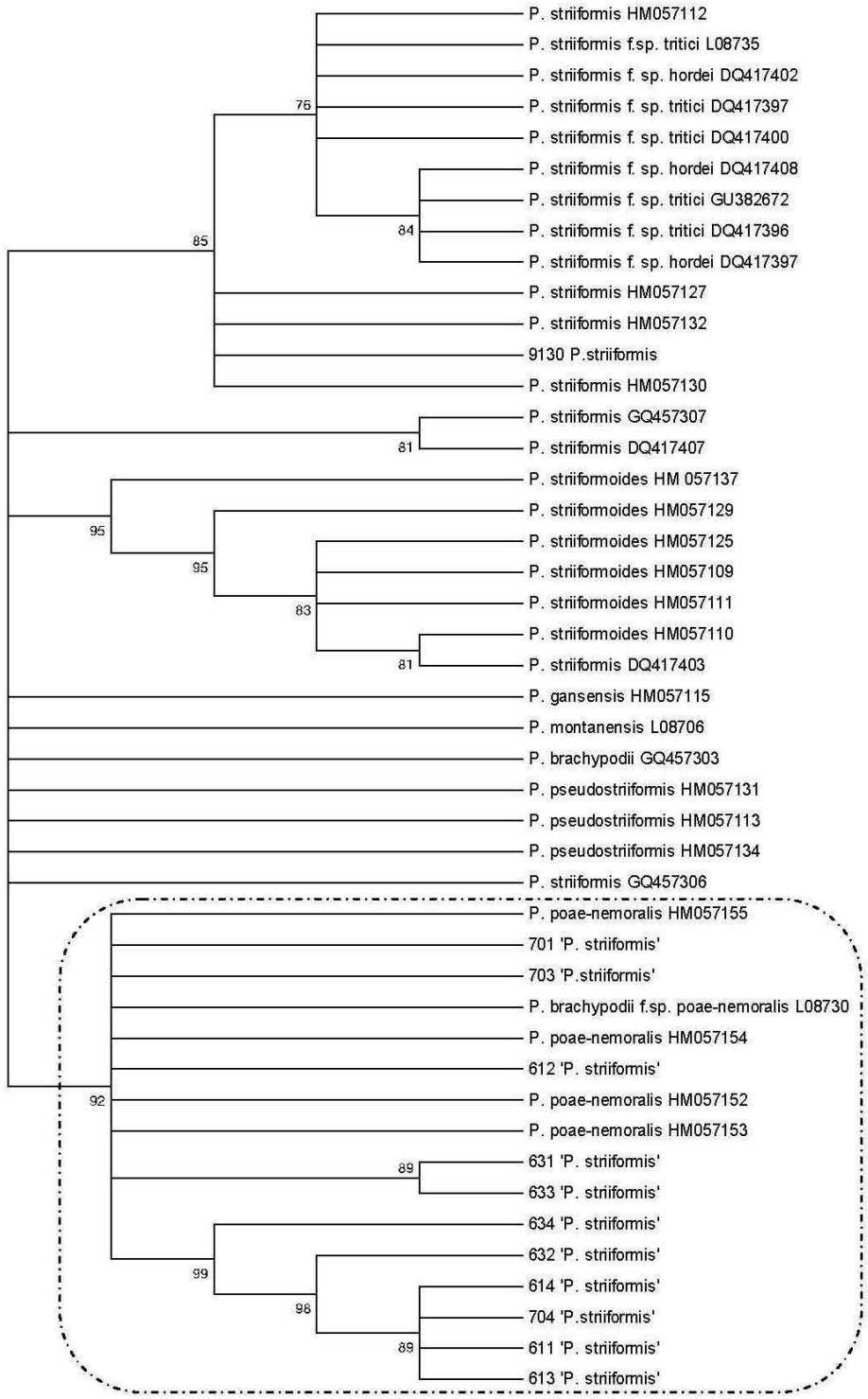


Figure 11. *Evolutionary relationships of taxa obtained using the ITS region and sequences downloaded from GenBank (details resented in Appendix A). Dotted line is highlighting the area in which sequences acquired in this study are presented.*

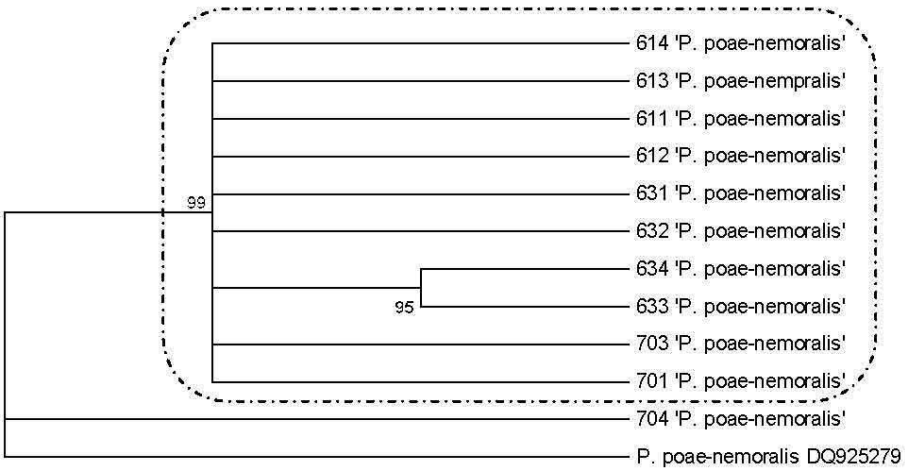


Figure 12. *Evolutionary relationships of taxa obtained using the EF-1α gene and sequences downloaded from GenBank (details are presented in Appendix A). Dotted line is highlighting the area with sequences obtained in this study. The sample 704 was not included into the highlighted area because it is cluster separately from the clade with sequences obtained in this study.*

According to the Bayesian phylogeny, two clades are formed in all presented trees (Figure 14 - 16). One clade consists of *'P. striiformis'* (in the ITS tree), *'P. poae-nemoralis'* (in the β-tubulin tree) and of *'P. striiformis'* and *P. coronata* in the EF-1α tree. Another clade contains *P. graminis* f.sp. *tritici*, *P. graminis* f.sp. *avenae* and *P. coronata* (in the ITS tree), and *P. graminis* f.sp. *tritici* and *P. graminis* f.sp. *avenae* (in the EF-1α and β-tubulin tree β-tubulin trees). In all trees, different species (group A-D) are separated from each other by well-supported clades with ~~the~~ high posterior probabilities.

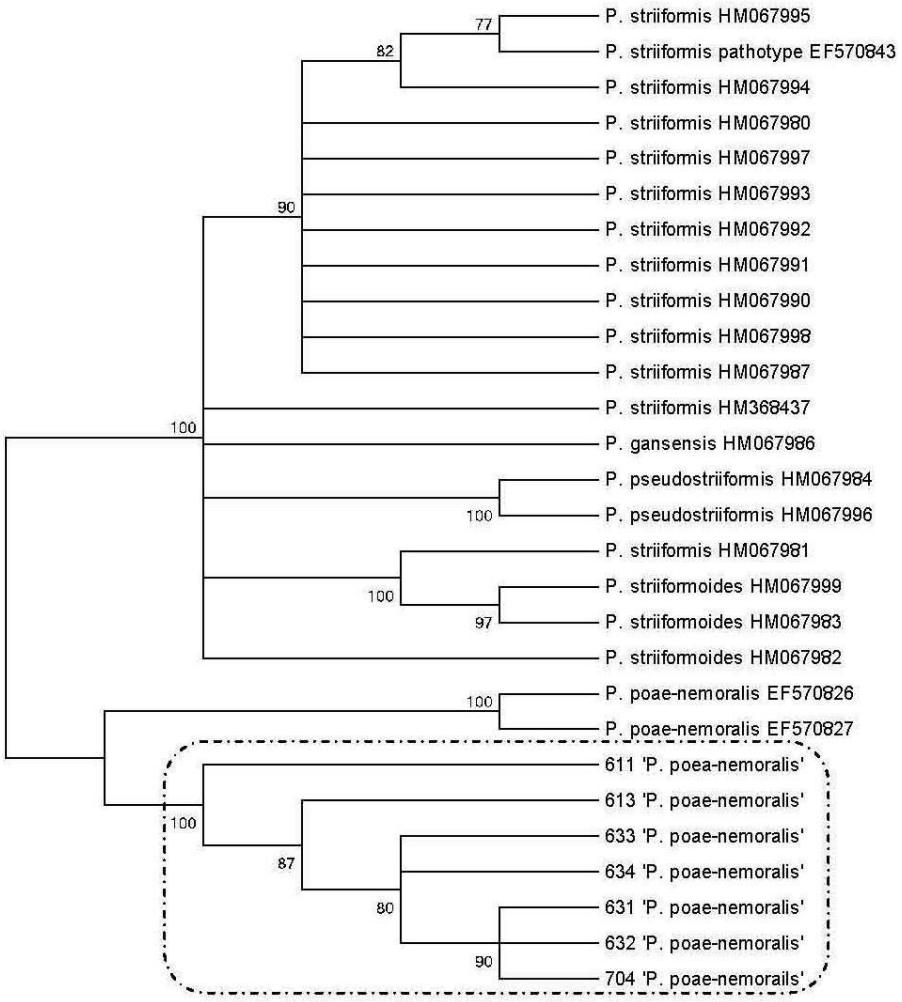


Figure 13. *Evolutionary relationships of taxa obtained using the β-tubulin gene and sequences downloaded from GenBank (details are presented in Appendix A). Dotted line is highlighting the area with sequences from this study.*

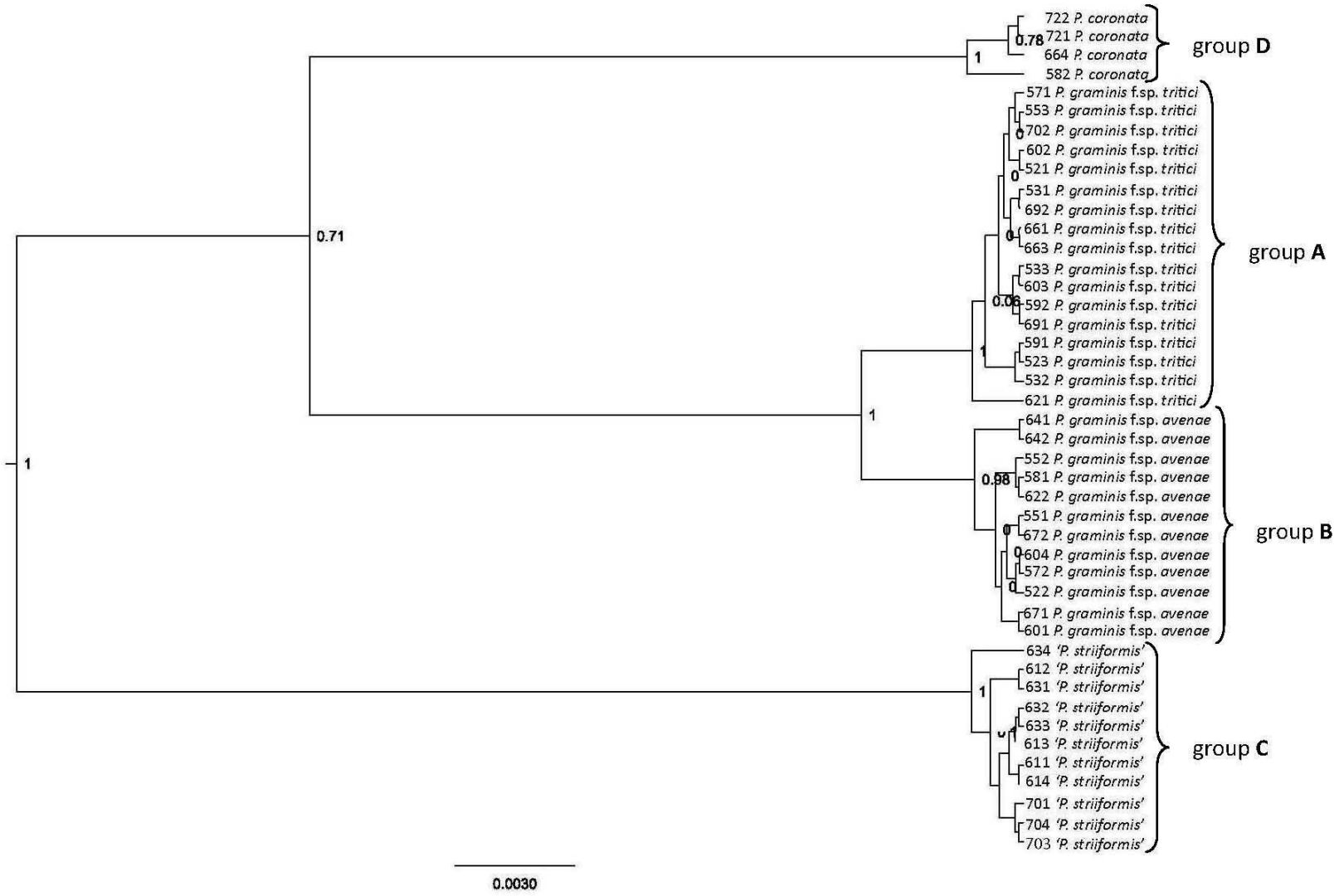


Figure 14. *Bayesian phylogeny for ITS region assessed by BEAST (using HKY substitution model). Numbers represent posterior probabilities.*

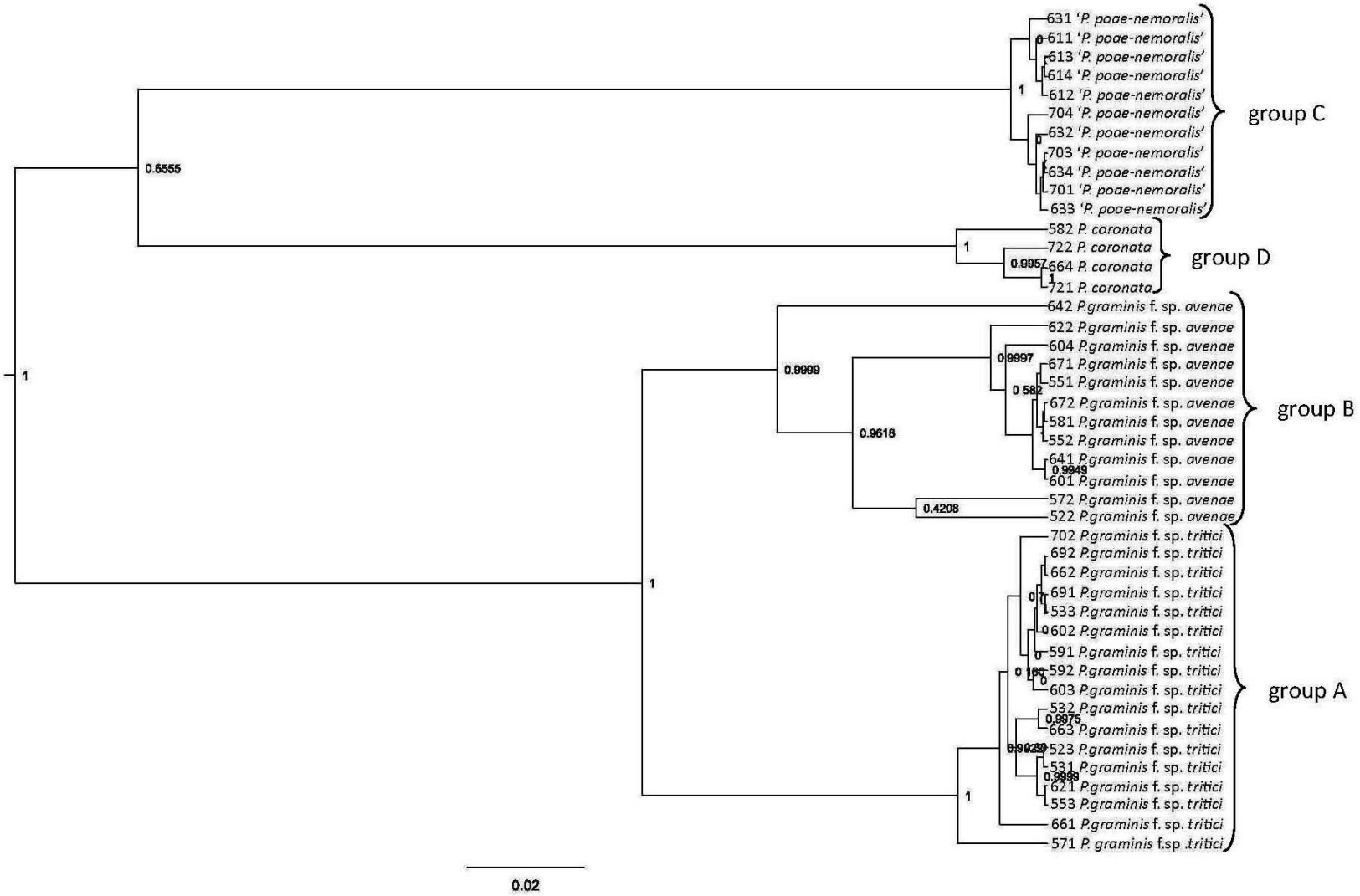


Figure 15. *Bayesian phylogeny for EF1-α gene assessed by BEAST (using HKY substitution model). Numbers represent posterior probabilities.*

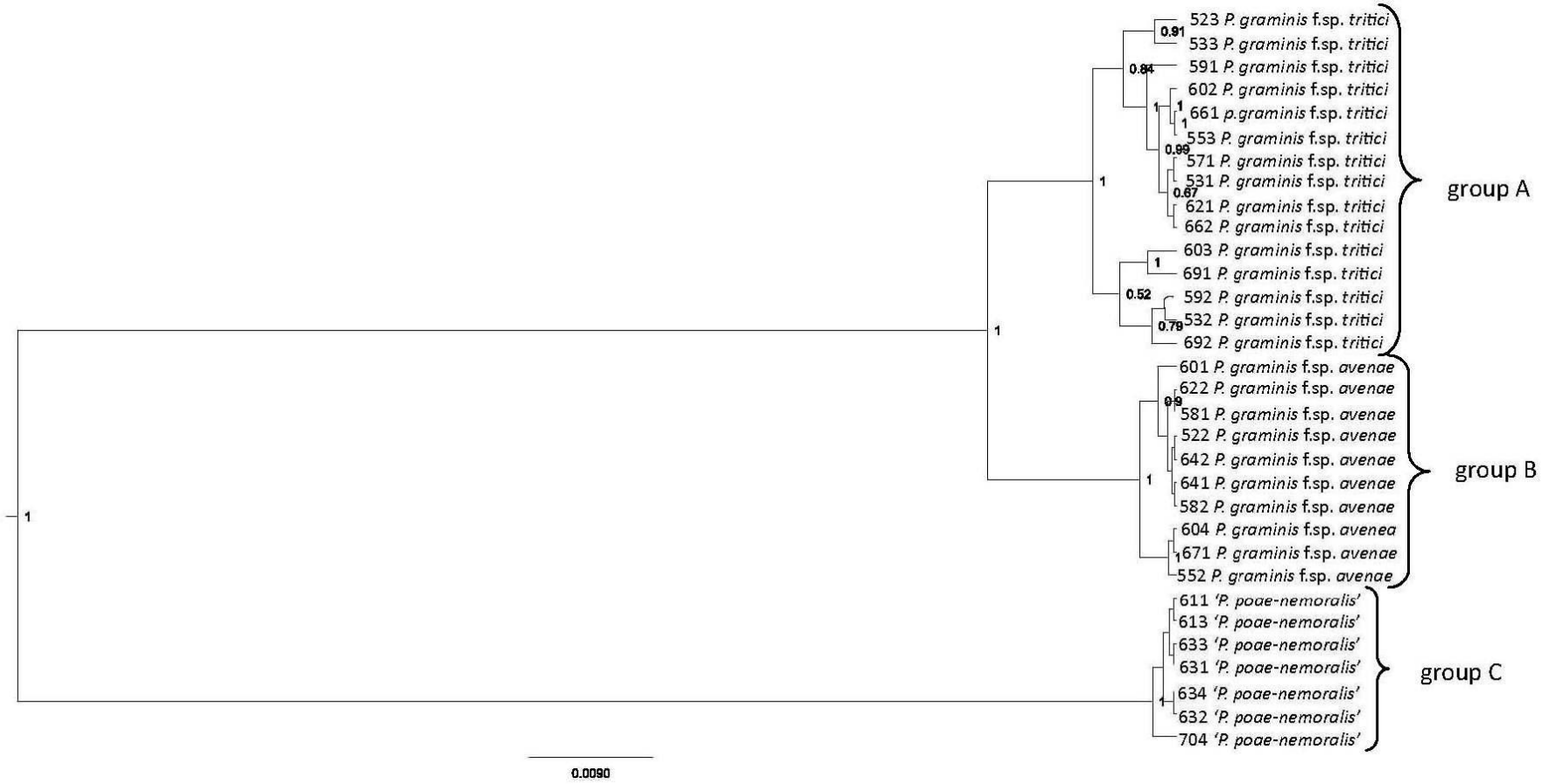


Figure 16. *Bayesian phylogeny for β-tubulin gene assessed by BEAST (using HKY substitution model). Numbers represent posterior probabilities.*

4 Discussion

4.1 Morphology

In this study combinations of morphological and molecular tools for identification of different rust fungi species were implemented. In general, these two techniques gave similar result: four species were distinguished using spore image analysis and five species were identified according to sequence data. This is well represented in tables 4 to 6, where correlation between spores size and the species identity based on DNA sequences obtained from three different genes could be observed. Therefore these two tools could be successfully used for species identification as a complement to each other. In the taxonomic study of *P. graminis* (Abbasi *et al.* 2005), the authors suggests that in species identification, only sequence data is not enough because morphological features can give much more information about species and their biology; at the same time they claim that the aecieal stage from *Berberis* spp. could not be used for rusts intraspecific classification, and only urediniospores are required for such morphological analysis (Abbasi *et al.* 2005).

Spore image analysis included measurements of spore width, length, pixels and projection area and indicated the presence of four different rust species. The difference in spore size was statistically significant (P-value < 0,0001) and could easily be observed on obtained spore images.

The approach in spore measurements utilized in this study has earlier been used by Anikster *et al.* (2005). Despite the fact that the same technique was used (using the best fitting ellipse for spore projection area measurement; measuring length and width as the larger and smaller axis of the ellipse respectively; random selection of an observation field under microscope and measurement at least 20 spores for each sample), the results obtained here differ from those received by Anikster *et al.* (2005). In their study, Anikster *et al.* (2005) measured all spore stages for five different species (*P. triticina*, *P. graminis*, *P. coronata*, *P. recondita*, *P. hordei* and *P. striiformis*); at the time this article was published aecial host for *P. striiformis* was not known, thus the aeciospores measurements are missing. That is why spore size of only two species could be compared (their *P. graminis* with ours *P. graminis* f.sp. *avenae* and *P. graminis* f.sp. *tritici* and their *P. coronata* with our *P. coronata*). According to these comparisons, we have received smaller spore's dimensions in all parameters. This could be explained by difference in used software and also by such factor as subjective view on the best fitting ellipse as well as spore boundaries.

Besides analysis of spore images, the description of aecia was also involved in the identification of the rust fungi species on the barberry plants. Aecium images of *P. graminis* f.sp. *avenae*, *P. graminis* f.sp. *tritici* and *P. striiformis/P. poae-nemoralis* were obtained and in accordance with Sato and Sato (1985) classification, all of them exhibit the morphological type *aecidium*. At the same time, figure 6 shows that aecia of different species has distinguishable features (shape, disposition to each other, location in the leaf tissue and peridium ends direction) which make it possible to use it as an additional tool in species identification. Moreover, according to Sato and Sato (1985), related genera usually have the same type of aecia thus it can be used in the natural classification and phylogenetic studies to reflect relationship within the *Uredinales* genera.

Additionally Leppik (1953) in the phylogenetic study of conifer rusts states that aecium is a valuable tool in phylogeny while aeciospores morphology and cell-wall structure have no special significance for phylogenetic distinction. In another taxonomical study of rust fungi on spruce (Crane, 2001), the author argue that in addition to aeciospore description certain morphological characteristics of aecia (the ornamentation of inner and outer peridial surfaces of the aecia, size and whether sori are confluent) may be a useful characters since it is difficult to identify *Chrysomyxa* to the species level. Cummins and Green (1966) describe morphology of urediospores, teliospores and aeciospores as well as morphological characteristics of uredia and telia while the description of aecia morphology is lacking in their review of the grass rust fungi that have *Berberis* and *Mahonia* spp. as an alternative host. Also in the morphological and taxonomical study of the *Puccinia* spp. on corn and sorghum (Pavgi, 1972) morphological distinctions of the pycnia and aecia at generic level were defined but assumed not to be much helpful in the species identification.

Summarizing all the above it is obvious that most taxonomic and phylogenetic studies involving aecia and aeciospores morphology were made on conifer rusts; there is a lack of studies on aecia on other rust species in general and on *Berberis* spp. in particular; also there is no common view on importance of morphological features of this structures in taxonomic and phylogenetic studies. This study contributes the knowledge about aecia and aeciospore morphology in different rust species, proves the possibility of rusts intraspecific classification not only on uredinial (Abbasi *et al.* 2005) but also on aecial stage, and shows how the information could be used as a good supplement to existing knowledge in this area.

4.2 DNA sequences analysis and species identification

Another part of this study focused on DNA sequences analysis and phylogenetic relationship between the species. Three different partial genes (ITS region, β-tubulin and EF1-α) were used. In general it was more difficult to obtain sequences of partial β-tubulin gene, even when different primers and primer combinations were used, still less sequences were obtained in the end. This tendency was also noticed in other studies of rust fungi. In the taxonomic study of *P. striiformis* Liu and Hambilton (2010) successfully amplified 48 sequences from ITS and 33 sequences from β-tubulin; in the study about evolutionary relationship among *Puccinia* spp. and *Uromycetes* (van der Merwe *et al.* 2007) 66 sequences from EF1-α and only 31 β-tubulin were amplified. The lengths of obtained sequences vary between different genes: the shortest sequences were obtained from EF1-α partial gene and the length of sequences from β-tubulin partial genes and ITS region had about the same length as previous studies.

All obtained sequences from the ITS region, and EF1-α and β-tubulin genes were compared with the BLAST database and the following rust species were identified: *P. graminis* f.sp. *avenae, P. graminis* f.sp. *tritici* and *P. coronata*. The sequence's matching (maximum identity) to those in the BLAST database was quite high and varied between 97 and 100% (two samples from EF1-α had 96% and one had only 90%). Moreover, morphology data perfectly fitted to this species identification.

Puccinia coronata was identified in four samples (582, 664, 721 and 722). The ITS sequences' matching to the BLAST database for all these samples was 99 %; the EF1-α sequences matched

little bit less (96 % for sample number 582, 98 % - for samples number 664 and 722, and 99 % - for sample 721). This identification was not confirmed by β-tubulin sequences because we failed to amplify samples number 664, 721 and 722 while amplified sequence from sample number 582 was identified as *P. graminis* f.sp. *avenae* (with 99 % of maximum identity). Despite this inconsistency the species name *P. coronata* was kept because of the strong support from sequences from ITS region and EF1-α gene, and also because of correspondence to the morphology data.

In total 12 samples (611-614, 631-364, 701, 703, 704) were identified differently. According to the sequences from ITS region they were identified as *Puccinia striiformis* and according to the sequences form β-tubulin and EF1-α genes – as *P. poae-nemoralis*. The most obvious explanation of this disagreement could be that the sequence's matching to BLAST database in general was rather low in quality for these sequences. First and foremost it concerns the EF1-α sequences since they had the lowest maximum identity that varied from 90 to 91 % (except 93 % for sample number 704). Another important factor was that all these sequences were identified based on only one reference sequences (Appendix B), which makes obtained species identification less reliable. This is followed by the ITS sequences that got 94 % of maximum identity (except 96 % for sample number 701 and 97 % - for sample number 703). In this case, there were more reference sequences available that were submitted by different authors (Zambino and Szabo 1993; Barnes and Szabo, 2007; Jin, Szabo and Carson, 2010; Liu and Hambleton, 2010) (Appendix A). Nevertheless all these reference sequences did not helped to identify these particular species and this will be discussed later in the text. On the other hand, all β-tubulin sequences have got quite high maximum identity (97 and 98 %) even though only two reference sequences submitted by one author (van der Merwe *et al.* 2008) were found in BLAST database (Appendix C). The spore image analysis indicated that all these samples are belonging to one species.

For the barberry plants species identification, the ITS region was sequenced for 18 samples and identified using the BLAST database. Unfortunately this did not allow us to identify exactly the barberry species. For each sample, three different *Berberis* spp. were found (*B. aetnensis, B. croatica* and *B. vulgaris*) and they had the same maximum score and maximum identity (it varied between the samples from 88 to 100 %). The probable explanation of this could be short effective length of the obtained sequences (it varied from 206 and 368 bp) as well as the lack of studies on barberry. According to the DNA sequences analysis and spore morphology *P. coronata* was identified (locations number 58, 66 and 72). That is why in this study the alternative host of *P. coronata* (*Rhamnus cathartica*) was also identified (at least for location 72). In the other two location *R. cathartica* was not identified since only one sample from each location was taken for DNA analysis, (in location 58 and 66 barberry and buckthorn plants were put in the same bag) and they happened to be *Berberis* spp.

The ITS region has been suggested as a possible plant barcode locus because of its broad utility across photosynthetic eukaryotes and fungi (with the exception of ferns) (Stoeckle, 2003) and currently it is the most commonly sequenced locus for plant molecular systematic investigations at the species level (Alvares and Wendel, 2003). Most phylogenetic studies that employed ITS have proved its high species-level discrimination and technical ease, and a large sequence data already exists for this region (Kress *et al.* 2003). Several other studies have also reported ITS as

one of the suitable markers for barcoding in plants (Kress *et al.* 2005, Edwards *et al.* 2008). But there are also other studies that indicate ITS' inherent difficulties, e.g. low PCR success (Chase *et al.* 2007, Kress *et al.* 2004), problem of secondary structure formation, resulting in poor quality sequence data (Desalle, 2007; Waugh 2007) and multiple copy numbers (Alvarez and Wendel 2003), etc. This also concerns *Berberis* spp. identification. Roy *et al.* (2010) in his study, the ITS region was amplified for all the tested species of *Ficus* and *Gossypium* but only one species of *Berberis*. Also according to Bottini *et al.* (2007), the taxonomy of *Berberis* is considered to be uncertain. This complexity in *Berberis* taxonomy has been attributed mainly to hybridization (Bottini *et al.* 1999; Bottini *et al.* 2007; Lubell *et al.* 2008a; Lubell *et al.* 2008b). Furthermore Kim *et al.* (2004) claims that divergence of the ITS sequence in *Berberis* is not high enough to provide a reliable relationship at the species level.

Considering obtained results and information mentioned above it is obvious that *Berberis* spp. identification could be difficult due to several reasons. Nevertheless in this study approximate identification of the *Berberis* spp. was assumed to be sufficient and no other attempts were made in this direction.

4.3 Phylogenetic analysis

4.3.1 Neighbor-joining analysis

Three different phylogenetic analysis were conducted in order to explore phylogenetic relationship of taxa obtained using sequences of the ITS region, EF1-α and β-tubulin genes. In the first analysis the neighbor-joining methods was used for tree construction. They share a common structure: different species in all trees have clustered apart and formed separate well-distinguishable groups.

In the phylogenetic tree of rusts taxa obtained from the ITS sequences, *P. graminis* f.sp. *tritici*, *P. graminis* f.sp. *avenae*, '*P. striiformis'* and *P. coronata* have clustered together with the high level of branch support. Clade 1 has 80 % of support and consists of three groups (A, C and D). In group A, almost all samples that were identified as *P. graminis* f.sp. *tritici* are phylogeneticaly very close to each other. This group has two sub-clades: A1 (with 79 % of support) and A2 (with 75 % of support) that separate samples number 521, 532, 602 and 692 from the others. In group D, all four samples (582, 664, 721 and 722) are separated from each other. Sample number 582 is clustered apart with the support of 100 %, sample 721 – with support of 79 % and samples 664 and 722 – with 91 %. This result probably could be explained by different evolutionary history of the sequenced *P. coronata* samples. Group C also belongs to Clade 1. Within this group, samples number 612, 701 and 703 are clustered separately but only sample 612 has strong support (81 %). Finally, group B forms a perfect clade with 100 % of support and it has no sub-clades.

The most interesting feature of this ITS tree is that *P. graminis* f.sp. *avenae* is thought to be more closely related to *P. graminis* f.sp. *tritici* than other obtained species, is actually placed distant from each other. At the same time, *P. graminis* f.sp. *tritici* could be found in one clade with *P. coronata* and '*P. striiformis'*. The distance of *P. graminis* f.sp. *avenae* and *P. graminis* f.sp. *tritici* samples could be explained by the following. Zambino and Szabo (1993) found that DNA

sequences of ITS region of some *formae specialis* of *Puccinia graminis* were identical (ff.spp. *avenae, dactylis, lolii* and *poae*) while f.sp. *avenae* and f.sp. *lolii* appeared to have different ITS sequences. In addition Anikster (1984) claims that some *formae specialis* are more closely related even though they belong to different host varieties; at the same time *formae specialis* that share the same variety appeared to be less related to each other. According to Savile (1984) *P. graminis* f.sp. *tritici* and P. *graminis* f.sp. *secalis* belong to two different varieties (var. *graminis* (on barley, rye, and oats) and var. *stakmanii* (predominantly on wheat, but also on rye, barley, and *Agropyron*) and crossing experiments have shown that they are more closely related to each other than to *P. graminis* f.sp. *avenae* (Anikster, 1984).

As it was mentioned earlier, the ITS region has been considered as one of the suitable regions for barcoding in plants (Kress *et al.* 2005, Edwards *et al.* 2008). In addition, the ITS region has become commonly used in fungi research as well (Peay *et al.* 2008), mainly because of the high number of copies and thus the ability to amplify from small and targeted DNA samples as well as its high variation between morphologically distinct fungal species (Gardes and Bruns, 1993).

The phylogenetic tree of rusts species acquired from EF1-α sequence represents *P. graminis* f.sp. *tritici*, *P. graminis* f.sp. *avenae*, '*P. poae-nemoralis'* and *P. coronata* clustered differently than in the ITS tree. The presence of three clades is the main distinction. Well-supported (99 %) Clade 1 represents only *P. graminis* f.sp. *tritici*. Within this clade, two samples (523 and 553) are clustered separately from the other *P. graminis* f.sp. *tritici*. The location of *P. graminis* f.sp. *avenae* is different compares with ITS tree (it is more close to *P. graminis* f.sp. *tritici*) but is still in individual clade with 100 % support. This clade has three sub-clades (it is also differ from ITS tree), which isolate samples number 522, 572, 622 and 642 from the others. Another distinction from the ITS tree is that Clade 3 consists of *P. coronata* and *'P. poae-nemoralis'* (group D and C respectively). The appearance of group D is slightly similar with the ITS tree (samples number 664 and 721 are clustered apart from the samples number 582 and 722). '*P. poae-nemoralis'* formed one well-supported clade except for the sample number 704, which is clustered separately with 100 % support. The ITS tree did not indicate the same pattern for this sample, but showed that samples from the same location (701 and 703) also are different from the others.

The EF-1a gene has been used for a variety of phylogenetic studies in fungi (Gentile *et al.* 2005; Kristensen *et al.* 2005). Also, the EF1-α gene that is characterized by the high discriminating power at the species level, and has been used as a genetic marker for phylogenetic studies for several *Fusarium* species for the accurate distinction of *formae specialis* (O'Donnell *et al.* 1998; O'Donnell, 2000). As a whole, it is assumed that EF1-α gene is suitable for studies of closely related species because of its highly variable introns (van der Merwe *et al.* 2007).

The last phylogenetic tree from the neighbor joining (NJ) analysis is obtained from β-tubulin sequences. It also has three cladeseach of which consists of one group. Clade 1 (88 % of support) contains *P. graminis* f.sp. *tritici* (group A). In this group, three sub-clades are present: one of which sets apart samples number 553 and 661, another separates samples 531 and the last one detaches samples number 523 and 533 from the rest of the *P. graminis* f.sp. *tritici*. In Clade 2 (branch support 99 %) only one sub-clade is present (that separates samples number 522 and 582). In the two previous trees, sample 582 was identified as *P. coronata*. The last clade represents group C (with the 100 % branch support) without any sub-clades. Sample number 704

do not clustered separately (as in the EF1-α tree) and samples number 701 and 703 were not amplified and thus cannot be compared with those in the ITS tree. It might be explained by mutation at one or both of the primer sites.

The β-tubulin tree has more common features with the EF1-α tree (compared with ITS tree) such as three clades, detached samples number 523 and 553 in the group A, as well as sample number 522 in group B, and close location of *P. graminis* f.sp. *avenae* and *P. graminis* f.sp. *tritici*.

The β-tubulin gene has been used successfully used in fungal phylogenetic analysis (Ayliffe *et al.* 2001; Craven *et al.* 2001; Begerow *et al.* 2004; Wirsel *et al.* 2004). β-tubulin sequence together with the ITS region were assume to be suitable for exploring relationships at the species level (White *et al.* 1990; Schardl *et al.* 1994) and also provided a good resolution of relationships within the *Puccinia* and *Uromyces* group (van der Merve *et al.* 2008). Moreover the β-tubulin gene has been successfully used in phylogenetic studies of rust fungi as a support for the EF1-α trees (van der Merve *et al*. 2007; van der Merve *et al.* 2008) as well as a support to the ITS trees (Liu & Hambelton, 2010).

By comparison of all phylogenetic trees mentioned above, some common features can be distinguished. At first, *P. graminis* f.sp. *avenae* forms one group within separate clades in all trees and (in case of ITS region) without any sub-clades. Secondly, for both EF1-α and β-tubulin genes, the phylogeny forms three well-supported clades, one of which consists only *P. graminis* f.sp. *tritici*; also for all relevant samples they both indicate *P. poae-nemoralis* instead of *P. striiformis* (as it stated by ITS). Finally *P. coronata* and *P. striiformis/P. poae-nemoralis* are found in one clade (in the ITS and EF1-α trees).

4.3.2 Neighbor-joining analysis comprising sequences downloaded from GenBank

In order to clarify the species of the sequenced *P. striiformis* or *P. poae-nemoralis*, new trees were constructed (using the same NJ method) in which sequences from taxonomic studies concerning this were included (Zambino and Szabo, 1993; Barnes and Szabo, 2007; van der Merwe *et al.* 2008; Jin, Szabo and Carson, 2010; Liu and Hambleton, 2010) (Appendix A to C).

The ITS tree contained *P. striiformis*, *P. striiformis* f.sp. *tritici*, *P. striiformis* f.sp. *hordei*, *P. striiformoides*, *P. pseudostriiformis*, *P. poae-nemoralis*, *P. gansesnsis*, *P. montanensis* and *P. brachypodii* f.sp. *poae-nemoralis*. Most of the sequences clustered separately from the reference sequences as in the previous ITS tree, samples number 612, 701 and 703 clustered separately. *P. striiformis* f.sp. *tritici* and *P. striiformis* f.sp. *hordei* have grouped separately as well as *P. striiformis*. *P. striiformoides* has also clustered separately but within this clade several sub-clades are formed. The other species (*P. montanensis*, *P. gansesnsis*, *P. pseudostriiformis* and *P. brachypodii*) have not formed any clusters and are located separately from each other. The most interesting thing in this tree is that sequences from this study were more closely located to *P. poae-nemoralis* and *P. brachypodii* f.sp. *poae-nemoralis* than to any other species, including the reference sequences of *P. striiformis*.

For the EF1-α tree, only one reference sequence of *P. poae-nemoralis* sequence was found in the GenBank database. According to this tree, all sequences are misidentified but one reference sequences probably is not enough to make the final conclusion about the species name.

The β-tubulin tree also did not help to solve the problem with the species identification because obtained sequences have formed well-supported clades and are isolated from the clade of reference sequences (such as *P. striiformis*, *P. pseudostriiformis*, *P. poae-nemoralis* and *P. striiformoides*). Concerning downloaded sequences, they also grouped separately with high branch support, and each clade consists of different species.

These three trees have some common features: obtained *P. striiformis* or *P. poae-nemoralis* sequences grouped separately from the reference sequences and formed well-supported clades (with 92 %, 99% and 100% of branch support for the ITS region, EF1-α and β-tubulin genes respectively). In the main tree, downloaded sequences of different species also have formed well-supported clades. Despite the fact that it is a great number of sequences available for the ITS region and β-tubulin gene in the GenBank database, still all controversial sequences (*P. striiformis/P. poae-nemoralis*) were not identified. There is also a lack of submitted EF1-α sequences.

In general, from 56 downloaded sequences only 5 have clustered close to the sequences obtained in this study. The main reason for this might be the difference in the sequences length. Some sequences were much shorter (in the β-tubulin alignment) but some were much longer (in the ITS alignment) than obtained sequences in this study. In the EF1-α alignment the sequence length was almost the same.

4.3.3 Bayesian phylogeny

The last three trees in this study were conducted by Bayesian MCMC method. The main purpose of this analysis was to confirm the trees obtained by the NJ method.

In general, all obtained trees have confirmed our previous result even though some differences exist. If compare Bayesian and NJ trees some common features could be observed: *P. graminis* f.sp. *tritici* (group A), *P. graminis* f.sp. *avenae* (group B) f *P. coronata* (group D), and '*P. striiformis'/'P. poae-nemoralis'* (group C) have clustered separately with posterior probability 1 (except 0,9999 for *P. graminis* f.sp. *avenae* in the EF-1α tree); in the *P. graminis* f.sp. *tritici* clade, samples 523 and 533, 532 and 633 have formed a well-supported clade (in the ITS and β-tubulin tree respectively); in the *P. graminis* f.sp. *avenae* clade, samples number 622 and 642 have formed sub-clade (in the EF-1α tree); within the *P. coronata* cluster samples number 664 and 721, 582 and 721 located separately from each other with posterior probability from 0,78 to 1 (in the EF-1α and ITS tree respectively).

There are also some differences in the Bayesian trees compared with the trees obtained by NJ analysis. At first, for all genes, only two main clades have been formed. Secondly, *P. graminis* f.sp. *tritici* and *P. graminis* f.sp. *avenae* samples are always present in the same clade. *P. coronata* and '*P. striiformis'/'P. poae-nemoralis'* are also found in one clade.

5 Conclusions

The results of this study indicate that morphology (spore measurement and aecia description) is an important tool in identification of rust fungi. Even with the employment of PCR technologies in taxonomic and phylogenetic studies on fungi in general and on rust fungi particular, morphological characteristics have not lost their significance because before any further investigation e.g. sequence analysis one should consider species morphology.

We also tried to identify rust species using molecular tools. For this purpose DNA sequences obtained from ITS region, EF1-α and β-tubulin partial genes were compared with the BLAST database. In our study, the BLAST database appeared to be very useful because it indicates if the obtained sequences correspond to the assumed species. With its help, the species identity problem was solved almost completely but not entirely. That is why the main conclusion that could be made from this part of the study is that one should be very careful when identifying species only according to BLAST database.

Even with the help of sequences obtained in other taxonomic research (Zambino and Szabo, 1993; Barnes and Szabo, 2007; van der Merwe *et al.* 2008; Jin, Szabo and Carson, 2010; Liu and Hambleton, 2010), we were not able to identify one species and answer the question whatever it is *P. striiformis* or *P. poae-nemoralis*. According to the ITS phylogenetic trees, this controversial samples were clustered close to *P. poae-nemoralis* and *P. brachypodii* f.sp. *poae-nemoralis* and therefore it is not *P. striiformis*; but two other trees showed that these samples clustered separately from the rest of the sequences which indicate that it is neither *P. poae-nemoralis* nor *P. striiformis*. This inconsistency might be explained by the differences in the sequence length as well as their quality. Future work to resolve this unclarity could be to investigate other spore stages such as teliospores or urediniospores and include more sequence data.

The last part of this study concerned identification of phylogenetic relationship between obtained species using different methods. They showed that all four species *P. graminis* f.sp. *tritici*, *P. graminis* f.sp. *avenae*, *P. coronata*, and '*P. striiformis'/'P. poae-nemoralis'* are phylogeneticaly distant from each other. The majority of the trees showed that *P. graminis* f.sp. *tritici* and *P. graminis* f.sp. *avenae* are more close to each other than to others. Also most trees showed that certain samples within one species clustered separately. These samples are 582, 664 and 721 in the *P. coronata* group; 522, 642 and 622 in the *P. graminis* f.sp. *avenae* group, and 523, 532, 533, 555, 663 and 661 in the *P. graminis* f.sp. *tritici* group.

To summarize, the combination morphological and molecular tools enables reliable identification of rust fungi. Those species that was found on barberry plants were more or less expected, but one species (or species complex) remained unidentified. DNA sequence analysis did not help to identify *Berberis* spp. Phylogenetic analysis using both neighbor-joining and Markov chain Monte Carlo methods gave in general the same result.

Acknowledgements

I would like to thank those who made this thesis possible.

I am grateful to the Swedish Institute (SI) for funding my Master's studies at SLU.

I want to thank my supervisor Prof. Jonathan Yuen for his advices, helpful discussions, help with the map, and reviewing the report. I want to say many thanks to my supervisor Anna Berlin for sample collection and nice images, for her help during my laboratory work, statistical analysis and report writing. I also want to thank Kerstin Dalman who helped me with phylogenetic analysis and for her guidance through MEGA program.

Last but not the least, I would like to thank my family for their support and to my husband Yevgeny for his care and understanding.

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Appendix A

Origin of reference sequences included in the figure 11, GenBank accession number, host plant and location of collection that have been used for construction of phylogenetic tree for taxa obtained using partial gene sequence of the coding regions of the ITS region. Sample 9130 was not deposited in GenBank

GenBank Accession number	**Fungus name**	**Host plant**	**Location (origin)**	**Deposited by**
M057109	*P. striiformoides*	*Dactylis glomerata*	USA	Liu and Hambleton, 2010
HM057110	*P. striiformoides*	*Dactylis glomerata*	Canada	Liu and Hambleton, 2010
HM057125	*P. striiformoides*	*Dactylis glomerata*	UK	Liu and Hambleton, 2010
HM057111	*P. striiformoides*	*Dactylis glomerata*	USA	Liu and Hambleton, 2010
HM057129	*P. striiformoides*	*Dactylis glomerata*	Chile	Liu and Hambleton, 2010
HM057137	*P. striiformoides*	*Dactylis glomerata*	China	Liu and Hambleton, 2010
HM057113	*P. pseudostriiformis*	*Poa pratensis*	Canada	Liu and Hambleton, 2010
HM057131	*P. pseudostriiformis*	*Poa pratensis*	Canada	Liu and Hambleton, 2010
HM057134	*P. pseudostriiformis*	*Poa nemoralis*	USA	Liu and Hambleton, 2010
HM057112	*P. striiformis*	*Triticum aestivum*	USA	Liu and Hambleton, 2010
HM057115	*P. gansensis*	*Achnatherum inebrians*	China	Liu and Hambleton, 2010
HM057127	*P. striiformis*	*Aegilops ligustica*	Turkey	Liu and Hambleton, 2010
HM057130	*P. striiformis*	*Elymus elymoides*	USA	Liu and Hambleton, 2010
HM057132	*P. striiformis*	*Triticum aestivum*	USA	Liu and Hambleton, 2010
HM057152	*P. poae-nemoralis*	*Poa pratensis*	Canada	Liu and Hambleton, 2010
HM057153	*P. poae-nemoralis*	*Arctagrostis latifolia*	Canada	Liu and Hambleton, 2010
HM057154	*P. poae-nemoralis*	*Poa annua*	New Zealand	Liu and Hambleton, 2010
HM057155	*P. poae-nemoralis*	*Koeleria litvinowii*	China	Liu and Hambleton, 2010
L08706	*P. montanensis*	*Berberis fendlery*	USA	Zambino and Szabo, 1993
L08730	*P. brachypodii* f.sp. *poae-nemoralis*	*Poa nemoralis*	Netherlands	Zambino and Szabo, 1993

GenBank Accession number	Fungus name	Host plant	Location (origin)	Deposited by
L08735	*P. striiformis* f.sp. *tritici*	*Triticum aestivum*	USA	Zambino and Szabo 1993
GQ457303	*P. brachypodii*			Jin, Szabo and Carson, 2010
GQ457306	*P. striiformis*			Jin, Szabo and Carson, 2010
GQ457307	*P. striiformis*			Jin, Szabo and Carson, 2010
GU382672	*P. striiformis* f.sp. *tritici*			Jin, Szabo and Carson, 2010
DQ417408	*P. striiformis* f.sp. *hordei*			Barnes and Szabo, 2007
DQ417407	*P. striiformis*			Barnes and Szabo, 2007
DQ417403	*P. striiformis*			Barnes and Szabo, 2007
DQ417402	*P. striiformis* f.sp. *hordei*			Barnes and Szabo, 2007
DQ417400	*P. striiformis* f.sp. *tritici*			Barnes and Szabo, 2007
DQ417397	*P. striiformis* f.sp. *tritici*			Barnes and Szabo, 2007
DQ417396	*P. striiformis* f.sp. *tritici*			Barnes and Szabo, 2007
DQ417397	*P. striiformis* f.sp. *tritici*			Barnes and Szabo, 2007
9130	*P. striiformis*	*Triticale,* variety *Dinaro*	Öland, Sweden	Collected by Anna Berlin in 2010

Appendix B

Origin of reference sequences included in the figure 12,, GenBank accession number, host plant and location of collection that have been used for construction of phylogenetic tree for taxa obtained using partial gene sequence of the coding regions of the EF1-α gene

GenBank Accession number	Fungus name	Host plant	Location (origin)	Deposited by
DQ925279	*P. poae-nemoralis*	*Anthoxanthum odoratum*	Europe	van der Merwe *et al.* 2008

Appendix C

Origin of reference sequences included in the figure 13,, GenBank accession number, host plant and location of collection that have been used for construction of phylogenetic tree for taxa obtained using partial gene sequence of the coding regions of the β-tubulin gene

GenBank Accession number	**Fungus name**	**Host plant**	**Location (origin)**	**Deposited by**
HM067991	*P. striiformis*	*Triticum aestivum*	China	Liu and Hambleton, 2010
HM067990	*P. striiformis*	*Triticum aestivum*	China	Liu and Hambleton, 2010
HM067986	*P. gansensis*	*Achnatherum inebrians*	China	Liu and Hambleton, 2010
HM067984	*P. pseudostriiformis*	*Poa pratensis*	Canada	Liu and Hambleton, 2010
HM067996	*P. pseudostriiformis*	*Poa pratensis*	Canada	Liu and Hambleton, 2010
HM067999	*P. striiformoides*	*Dactylis glomerata*	China	Liu and Hambleton, 2010
HM067982	*P. striiformoides*	*Dactylis glomerata*	Canada	Liu and Hambleton, 2010
HM067983	*P. striiformoides*	*Dactylis glomerata*	USA	Liu and Hambleton, 2010
HM067981	*P. striiformoides*	*Dactylis glomeratae*	USA	Liu and Hambleton, 2010
EF570843	*P. striiformis*	*Triticum aestivum*	Australia	van der Merwe *et al.* 2008
EF570826	*P. poae-nemoralis*	*Anthoxanthum odoratum*	Europe	van der Merwe *et al.* 2008
EF570827	*P. poae-nemoralis*	*Anthoxanthum odoratum*	Europe	van der Merwe *et al.* 2008