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FULL PAPER

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Taxonomy, phylogeny, and distribution of *Puccinia graminis*, the black stem rust: new insights based on rDNA sequence data

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Abstract *Puccinia graminis* (Uredinales) is an economically important and common host-alternating rust species on Berberidaceae/Poaceae (subfamilies Pooideae and Panicoideae) that has been spread globally by human activities from an unknown center of origin. To evaluate the taxonomic implications, phylogenetic relationships, and distribution/spread of this complex species, we sequenced and cladistically analyzed the ITS1, 5.8S, and ITS2 regions from herbarium specimens on various host plants from Iran (17), Europe (1), and North America (4). The ITS region plus the 5.8S gene ranged from 686 to 701 bp, including the flanking partial sequences of the 18S and 28S rDNA. Our phylogenetic analysis included 54 bp of the 18S sequence, the entire ITS1 + 5.8S + ITS2, and 58 bp of the 28S sequence. A second analysis used only the last 42 bp of ITS1, and all the 5.8S and ITS2, to incorporate data from additional sequences downloaded from GenBank. In addition to variation in sequence length, there was variation in sequence content. The analysis does not support classical morphology-based taxonomic concepts of the *P. graminis* complex. Also, host range, host taxonomy, and geographic origin provide minor information on taxonomic relationships. *Puccinia graminis* is most probably monophyletic. Coevolutionary aspects can hardly be discussed because of lack of sequence data from alternate host specimens. The occurrence of unrelated fungal taxa on the same host species suggests that, besides coevolution with the host, host jumps and hybridization may have played an important role in the evolution of *P. graminis*. From rDNA data we conclude that the pathogen was introduced to North America at least twice independently. For a new taxonomic concept, we think the complex has to be split into at least two species. New morphological features and further features other than sequence data, however, must be checked for taxonomic value first and, if necessary, be considered.

Key words Coevolution · Collective species · Species concept · Taxonomy

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Introduction

The black stem rust, *Puccinia graminis* Pers. (Uredinales), is a common heteroecious species with plant species of Berberidaceae as aecial hosts and members of the Poaceae as telial hosts. Cummins (1971) lists hosts of 77 genera of Poaceae (primarily in subfamily Pooideae but also a few in the Panicoideae) containing species that harbor *P. graminis*. In addition, more than 70 species of *Berberis* and some of *Mahonia* are listed as aecial hosts (Gäumann 1959; Cummins 1971; Anikster and Wahl 1979). *Puccinia graminis* is a complex species consisting of numerous biologically specialized *formae speciales*, and has been divided into infraspecific taxa that differ mainly in urediniospore length. Urban's (1967) morphological species concept is generally acknowledged as definitive. For example, Abbasi et al. (2002) classified specimens according to urediniospore length and also found differences in the number of germ pores. Urban further divides *P. graminis* into the two subspecies *P. graminis* subsp. *graminis* and *P. graminis* subsp. *graminicola* Z. Urb. The first subspecies infects mainly cultivated cereals and related hosts, whereas the latter occurs primarily on wild grasses. Urban also divides the type subspecies into two varieties, namely *P. graminis* subsp. *graminis* var. *graminis* (mainly on *Triticum*, *Aegilops*, *Elymus*) and *P. graminis* subsp. *graminis* var. *stakmannii* A.L. Guyot et al. (mainly on *Avena*, *Hordeum*, *Secale*). Because of overlapping sizes of urdiniospores and subsequent problems in delimiting the infraspecific taxa, recent

Table 1. Specimens of *Puccinia graminis* included in the sequence analysis

Reference number	Host	Locality	Length of the ITS region (bp)	Herbarium number	Infraspecific classification (Urban 1967; Abbasi et al. 2002)	GenBank accession number
3	*Berberis* sp.	Iran, Dizin	695	IRAN 11459F	nd	AY874145
5	*Triticum aestivum*	Iran, Shahrud	696	IRAN 9803F	gg	AY874142
11	*Elymus hispidus*	Iran, Alburz, Dizin	701	IRAN 11082F	gg	AY874148
12	*Elymus libanoticus*	Iran, Alburz, Dizin	700	IRAN 11083F	gs	AY874135
13	*Elymus elongatiformis*	Iran, Alburz, Dizin	695	IRAN 11084F	gs	AY874136
14	*Elymus elongatiformis*	Iran, Alburz, Khor	701	IRAN 11089F	gg	AY874134
20	*Triticum aestivum*	Iran, Shavur	697	IRAN 10839F	gg	AY874146
26	*Aegilops crassa*	Iran, Bakhtaran	701	IRAN 6646F	gg	AY874138
29	*Avena sativa*	Iran, Gorgan	695	IRAN 6832F	gg	AY874137
31	*Poa trivialis*	Iran, Javaherdeh	694	IRAN 11086F	gr	AY874147
33	*Taeniathrum crinitum*	Iran, Golestan National Park	696	IRAN 9261F	gs	AY874144
34	*Boissiera squarrosa*	Iran, Golestan National Park	695	IRAN 9260F	gs	AY874143
35	*Eremopyrum distans*	Iran, Golestan National Park	699	IRAN 9258F	gs	AY874140
39	*Hordeum spontaneum*	Iran, Golestan National Park	697	IRAN 11087F	gs	AY874133
40	*Leucopoa sclerophylla*	Iran, Mount Saluk	695	IRAN 10838F	gs	AY874141
96	*Bromus tomentosus*	Iran, Kelardasht	695	IRAN 11088F	gs	AY874139
105	*Avena ludoviciana*	Iran, Amarlu	686	IRAN 11453F	gs	AY874149
SC	*Secale cereale*	Germany, Mecklenburg-Vorpommern	701	PUR N1125	gs	AY874151
CDL	*Triticum aestivum*	USA	697	—	nd	AY114289
PGI	*Triticum aestivum*	USA, Arkansas	698	PUR 89472	gg	AY874153
PGII	*Triticum aestivum*	USA, Indiana	697	PUR	gg	AY874154
PGIII	*Poa pratensis*	USA, Indiana	694	PUR N1189	gr	AY874155

nd, not determined; ITS, internal transcribed spacer; gg, *P. graminis* subsp. *graminis* var. *graminis*; gs, *P. graminis* subsp. *graminis* var. *stakmanii*; gr, *P. graminis* subsp. *graminicola*

authors have considered additional characters and techniques such as germling morphology (Swertz 1994), isozyme banding patterns (Burdon and Marshall 1981; Swertz 1994), and DNA sequence data (Zambino and Szabo 1993). Those studies, however, included only a small part of the full range of genetic variation present within the species so were not sufficient to propose new taxonomic concepts.

Zambino and Szabo (1993) analyzed the internal transcribed spacer (ITS) region of the ribosomal DNA and concluded that the *P. graminis* is monophyletic, but that study was not comprehensive enough (only 13 specimens, mainly from the United States, were sequenced) to draw further conclusions on the phylogeny and (co-)evolution of this pathogen. According to most authors (Anikster and Wahl 1979; Leppik 1961, 1970; Savile and Urban 1982; Urban and Markova 1983, 1984), Asia or North Africa (Central Asia, the Middle East, Near East, Ethiopia, Azerbaijan, or Iran depending on the author) is the origin from which the pathogen has been introduced to other continents by man. This assumption is based mainly on the fact that these regions have floras with a high diversity of potential telial and aecial hosts (i.e., grasses in the Pooideae and members of the Berberidaceae, respectively). In the following, we provide a phylogenetic study based on rDNA sequence data of 28 specimens (6 of which were downloaded from GenBank) on 19 different host species to obtain more information about phylogeny, evolutionary aspects, and spread of *P. graminis*. Furthermore, it should help to provide better arguments for a future taxonomic revision of the complex species.

Materials and methods

The samples used for DNA sequencing were 17 herbarium specimens collected by M. Abbasi in Iran during the past 15 years (voucher specimens are deposited in IRAN and further duplicates in PUR), 1 specimen from Germany, and 4 from the USA (Table 1). DNA was purified from dried herbarium material by the extraction protocol of Taylor and Swann (1994) and by the grinding method. For the latter method, spores (teliospores, urediniospores, or aeciospores) were scraped from the herbarium specimens and suspended in 50 µl low ethylenediaminetetraacetic acid (EDTA) TE (0.089 M Tris base, 0.045 M boric acid, 0.05 µM EDTA) + 1% 2-mercaptoethanol buffer in 1.5-ml plastic tubes and ground with a mini-pestle mounted in an electric drill. The complete ITS region (3′-end of the 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and 5′-end of the 28S rRNA gene) of each specimen was amplified with primers ITS4 and ITS5 of White et al. (1990). Amplification was as described by Zambino and Szabo (1993) with the following cycling parameters: 40 cycles of 94°C for 30 s, 50°C for 1 min, 72°C for 2 min, and a final extension of 10 min at 72°C. The size and quantity of amplification products were

verified on 1% agarose gels. DNA bands of sufficient quantity and of the expected size were excised from the gels, and the DNA was purified with the GeneClean spin kit (BIO 101, Vista, CA, USA) according to the manufacturer's instructions. Purified products were quantified with a Hoefer DyNAQuant 2000 fluorometer (Hoefer, San Francisco, CA, USA) and cloned with the TA cloning kit (Invitrogen, Carlsbad, CA, USA). The presence of inserts was confirmed by digestion with *Eco*RI and agarose electrophoresis. Plasmid DNA was prepared with the Wizard miniprep kit (Promega, Madison, WI, USA), and DNA concentration was estimated with a fluorometer. DNA samples were prepared for sequencing with the Thermo-Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech) and sequenced on an ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech) as described by Goodwin and Zismann (2001). Each clone was sequenced in both directions with the M13 reverse and M13-40 primers. For the majority of specimens, more than one clone was sequenced to minimize errors caused by polymerase chain reaction (PCR) amplification.

DNA sequences were aligned with the profile mode of Clustal X 1.81 (Thompson et al. 1997) with default settings as described elsewhere (Goodwin et al. 2001), and were edited manually when necessary. Following alignment, genetic distances among all isolates were calculated and neighbor-joining trees were prepared with the Draw N-J Tree option of Clustal X. This option uses Kimura's two-parameter method for estimating evolutionary distances (Kimura 1980) and the neighbor-joining algorithm of Saitou and Nei (1987). Bootstrap analyses (1000 replications) were performed on the resulting trees with the Bootstrap N-J Tree option of Clustal X, and the final trees were visualized and printed with Njplot (Perrière and Gouy 1996).

Two analyses were performed. The first was on the complete ITS region of the 22 herbarium specimens listed in Table 1 plus single representatives of three related species as outgroups. In the second analysis, the ITS database was augmented with six sequences representing different *formae speciales* of *P. graminis* that were downloaded from GenBank (Table 2). Because sequences downloaded from GenBank included only the last 42 bases at the 3′-end of ITS1, the second analysis used only the alignable data common to all specimens (i.e., the last 42 bp of ITS1 plus the complete 5.8S and ITS2 sequences). For both analyses, sequences of *P. striiformis* (GenBank AY874152) and *P. recondita* (GenBankAY880845) from *Triticum aestivum* and of *P. hordei* (GenBankAY874150) from *Hordeum vulgare* were used as outgroups. All outgroup specimens were new sequences obtained as described above from herbarium material collected in Iran.

Morphological analysis of the specimens and their identification to subspecies and variety according to the criteria of Urban (1967) were published previously (Abbasi et al. 2002). Infraspecific classification could not be determined for sequences downloaded from GenBank because the original specimens were not available for examination. Infraspecific classification also could not be determined for the specimen from *Berberis* sp. because it included only the aecial state and, therefore, did not include the urediniospores required for morphological analysis (see Table 1).

Results

Nucleotide sequence analysis of the ITS region of *P. graminis* revealed a high level of molecular variation for both sequence length and content. The boundaries of the internal transcribed spacers ITS1 and ITS2 were determined by comparison with several published sequences in the rust fungi. The complete amplified region ranged from 686 to 701 bp, including flanking partial sequences of the 18S (54 bp) and 28S (58 bp) rDNA. Therefore, the length of the ITS region itself ranged from 574 to 589 bp. As expected, sequences of the 5.8S gene were highly conserved among the 22 specimens, whereas those for ITS1 and ITS2 exhibited polymorphisms due to base substitutions, insertions, or deletions of up to 36 nucleotides. Both ITS1 and ITS2 contained phylogenetically informative sites. Two informative sites were also found near the 5′-end of the 18S sequence.

Neighbor-joining analyses of the entire ITS1+5.8S+ITS2 region of the 22 specimens plus the three outgroup species revealed that *P. graminis* as a whole is monophyletic (Fig. 1). However, the species was separated clearly into three clades, each with bootstrap support of 96% or higher. Clade 1 contained specimens from a wide array of wild hosts that were collected only in Iran (Fig. 1). This clade also contained specimens from cultivated hosts (oats and wheat) as well as the aecial-stage sample from *Berberis* sp., confirming that *Berberis* is an alternate host for members of this clade. Specimen 26 from *Aegilops crassa* was clearly distinct, but

Table 2. DNA sequences[a] of *Puccinia graminis* downloaded from GenBank

GenBank accession no.	Host	Country, state	*formae speciales*
L08696	*Avena sativa*	USA	f. sp. *avenae*
L08698	*Dactylis glomerata*	USA, Minnesota	f. sp. *dactylidis*
L08699	*Lolium perenne*	USA, Minnesota	f. sp. *lolii*
L08701	*Poa pratensis*	USA, Minnesota	f. sp. *poae*
L08703	*Secale cereale*	USA, New York	f. sp. *secalis*
L08708	*Triticum aestivum*	USA, Kansas	f. sp. *tritici*

[a] Includes the last 42 bp of ITS1 and all the 5.85 and ITS2 region

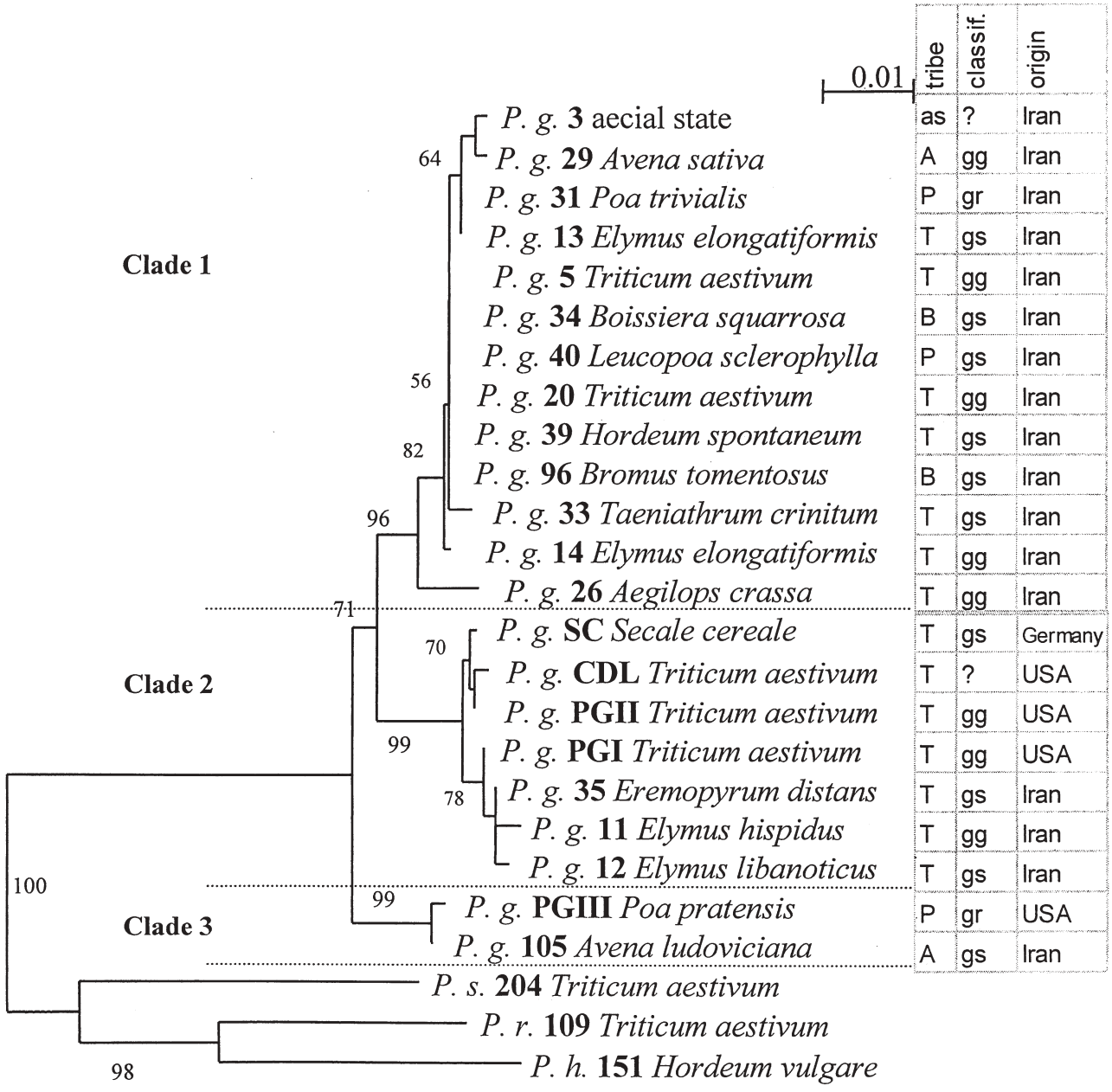


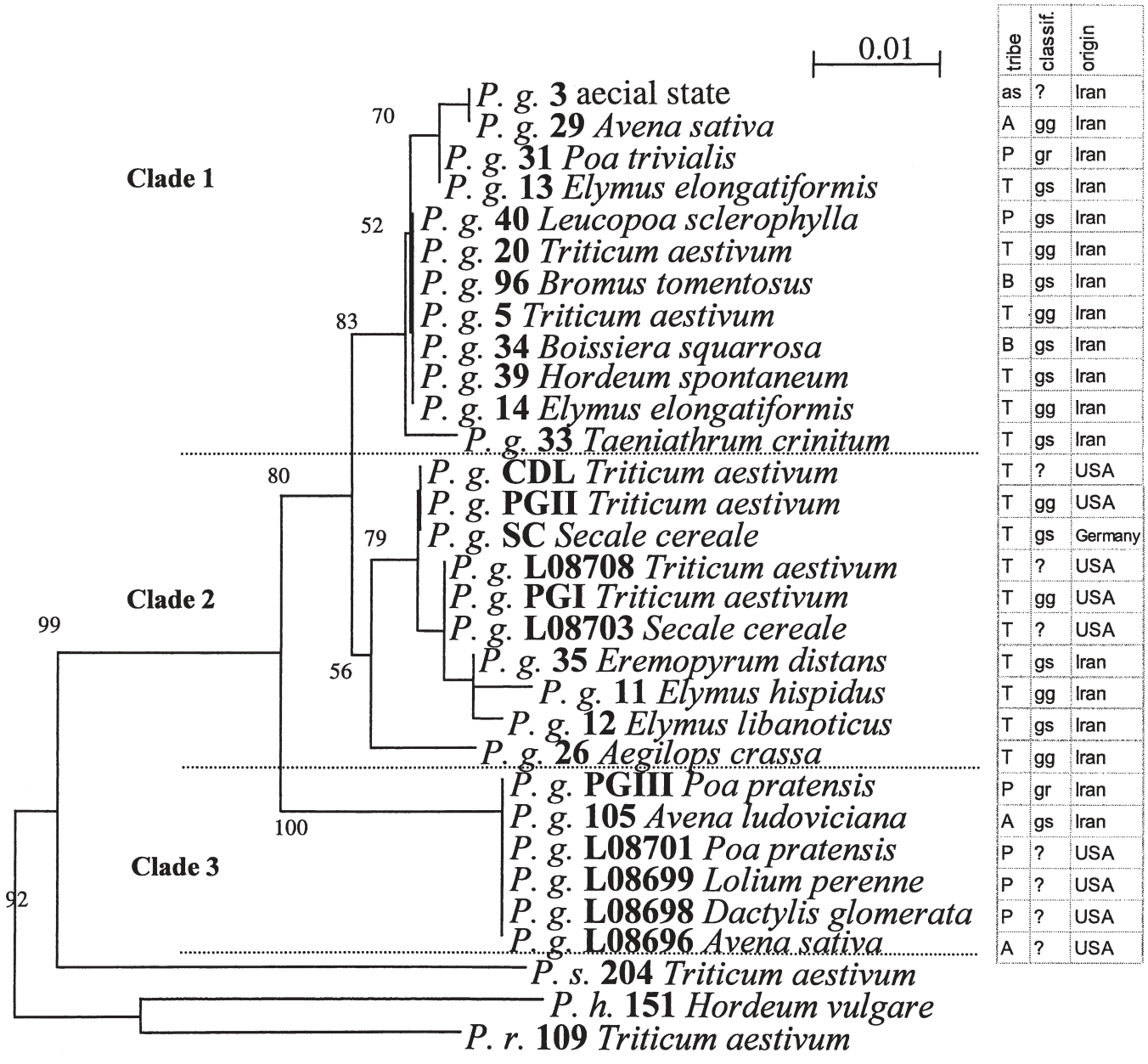
Fig. 1. Phylogram from neighbor-joining analysis of *Puccinia graminis* DNA data including some cereal rusts as an outgroup. The topology and bootstrap analysis were based on the entire internal transcribed spacer (ITS)1, 5.8, and ITS2 regions. *P. g.*, *Puccinia graminis*; *P. s.*, *Puccinia striiformis*; *P. h.*, *Puccinia hordei*; *P. r.*, *Puccinia recondita*; *gg*, *P. graminis* subsp. *graminis* var. *graminis*; *gs*, *P. graminis* subsp. *graminis* var. *stakmanii*; *gr*, *P. graminis* subsp. *graminicola*; *A*, Avenae; *B*, Bromeae; *P*, Poeae; *T*, Triticeae

also clustered with the other members of clade 1 in this analysis (Fig. 1).

Clade 2 contained specimens from rye and wheat from Germany and the USA, respectively, plus specimens from *Eremopyrum distans* and two species of *Elymus* from Iran (Fig. 1). Thus, clade 2 contained specimens from both cultivated and wild hosts spanning three continents. All the hosts infected by members of this clade were in the tribe Triticeae.

Clade 3 contained two specimens, one from wild *Avena* in Iran and the other from *Poa pratensis* in the USA (Fig. 1). Therefore, members of this clade also occurred on at least two continents.

The same three clades were obtained when the analysis was augmented with sequences from six *formae speciales* obtained from the USA (Fig. 2). Two of the additional specimens (*formae speciales secalis* and *tritici*) clustered within clade 2, while the remaining four specimens clustered within clade 3. Clade 1 remained composed solely of specimens from Iran that were collected from four host tribes. Bootstrap values for each clade in the second analysis remained high and ranged from 79% for clade 2 to 100%



tribe	classif.	origin
as	?	Iran
A	gg	Iran
P	gr	Iran
T	gs	Iran
P	gs	Iran
T	gg	Iran
B	gs	Iran
T	gg	Iran
B	gs	Iran
T	gs	Iran
T	gg	Iran
T	gs	Iran
T	?	USA
T	gg	USA
T	gs	Germany
T	?	USA
T	gg	USA
T	?	USA
T	gs	Iran
T	gg	Iran
T	gs	Iran
T	gg	Iran
P	gr	Iran
A	gs	Iran
P	?	USA
P	?	USA
P	?	USA
A	?	USA

Fig. 2. Phylogram from neighbor-joining analysis of *Puccinia graminis* DNA data including some cereal rusts as an outgroup. The topology and bootstrap analysis were based on the last 42 bp of the ITS1 plus the entire 5.8S and ITS2 regions. *P. g.*, *Puccinia graminis*; *P. s.*, *Puccinia striiformis*; *P. h.*, *Puccinia hordei*; *P. r.*, *Puccinia recondita*; *gg*, *P. graminis* subsp. *graminis* var. *graminis*; *gs*, *P. graminis* subsp. *graminis* var. *stakmanii*; *gr*, *P. graminis* subsp. *graminicola*; *A*, Avenae; *B*, Bromeae; *P*, Poeae; *T*, Triticeae

for clade 3. The slightly lower bootstrap values for clades 1 and 2 compared to those in Fig. 1 probably occurred because polymorphic sites at the 3′-end of the 18S gene and the first half of ITS1 were excluded from the second analysis. Tree topology was almost identical between the two analyses except for the position of specimen number 26, which clustered as a sister to clade 1 in the first analysis but as a sister to clade 2 in the second analysis.

There was no correlation between the three clades identified by the ITS sequences and morphology-based infraspecific taxa, as all three infraspecific taxa were distributed widely among the three clades. For example, clade 1 contained representatives of all three infraspecific taxa, clade 2 contained *P. graminis* subsp. *graminis* varieties *graminis* and *stakmanii*, and clade 3 contained *P. graminis* subsp. *graminis* and *P. graminis* subsp. *graminicola*.

Discussion

Phylogeny

This analysis of diverse specimens from three continents confirms the conclusion of Zambino and Szabo (1993) that

P. graminis is monophyletic. A high bootstrap value (100%) separates *P. graminis* clades from the three species of grass rusts used as outgroups. The results also confirmed the utility of herbarium specimens for phylogenetic analyses of rust fungi. The complete ITS region was amplified without difficulty from specimens up to 15 years old, and analysis of older specimens may be possible if the region is amplified in smaller, overlapping segments. This method could greatly expand the range of specimens available for analysis to include the type specimens on which the morphological classifications were based.

Although *P. graminis* as a whole is monophyletic, it clearly is a compound species with high genetic variability. There are three different clades supported by high bootstrap values. Differences between clades 1 and 2 specimens on the one side and clade 3 on the other are particularly high. So, considering clade 1 and 2 as a single clade would be reasonable as well. The analyses provides some interesting information on the phylogeny of the complex species that is not typical for obligate plant parasitic fungi. Host range and subspecific classification seem not to provide very much phylogenetically relevant information. In contrast to many other obligate fungal plant parasites (see Scholler 1998), related host plants within the *P. graminis* complex do not indicate related fungi and vice versa. *Triticum aestivum* (clades 1, 2) and *Avena sativa* (1, 3) are even represented in two different clades (Figs. 1, 2). This result indicates that there was no continuous coevolution and permanent binding to certain host plants; we assume that hybridization (as suggested by Johnson 1949; Johnson et al. 1932; Green 1971) and jumps to different hosts have played a major role in the evolution of the fungus. Host jumps may have taken place from a telial host to new telial hosts via urediniospores, from telial hosts to aecial hosts via basidiospores, or from an aecial host to a new telial host via aeciospores. The role of the aecial host in the phylogeny of *P. graminis* remains unclear, as it is in other heteroecious rust fungi. On one hand, *P. graminis* is facultatively heteroecious (McAlpine 1906: 121), i.e., the fungus does not need the aecial host for reproduction and survival, but on the other hand, no sexual recombination is possible without aecia and the aecial host. This finding indicates that new hosts may have been "conquered" via aeciospores and that the *Berberis* may have played an important role in phylogenetic history of the rust. Consequently, more specimens on the aecial host need to be studied. Furthermore, it would be helpful to know more about the host range of a certain strain before sequencing it. We do not know the host range of all the strains from Iran, except for the host plant species on which we found it. As already mentioned, most of the host plants of *P. graminis* belong to subfamily Pooideae, including all material we evaluated for this study. From the phylogenetic point of view, it would be interesting to sequence *P. graminis* of species on Panicoideae (e.g., on *Echinochloa* spp. or *Setaria* spp.; Cummins 1971) to answer the question whether *P. graminis* is polyphyletic (on at least two different subfamilies) or monophyletic, switching from Pooideae to Panicoideae or vice versa.

The subspecific taxa delimited mainly using morphological data as proposed by Urban (1967) and Abbasi et al. (2002) are not natural groups and, consequently, do not reflect the phylogeny of the rust. Although information on the subspecific placement of eight specimens was not available, Figs. 1 and 2 provide sufficient evidence for a polyphyletic origin (all three taxa occur in at least two different clades). Later, we discuss this again from the taxonomic point of view.

Distribution and spread

We evaluated 28 specimens from three different countries and continents, namely from Asia/Iran (17), from Europe/Germany (1), and from North America/USA (9). As noted earlier, *P. graminis* is introduced in Germany and the United States but is a native of Iran. In Figs. 1 and 2, Iranian specimens represent all specimens in clade 1 and are represented in clade 2 and 3 as well. USA specimens are in clade 2 and 3 and the European (German) specimen clusters in clade 2. As mentioned, *P. graminis* is a species of Asiatic origin and was introduced to other countries (Leppik 1961; Anikster and Wahl 1979). The question whether Iran is the center or one of the centers of origin for the black stem rust (as it assumed for wheat and related species; see Vavilov 1992) cannot be answered yet. Variability of the Iranian specimens is high, but we have no comparative data from regions/countries where *P. graminis* is native as well. Generally, genetic diversity of a spreading species is always highest in its geographic origin. In addition, the higher genetic diversity in Iranian specimens may also be a consequence of the number of species and of species on different host plants studied. The non-Iranian specimens were all from cereals or forage grasses. Definitely, *P. graminis* must have been introduced to North America at least twice independently because in this continent we found genetically strongly different clade 2 and clade 3 specimens.

Taxonomy

Our study shows that *P. graminis* is a complex species. Certain morphological characters proposed for subspecific classification (Urban 1967; Abbasi et al. 2002) do not represent natural groups and turned out to be polyphyletic. Interestingly, host range or *forma speciales* provide no taxonomic information, either. Therefore, a new taxonomic concept is urgently required. There are, however, two major questions and problems involved with a new concept (including new or unknown scientific names) of such an important pathogen.

The first question is whether the complex should be reclassified just by using sequence data. In our opinion, it should not. In general we think that especially morphological and ontogenetical data should be used to characterize a taxon, because these features tell us much more about a species and its biology than a variable sequence of the rDNA-ITS region as a small and nonfunctional part of the genome. Particularly, in *P. graminis*, the "molecular

way" only would be hardly accepted because many nontaxonomists and nonmicrobiologists, such as plant pathologists, extension people, growers, farmers, etc., who permanently deal with this fungus and who need a classification based on features that can be traced within a short time and without major technical effort. Therefore, taxonomists should invest some time and resume looking for additional morphological or ontogenetical features starting with those specimens used for sequencing. Other simply determinable and taxonomically valuable features such as the production of taxon-specific chemicals (e.g., for carotene type and contents in rust fungi; see Zwetko and Pfeifhofer 1991) also could be tested for the *P. graminis* complex. Second, into how many species (or subspecies) should the complex be split? This question cannot be answered yet. As mentioned, we cannot base a classification on sequence data only and urgently need further non-molecular data. Based on the present rDNA sequence data available, it seems reasonable to split *P. graminis* into at least two species, the first species consisting of taxa belonging to clade 1 and 2 and the second species belonging to clade 3. However, even the sequence data are still not sufficient to carry out major taxonomic changes. Especially, specimens on Panicoideae and type specimens should be integrated in the study.

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