# PCR Detection of *Pseudoperonospora humuli* and *Podosphaera macularis* in *Humulus lupulus*

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**Abstract**

Patzak J. (2005): **PCR detection of** *Pseudoperonospora humuli* **and** *Podosphaera macularis* **in** *Humulus lupulus*. Plant Protect. Sci., **41**: 141–149.

Hop downy mildew (*Pseudoperonospora humuli*) and hop powdery mildew (*Podosphaera macularis*) are the most important pathogens of hop (*Humulus lupulus*). The early detection and identification of these pathogens are often made difficult by symptomless or combined infection with another pathogens. Molecular analysis of internal transcribed spacer (ITS) regions of rDNA is a novel and very effective method of species determination. Therefore, specific PCR assays were developed to detect the pathogens *Pseudoperonospora humuli* and *Podosphaera macularis* in naturally infected hop plants. The specific PCR primer combinations P1 + P2 and S1 + S2 amplified specific fragments from *Pseudoperonospora humuli* and *Podosphaera macularis*, respectively, and did not cross-react with hop DNA nor with DNA from other fungi. PCR primer combinations R1 + R2 and R3 + R4 could be used in multiplex PCR detection of *Pseudoperonospora humuli*, *Podosphaera macularis*, *Verticillium albo-atrum* and *Fusarium sambucinum*. Phylogenetic relationships were inferred for 42 species of the *Erysiphales* from nuclear rDNA (ITS1, 5.8S, ITS2). The molecular characterisation and phylogenetic analyses confirmed the species identification of hop powdery mildew. The PCR assays used in this study proved to be accurate and sensitive for detection, identification, classification and disease-monitoring of the major hop pathogens.

**Keywords**: hop powdery mildew; hop downy mildew; internal transcribed spacers (ITS); PCR detection; phylogenetic analysis

Hop (*Humulus lupulus* L.) is a dioecious, perennial, climbing plant and only female individuals are cultivated. Female inflorescences, referred to as cones, are commercially used in brewing, and the pharmaceutical and cosmetics industry. Unfortunately, the plants are regularly subjected to infection by oomycetal and fungal pathogens. The infection sometimes results in severe loss of production as the plants wither (Neve 1991).

*Pseudoperonospora humuli* (Miabe and Takah.) Wilson is the causal agent of hop downy mildew, a disease occurring worldwide. The pathogen infects young shoots, leaves, flowers and cones, causing "basal spikes", angular black leaf spots and brown discolouration of cones (Neve 1991). Oospores are formed in infected shoots and cones (Chee & Klein 1998). Although hop downy mildew has not been extensively studied, the fine structure of the sporangium was described by Constantinescu (2000).

*Podosphaera macularis* (Braun) syn. *Sphaerotheca macularis* (Wallr.:Fr) Lind. (synonym *S. humuli*

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 1486434701.

(DC.) Burrill) is the causal agent of hop powdery mildew, which has become a serious problem in almost all hop growing regions (Seigner *et al*. 2002). This pathogen infects leaves and cones, causing humps, blisters, pale spots, distortion of cones and white mildew colonies. Conidia grow up from a surface mycelium (Neve 1991). The pathogen has not been extensively studied.

Early detection and identification of pathogens are crucial for the implementation of efficient control strategies. Disease symptoms are rarely discernible on growing crops before mass infection occurs and they can sometimes be confused with those of another species. Therefore, rapid, specific and sensitive methods for the detection of all important pathogens are required. Classical identification methods, based on morphological or physiological characters, are time-consuming, labour intensive and require considerable knowledge of the genera involved (Grote *et al*. 2002). Molecular DNA techniques represent a novel and highly effective means of species determination, based mainly on the comparison of internal transcribed spacers (ITS) regions of rDNA (Cooke *et al*. 2000). Molecular data as a whole have been used extensively to reconstruct phylogenetic relationships among downy and powdery mildew fungi (Saenz & Taylor 1999; Voglmayr 2003).

In this paper, we report the development of specific PCR primers for the detection of *Pseudoperonospora humuli* and *Podosphaera macularis* in naturally infected hop plants using molecular sequencing of ribosomal ITS regions.

## MATERIALS AND METHODS

***Sample sources and DNA extraction***. Hop downy mildew (*Pseudoperonospora humuli*) and hop powdery mildew (*Podosphaera macularis*) were obtained as mycelia from infected hop (*Humulus lupulus*) plants in the experimental hopgarden of the Hop Research Institute Co. Ltd., Žatec, Czech Republic. Other species of fungi were collected on diseased hop plants and isolated by successive plating on potato dextrose agar (Sigma-Aldrich, U.S.A.). Individual isolates were separated and determined and grown 5 d at 23°C. The determination of species was confirmed by Prof. Ewa Solarska from the Institute of Soil Science and Plant Cultivation, Lublin, Poland. A *Verticillium albo-atrum* isolate from hop was obtained from Dr. S. Radišek, Institute of Hop Research and Brewing, Žalec, Slovenia.

DNA was isolated from pure cultures of fungal isolates, mycelia and infected plant materials with DNeasy Plant Mini kit (Qiagen, FRG), and from infected plants in field conditions with REDExtract N-Amp Plant PCR Kit (Sigma-Aldrich, USA), both according to manufacturer's instructions. Samples were collected between 2001 and 2004.

***PCR and sequencing***. The ITS regions of the nuclear rDNA, from 18S, 5.8S and 26S, were amplified using primers R1 (GTGAACCWGCGGARGGATCATT) derived from the conserved region of 16S rDNA, R2 (TTYGCTRCGTTCTTCATCGATG), R3 (GCATCGATGAAGAACGYAGCRA) derived from the conserved region of 5.8S rDNA and R4 (TATCTTAARTTCAGCGGGT) derived from conserved region of 26S r DNA. Taq PCR master mix kit (Qiagen, FRG) and REDExtract N-Amp Plant PCR Kit (Sigma-Aldrich, U.S.A.) were used for PCR reactions according to manufacturer's instructions. PCR amplifications were carried out in a Genius thermocycler (Techne, UK). After denaturation step 95°C 3 min, 35 cycles of amplification (94°C 30 s, 54°C 60 s, 72°C 90 s) and 10 min at 72°C were performed. A minimum of two amplifications was always performed in order to check consistency. Aliquots of 10 µl of PCR products were analysed by electrophoresis on horizontal 2% agarose gel, stained by ethidium bromide and visualised under UV light. PCR products were then excised from the gel, isolated with QIAquick Gel Extraction kit (Qiagen, FRG) and prepared for DNA sequencing. Both DNA strands were sequenced in the Laboratory of Plant Molecular Physiology, Masaryk University, Brno, Czech Republic, using ABI Prism 310 DNA sequencer (Applera Corporation, Applied Biosystems, U.S.A.) in 2001.

The specific rDNA of *Pseudoperonospora humuli* were amplified using the primers P1 (CTGAGGGGACGAAAGGCTCTG) derived from ITS1 and P2 (CTGGTCACATGGACAGCCTTCA) derived from ITS2. The specific rDNA of *Podosphaera macularis* were amplified using the primers S1 (CCCGAACTCATGTAGTTAGTGC) derived from ITS1 and S2 (GAGCACATCGGTACCGCCACTA) derived from ITS2. The PCR protocol was the same as described above. The experiments were completed during 3 years for determination of specificity, consistency and monitoring ability.

***Data analysis***. The sequence alignment of conserved rDNA regions of *Humulus lupulus*, *Peronospora* sp., *Podosphaera* sp., *Verticillium* sp. and *Fusarium* sp. was initially produced by MegAlign

Table 1. List of GenBank accession numbers for taxa studied in phylogenetic analyses

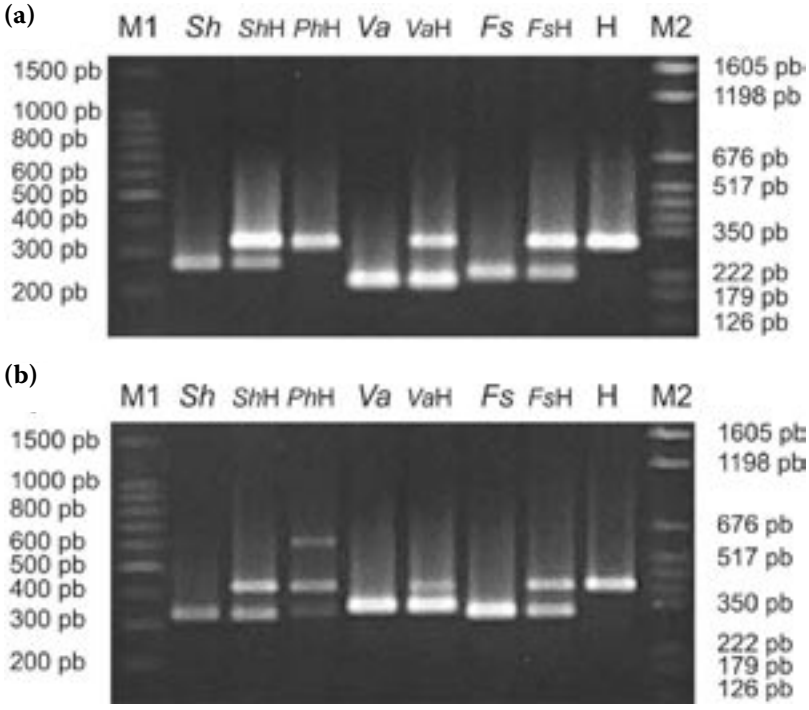
| Taxon | Source of sequence | Accession number |
|---|---|---|
| *Cystotheca lanestris* | Takamatsu *et al.* (2000) | AB000933 |
| *Cystotheca wrightii* | Takamatsu *et al.* (2000) | AB000932 |
| *Erysiphe convolvuli* | Cunnington *et al.* (2000) only in database | AF154327 |
| *Erysiphe glycines* | Takamatsu *et al.* (2002) only in database | AB078807 |
| *Erysiphe gracilis* | Mori *et al.* (2000) | AB022358 |
| *Erysiphe graminis* | Hirata and Takamatsu (1996) | D84379 |
| *Erysiphe pisi* | Cunnington *et al.* (1999) only in database | AF073348 |
| *Erysiphe prunastri* | Grigaliunaite and Takamatsu (2001) only in database | AB046984 |
| *Erysiphe tritici* | Mori *et al.* (2000) | AB022377 |
| *Microsphaera trifolii* | Cunnington *et al.* (2001) only in database | AF298542 |
| *Microsphaera alphitoides* | Rolland *et al.* (2002) only in database | AJ309201 |
| *Microsphaera juglandis* | Takamatsu *et al.* (1999) | AB015928 |
| *Microsphaera platani* | Cunnington *et al.* (1999) only in database | AF073349 |
| *Microsphaera sparsa* | Cunnington *et al.* (2001) only in database | AF298541 |
| *Oidium longipes* | Kiss *et al.* (2001) | AF250777 |
| *Oidium lycopersici* | Kiss et al. (2001) | AF229021 |
| *Podosphaera aphanis* | Cunnington *et al.* (1999) only in database | AF073355 |
| *Podosphaera balsaminae* | Hirata *et al.* (2000) | AB040344 |
| *Podosphaera cercidiphylli* | Takamatsu *et al.* (2000) | AB026140 |
| *Podosphaera clandestina* | Takamatsu *et al.* (2000) | AB026150 |
| *Podosphaera collomiae* | Cunnington *et al.* (2001) only in database | AF298546 |
| *Podosphaera cucurbitae* | Hirata *et al.* (2000) | AB040330 |
| *Podosphaera elsholtziae* | Takamatsu *et al.* (2000) | AB026142 |
| *Podosphaera euphorbiae-hirtae* | Hirata *et al.* (2000) | AB040306 |
| *Podosphaera ferrugunea* | Takamatsu *et al.* (2000) | AB027232 |
| *Podosphaera filipendulae* | Takamatsu *et al.* (2000) | AB022385 |
| *Podosphaera fugax* | Takamatsu *et al.* (2000) | AB026134 |
| *Podosphaera fuliginea* | Hirata *et al.* (2000) | AB040332 |
| *Podosphaera fusca* | Hirata *et al.* (2000) | AB040331 |
| *Podosphaera intermedia* | Takamatsu *et al.* (2000) | AB026145 |
| *Podosphaera leucotricha* | Cunnington *et al.* (1999) only in database | AF073353 |
| *Podosphaera longiseta* | Takamatsu *et al.* (2000) | AB000945 |
| *Podosphaera macularis* | Patzak (2001) only in database | AF448224 |
| *Podosphaera pannosa* | Takamatsu *et al.* (2000) | AB022348 |
| *Podosphaera phaseoli* | Hirata *et al.* (2000) | AB040297 |
| *Podosphaera pseudofusca* | Hirata *et al.* (2000) | AB040320 |
| *Podosphaera spiraeae* | Takamatsu *et al.* (2000) | AB026153 |
| *Podosphaera tridactyla* | Cunnington *et al.* (2000) only in database | AF154321 |
| *Podosphaera xanthii* | Hirata *et al.* (2000) | AB046985 |
| *Sawadaea polyfida* | Takamatsu *et al.* (2000) | AB000936 |
| *Sawadaea tulasnei* | Takamatsu *et al.* (2000) | AB022367 |
| *Uncinula bicornis* | Cunnington *et al.* (2001) only in database | AF298540 |

module of LASERGENE system v. 4.03 (DNAStar Inc., U.S.A.). The obtained contiguous sequences, from sequencing reactions, were assembled by SeqMan II module of LASERGENE system v. 4.03 (DNAStar Inc., U.S.A.). Searching for the most similar sequences in NCBI's integrated databases (GenBank, EMBL and DDBJ), Advanced BLAST 2.0 (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) was used. Nuclear rDNA sequences were registered with accession numbers AF448225 for *Pseudoperonospora humuli* and with AF448224 for *Podosphaera macularis* in GenBank in 2001. Specific PCR primers were designed using the PrimerSelect module of LASERGENE system v. 4.03 (DNAStar Inc., U.S.A.). Phylogenetic analyses were performed from CLUSTAL V multiple alignment of selected rDNA (ITS1, 5.8S, ITS2) sequences by the neighbor-joining method (MegAlign module of LASERGENE system v. 4.03, DNAStar Inc., U.S.A.). The sequences for analyses were obtained from GenBank and are listed in Table 1.

## RESULTS

PCR primers R1 and R2 amplified a fragment of 305 bp nuclear rDNA (mainly ITS1) region from *Humulus lupulus*, a fragment of 297 bp nuclear rDNA region from *Pseudoperonospora humuli*, a fragment of 248 bp nuclear rDNA region from *Podosphaera macularis*, a fragment of 204 bp nuclear rDNA region from *Verticillium albo-atrum* and a fragment of 222 bp nuclear rDNA region from *Fusarium sambucinum* (Figure 1a). PCR primers R3 and R4 amplified a fragment of 397 bp nuclear rDNA (mainly ITS2) region from *H. lupulus*, a fragment of 598 bp nuclear rDNA region from *Pseudoperonospora humuli*, a fragment of 312 bp nuclear rDNA region from *Podosphaera macularis*, a fragment of 331 bp nuclear rDNA region from *V. albo-atrum* and a fragment of 317 bp nuclear rDNA region from *F. sambucinum* (Figure 1b).

The nuclear rDNA (ITS1, 5.8S, ITS2) sequences for *H. lupulus*, *V. albo-atrum* and *F. sambucinum*, were similar to sequences registered in NCBI's integrated databases (AF223066, AF364015 and U38277, respectively). However, unknown nuclear rDNA sequences for *Pseudoperonospora humuli* and *Podosphaera macularis* were registered with accession numbers AF448225 and AF448224, respectively, in GenBank in 2001. A BLAST search for similar sequences in GenBank revealed that the most similar sequences to that of our *Pseudoperonospora humuli* were of *P. humuli* isolates HV 148 (AY198305) and HV 136 (AY198304), *P. cubensis* (AY198306) and *P. urticae* (AY198307), registered in 2004 and with 94%, 94% and 91% of homology, respectively. The most similar sequences to that of *Podosphaera macularis* are *P. filipendulae* (AB022385) and *P. ferrugunea* (AB027232) with 98% and 96% of homology, respectively. A phylogenetic




Lanes: *Sh* – *Podosphaera macularis*; *Sh*H – hop with *Podosphaera macularis;* *Ph*H – hop with *Pseudoperonospora humuli*; *Va* – *Verticillium albo-atrum*; *Va*H – hop with *Verticillium albo-atrum*; *Fs* – *Fusarium sambucinum*; *Fs*H – hop with *Fusarium sambucinum*; H – hop without infection; M1 – 100 bp Ladder; M2 – pGEM DNA marker (Promega, U.S.A.)

Figure 1. PCR detection of oomycetal and fungal pathogens in infected hop plants by non-specific primer combinations **(a)** R1 + R2 and **(b)** R3 + R4 on 2% agarose gel

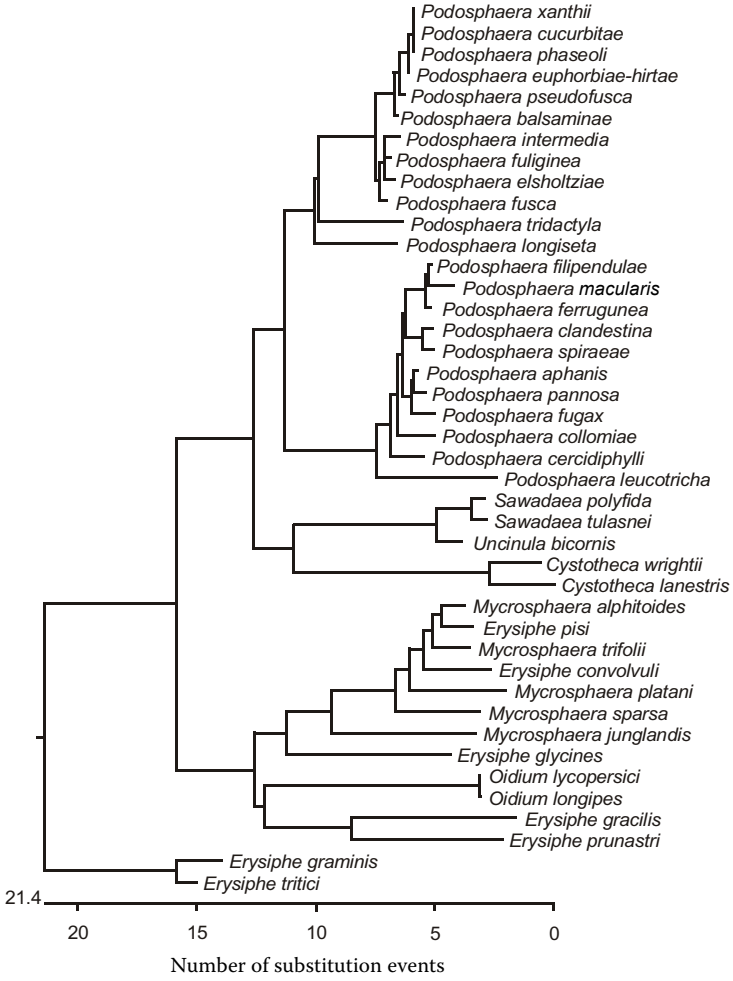
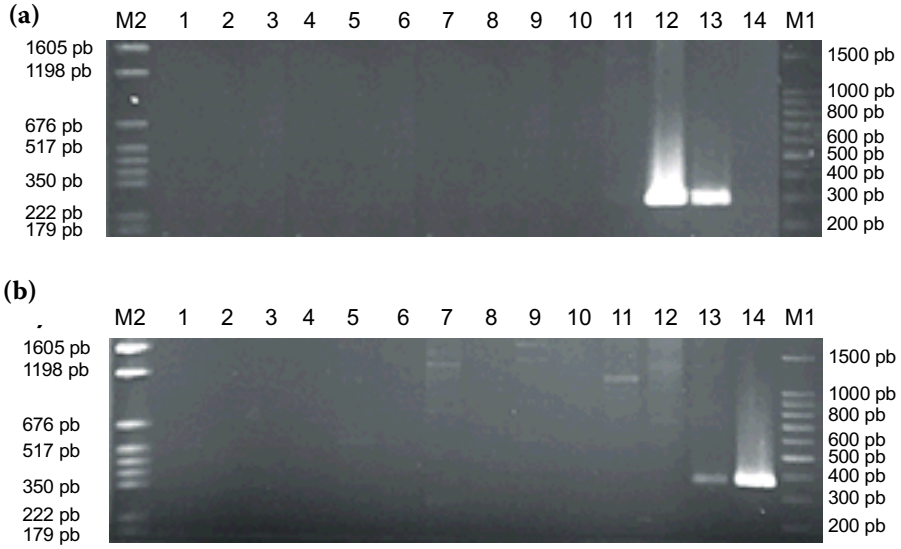
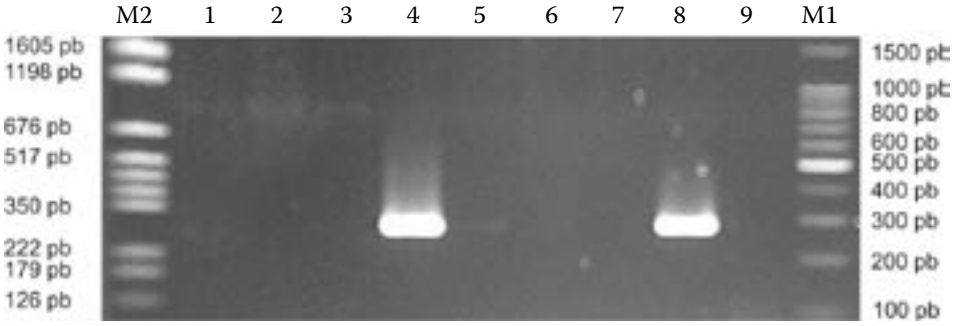



Figure 2. Phylogenetic analysis of nuclear rDNA (ITS1, 5.8S, ITS2) for 42 species of *Erysiphales* revealed by neighbor-joining method from CLUSTAL V multiple alignment (MegAlign module of LASERGENE system v. 4.03, DNAStar Inc., U.S.A.). Accession numbers of sequences are in Materials and methods




Lanes: 1 – *Verticillium albo-atrum*; 2 – *Fusarium sambucinum*; 3 – *Fusarium oxysporum*; 4 – *Fusarium equiseti*; 5 – *Alternaria alternata*; 6 – *Penicillium humuli*; 7 – *Phytophtora humicola*; 8 – *Epicoccum nigrum*; 9 – *Trichoderma* sp.; 10 – *Ascochyta* sp.; 11 – *Cladosporium* sp.; 12 – *Podosphaera macularis*; 13 – *Podosphaera macularis* and *Pseudoperonospora humuli*; 14 – *Pseudoperonospora humuli*; M1 – 100 bp Ladder; M2 – pGEM DNA marker (Promega, U.S.A.)

Figure 3. Analysis of specifity of PCR primer combinations **(a)** S1 + S2 and **(b)** P1 + P2 for detection of *Podosphaera macularis* and *Pseudoperonospora humuli*, respectively, in infected hop plants on 2% agarose gels




Lanes: 1, 2, 5, 6 – leaves from healthy plants; 3, 7 – leaves without spores and mycelia from infected plants; 4, 8 – leaves with mycelia and conidia from infected plants; 9 – negative control with water in PCR; M1 – 100 bp Ladder; M2 – pGEM DNA marker (Promega, USA)

Figure 4. Specific PCR detection of *Podosphaera macularis* from field samples of hop by using primer combinations S1 + S2 on 2% agarose gels

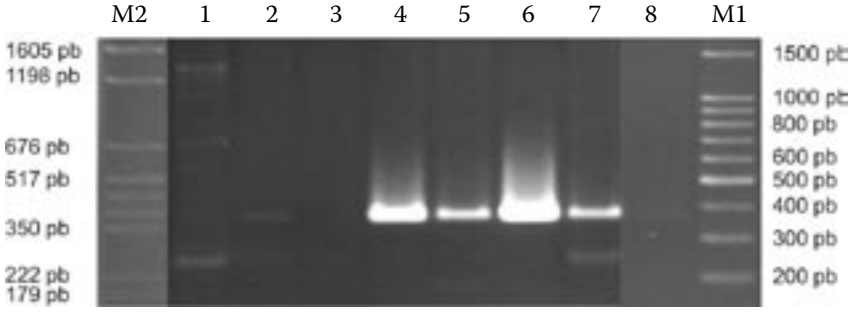
analysis of nuclear rDNA (ITS1, 5.8S, ITS2) for 42 species of *Erysiphales* (Figure 2) confirmed the BLAST searching, i.e. a species of identification and phylogenetic relationship of hop powdery mildew.

Specific PCR primers S1 and S2 amplified a fragment of 282 bp nuclear rDNA (ITS1, 5.8S, ITS2) region from *Podosphaera macularis* (Figure 3a), and specific PCR primers P1 and P2 amplified a fragment of 371 bp nuclear rDNA (ITS1, 5.8S, ITS2) region from *Pseudoperonospora humuli* (Figure 3b). None of the specific primer combinations cross-reacted with hop DNA or DNA from other fungi in hop. Because of the specificity in PCR products and the uniqueness of sequences used, the primer combinations S1 + S2 and P1 + P2 were considered suitable for specific detection of *Podosphaera macularis* and *Pseudoperonospora humuli*, respectively, from hop plants.

We also used these PCR methods to monitor the occurrence of infection in the field. Powdery mildew was detected only on infected leaves, but not in infected plants without visible symptoms (Figure 4). Downy mildew, however, was detectable not only in basal spikes, but also in infected plants without symptoms (Figure 5).

## DISCUSSION

ITS1 and ITS2 regions of ribosomal DNA units are more variable in sequences than rRNA genes and have potential for distinguishing fungal species (Grote *et al.* 2002). In fact, PCR primers designed from these regions allowed us to make species-specific identification of *Pseudoperonospora humuli* and *Podosphaera macularis* in hop plants. The PCR assays were reliable in detecting each of the pathogens from plant extracts exhibiting




Lanes: 1, 2, 3, – leaves from healthy plants; 4, 6 – basal spikes; 5, 7 – leaves without symptoms from infected plants; 8 – negative control with water in PCR; M1 – 100 bp Ladder; M2 – pGEM DNA marker (Promega, U.S.A.)

Figure 5. Specific PCR detection of *Pseudoperonospora humuli* from field samples of hop by using primer combinations P1 + P2 on 2% agarose gel

different degrees of disease severity, with positive amplification resulting even from symptomless infected plants (Figure 5). The detection of the target fungal DNA from heavily infected, as well as from weakly infected tissue without or with barely visible symptoms, indicates that the assays are sensitive and could be useful for monitoring and forecasting the diseases in field conditions. Similar systems were described for other oomycetal or fungal pathogens on different plants (Moricca *et al*. 1998; Chen *et al*. 1999; McKay *et al*. 1999; Grote *et al*. 2002).

A multiplex PCR is a valuable diagnostic tool for rapid detection of several pathogens in host plants (Fraaije *et al*. 2001). Our studies using PCR primers combinations R1 + R2 and R3 + R4 also revealed the possibility of their use in multiplex PCR detection of different oomycetal and fungal pathogens in hop. Thus, PCR primer R1 and R2 distinguished the species *Podosphaera macularis*, *V. albo-atrum* and *F. sambucinum*, but not *Pseudoperonospora humuli* from *H. lupulus* (Figure 1a). Further, PCR primer R3 and R4 distinguished the species *Pseudoperonospora humuli*, *V. albo-atrum* from *H. lupulus*, but not *Podosphaera macularis* and *F. sambucinum* (Figure 1b). Specific PCR primers (S1 + S2 and P1 + P2) also were reliable detecting double infection with *Pseudoperonospora humuli* and *Podosphaera macularis* in hop plants (Figure 3).

The phylogenetic analysis of the relationships between *Podosphaera macularis* and 41 related species of the *Erysiphales* (Figure 2) corresponded with phylogenies described by Saenz and Taylor (1999) and Takamatsu *et al*. (2000). The present study is the first report about molecular phylogenetic relationships of *Podosphaera macularis*, closely related to *P. filipendulae* and *P. ferruginea* based on sequence homologies. Both of these fungi were part of the subclade Sphaerotheca, section Sphaerotheca of Cystotheceae, that infected 806 plant species, including hop (Takamatsu *et al*. 2000). Phylogenetic differences of *Cystotheceae clade* from *Erysiphe clade* were similar to those published by Saenz and Taylor (1999). Both the molecular characterisation and phylogenetic analysis reported here confirmed species identification of *Podosphaera macularis*. The phylogenetic study of *Pseudoperonospora humuli* was not carried out, because detailed phylogenetic relationships of Peronosporaceae, based on ITS sequences, were published recently (Voglmayr 2003).

Our studies demonstrated that the specific PCR primers P1 + P2 and S1 + S2 are suitable for detecting the fungal pathogens *Pseudoperonospora humuli* and *Podosphaera macularis*, respectively, in naturally infected hop plants. The specificity of the PCR assay was also confirmed when compared with DNA from 11 other fungi occurring on hop leaves, stems and cones (Figure 3a, b). The efficacy of assays was high on plant tissues exhibiting symptoms and also on plants infected with downy mildew but without symptoms (Figures 4 and 5). The PCR based molecular method required less labour and time than did microscopic methods (Constantinescu 2000) or spore trapping (Mahafee *et al*. 2001). It was found to be accurate and sensitive and can be used for routine detection, classification, as well as disease monitoring.

***Acknowledgements***. The author wishes to give special thanks to Dr. František Veselý from the Hop Research Institute Co. Ltd., Žatec, Czech Republic, and Prof. Ewa Solarska, Institute of Soil Science and Plant Cultivation, Lublin, Poland, for their help with the identification of individual hop diseases and pathogens. The author expresses special thanks to Dr. Sebastjan Radišek, Institute of Hop Research and Brewing, Žalec, Slovenia, for the *Verticillium albo-atrum* isolate.

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Received for publication June 27, 2005
Accepted after corrections August 26, 2005

**Abstrakt**

Patzak J. (2005): **PCR detekce patogenů** *Pseudoperonospora humuli* **a** *Podosphaera macularis* **u chmele**. Plant Protect. Sci., **41**: 141–149.

Plíseň chmelová (*Pseudoperonospora humuli*) a padlí chmelové (*Podosphaera macularis*) jsou nejzávažnějšími patogeny chmele (*Humulus lupulus*). Včasná detekce a identifikace patogenů je často ztížena nepřítomností symptomů před hromadnou infekcí nebo záměnou s jiným patogenem. Molekulární analýza interních transkribovaných mezerníků (ITS) rDNA regionu je nová a velice účinná metoda determinace druhů. Specifická PCR metoda byla vyvinuta pro detekci patogenů *Pseudoperonospora humuli* a *Podosphaera macularis* v přirozeně infikovaných rostlinách chmele. Specifické kombinace PCR primerů P1 + P2 a S1 + S2 amplifikovaly specifické fragmenty z *Pseudoperonospora humuli* a *Podosphaera macularis* a nereagovaly s chmelovou DNA ani DNA z jiného oomycetálního druhu. Kombinace PCR primerů R1 + R2 a R3 +R 4 bylo možné použít k „multiplexové" PCR detekci *Pseudoperonospora humuli*, *Podosphaera macularis*, *Verticillium albo-atrum* a *Fusarium sambucinum*.

Fylogenetické vztahy byly odvozeny pro 42 druhů *Erysiphales* z nukleární rDNA (ITS1, 5.8S, ITS2). Molekulární charakterizace a fylogenetická analýza potvrdila s určitostí identifikaci druhu padlí chmelového. Tyto PCR metody se osvědčily jako přesné a citlivé pro detekci, identifikaci, klasifikaci a monitorování těchto patogenů.

**Klíčová slova**: padlí chmelové; plíseň chmelová; interní transkribované mezerníky (ITS); PCR detekce; fylogenetická analýza

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